KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

Lijana Dienaitė

INVESTIGATION OF CHEMICAL COMPOSITION, ANTIOXIDANT AND BIO ACTIVITY OF EXTRACTS OF VARIOUS *NEPETA* AND *PAEONIA* SPECIES

Master's Degree Final Project

Supervisor
Assoc. Prof. Dr. Audrius Pukalskas

KAUNAS, 2016

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CHEMINĖS TECHNOLOGIJOS FAKULTETAS

NEPETA IR *PAEONIA* GENČIŲ AUGALŲ EKSTRAKTŲ CHEMINĖS SUDĖTIES IR ANTIOKSIDACINIO BEI BIOLOGINIO AKTYVUMO TYRIMAI

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"Investigation of chemical composition, antioxidant and bio activity of extracts of various *Nepeta* and *Paeonia* species"

DECLARATION OF ACADEMIC INTEGRITY

03 June 2016
Kaunas

I confirm that the final project of mine, **Lijana Dienaitė**, on the subject. "Investigation of chemical composition, antioxidant and bio activity of extracts of various *Nepeta* and *Paeonia* species" is written completely by myself; all the provided data and research results are correct and have been obtained honestly. None of the parts of this thesis have been plagiarized from any printed, Internet-based or otherwise recorded sources. All direct and indirect quotations from external resources are indicated in the list of references. No monetary funds (unless required by law) have been paid to anyone for any contribution to this thesis.

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List of abbreviations, acronyms and symbols

Abbreviation	Full form
AA	Antioxidant Activity
AAPH	2',2'-Azobis (2-amidinopropane) dihydrochloride
ABTS$^{\bullet+}$	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
Ace	Acetone
ASE	Accelerated Solvent Extraction
C	*Nepeta cataria*
CAA	Cellular Antioxidant Activity
CAE	Caffeic Acid Equivalent
CPM	Crude Plant Material
DCF	Dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescin diacetate
DW	Dry Weight
ED_{50}	Effective Dose (cell viability 50 %)
ESI	Electro Spray Ionisation
FBS	Fetal Bovine Serum
F-C	Folin-Ciocalteu Reagent Assay
G	*Nepeta grandiflora*
GAE	Gallic Acid Equivalent
HEK	Hexane
HORAC	Hydroxyl Radical Adverting Capacity
HOSC	Hydroxyl Radical Scavenging Capacity
HPLC-DPPH$^{\bullet}$	High Performance Liquid Chromatography DPPH$^{\bullet}$ post column method
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
DPPH$^{\bullet}$	2,2-diphenil-1-picrylhydrazyl radical
IC_{50}	Inhibitory concentration
M	*Nepeta melissifolia*
MeOH	Methanol
Milli-Q water	Ultrapure water
m/o	microorganisms
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	*Nepeta nuda*
ORAC	Oxygen Radical Adverting Capacity

PBS	Phosphate Buffer Saline
PL	Peony leafs
PLASEM	Peony leafs extract after accelerated solvent extraction with methanol
PLTRM	Peony leafs extract after traditional extraction with methanol
PLTRW	Peony leafs extract after traditional extraction with water
PR	Peony roots
PRASEM	Peony root extract after accelerated solvent extraction with methanol
PRTRW	Peony root extract after traditional extraction with water
QE	Quercetin Equivalent
R	*Nepeta racemosa*
S	*Nepeta sibirica*
SE	Soxhlet Extraction
SPB	Sodium Phosphate Buffer
***spp*.**	Species
TE	Trolox Equivalent
TEAC	Trolox Equivalent Antioxidant Capasity
TPC	Total Polyphenol Content
TR	Traditional Extraction
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UPLC	Ultra Performance Liquid Chromatography
UPLC-MS	Ultra Performance Liquid Chromatography Mass Spectrometry
UPLC-Q-TOF-MS	Ultra Performance Liquid Chromatography Quadrupole Time Of Flight Mass Spectrometry

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Dienaitė, Lijana. *Investigation of Chemical Composition, Antioxidant and Bio Activity of Extracts of Various Nepeta and Paeonia Species*: *Master's* thesis in Food Science and Safety / supervisor Assoc. Prof. Dr. Audrius Pukalskas. The Faculty of Chemical Technology, Kaunas University of Technology.

Research area and field: Technological Sciences, Food Technology

Key words: *Nepeta*, peony, antioxidant activity, polyphenols, cytotoxicity, antiproliferative effect, chromatography.

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SUMMARY

Antioxidant testing of natural products (such as plant extracts) has attracted increasing interest in recent years, mainly due to the fact that antioxidants can neutralise harmful free radicals *in vitro*, thus suggesting that an antioxidant-rich diet might provide health benefits.

Nepeta and *peony* species presented in this work are insufficiently phytochemically characterized and poorly tested in a sense of their biological activity. Due to this fact the aim of this study was determined phytochemical composition and antioxidant also bio activity of *Nepeta* and peony species extracts.

Traditional multi-step soxhlet extraction and novel accelerated solvent extraction (ASE) were used to produce natural extracts of *Nepeta* and peony species. Antioxidant activity of extracts were assayed using $DPPH^{\bullet}$, $ABTS^{\bullet+}$, ORAC, HORAC and HOSC, as well as the cellular antioxidant (CAA) method. The total content of phenolic compounds in the extracts was determined using the *Folin-Ciocalteu* reagent method. Antioxidant activity of solid residues were tested by QUENCHER approach method. Antioxidant activity of individual compounds were analysed by HPLC-$DPPH^{\bullet}$ post column assay. Quantitative and qualitative analysis of *Nepeta* and peony species extracts were analysed by ultra performance liquid chromatography coupled with mass quadrupole time of flight detector (UPLC-Q-TOF). Inhibitory effect of investigated extracts were analysed using α-amylase assay and IC_{50} values was determined. Cytotoxic properties of obtained extracts after 4, 24 and 48 hours of incubation were evaluated using cytotoxicity assay and antiproliferative effect was evaluated in human colon carcinoma HT29 cell line.

The evaluation of the antioxidant activity of obtained extracts by various in vitro methods the highest activity showed extracts extracted by polar solvents, while the lowest activity possessed acetone extracts. Solid fractions evaluated by Quencher approach showed that peony leafs fractions have higher antioxidant activity than thus of rest.

Qualitative analysis showed that the main compounds of *Nepeta* species were phenolic acids such as rosmarinic, chlorogenic, caffeic, ferulic and etc., while in peony extracts were gallic acid derivatives such as methyl digallate, digallic acid, galloylhexose etc. Quantitative analysis of *Nepeta* species showed that ferulic acid was the main compound in water and acetone extracts,

while rosmarinic acid was the main compound in methanol extracts. The highest amount of reported compounds were found in *N. racemosa*, *N. nuda*, *N. sibirica* and *N. melissifolia* extracts.

In bio assays was determined that: peony leaf extract after traditional extraction with methanol possessed the highest inhibitory activity against α-amylase. In cytotoxicity studies *N. melissifolia* after 48 h of incubation have cytotoxicity on Caco-2 line cells. (IC_{50} 6.98 mg/mL) and the best antiproliferative effect was determined in water extract of *N. nuda (*ED_{50} 2.75 mg of extract/mL).

It may be concluded, that peony and *Nepeta* species are a good source of antioxidant compounds and may be promising as bioactive ingredients in food and pharmaceutical industries.

Dienaitė, Lijana. *Nepeta* ir *Paeonia* genčių augalų ekstraktų cheminės sudėties ir antioksidacinio bei biologinio aktyvumo tyrimai. *Magistro* baigiamasis projektas / vadovas doc. dr. Audrius Pukalskas; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

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Kaunas, 2016. 97 p.

SANTRAUKA

Natūralių produktų (pvz.: augalų ekstraktų) antioksidacinio aktyvumo tyrimai pastaraisiais metais sulaukia vis didesnio susidomėjimo daugiausia dėl to, kad antioksidantai gali neutralizuoti kenksmingus laisvuosius radikalus *in vitro* ir manoma, kad antioksidantais praturtinta dieta gali teikti naudą sveikatai.

Šiame darbe pateiktų katžolės ir bijūno rūšių cheminės sudėtys yra nepakankamai ištirtos tiek antioksidacinio, tiek biologinio aktyvumo prasme, todėl šio darbo tikslas buvo nustatyti skirtingų katžolės ir bijūno ekstraktų fitocheminę sudėtį bei antioksidacinį ir biologinį aktyvumą.

Katžolės ir bijūno rūšių ekstraktai buvo paruošti naudojant tradicinę daugiapakopę soksleto ekstrakciją ir pagreitintą ekstrakciją organiniais tirpikliais. Skirtingų ekstraktų antioksidacinis aktyvumas buvo įvertintas, naudojant $DPPH^{\bullet}$, $ABTS^{\bullet+}$, ORAC, HORAC ir HOSC metodus, taip pat antioksidacinis aktyvumas ląstelėse buvo įvertintas naudojant CAA metodą. Bendras fenolinių junginių kiekis nustatytas *Folin-Ciocalteu* metodu. Prieš ekstrakciją ir po skirtingų ekstrakcijų likusios kietosios frakcijos antioksidacinis aktyvumas tirtas $ABTS^{\bullet+}$, $DPPH^{\bullet}$ ir bendro fenolinių junginių kiekio metodais, pritaikius QUENCHER procedūrą.

Kiekybinė ir kokybinė katžolių ir bijūno ekstraktų analizė buvo atlikta taikant ultra efektyviąją skysčių chromatografiją su kvadrupoliniu skriejimo laiko masių detektoriumi (UPLC-Q-TOF). Nustatytų fenolinių junginių antiradikalinės savybės katžolės ir bijūno augalų ekstraktuose, įvertintos kombinuotu HPLC-$DPPH^{\bullet}$ pokolonėliniu metodu. Tirtų ekstraktų inhibitorinės savybės buvo patikrintos atlikus α-amilazės metodą. Taip pat nustatytos IC_{50} vertės. Citotoksinės ekstraktų savybės po 4, 24 ir 48 valandų inkubacinio periodo tirtos pritaikius citotoksiškumo metodą. Antiproliferatinis tirtų ekstraktų aktyvumas buvo įvertintas naudojant žmogaus storosios žarnos karcinomos HT29 ląsteles.

Įvertinus ekstraktų antioksidacinį aktyvumą įvairiais *in vitro* metodais nustatyta, kad didžiausiu aktyvumu pasižymėjo poliniais tirpikliais išgauti ekstraktai, o mažiausią aktyvumą turėjo acetoniniai ekstraktai. Ištyrus kietų frakcijų antioksidacinį aktyvumą QUENCHER metodu nustatyta, kad bijūnų lapų kietosios frakcijos turėjo didžiausią antiksidacinį aktyvumą, lyginant su kitomis augalų frakcijomis.

Tiriant ekstraktų kokybinę sudėtį buvo nustatyta, kad pagrindiniai junginiai katžolėse yra fenolinės rūgštys (pvz.: rozmarinų, chlorogeno, ferulio ir kt.), o bijūnuose – galo rūgšties dariniai kaip antai, metildigalatas, digalo rūgštis, galoheksozė ir kt. Katžolės augalų kiekybinė analizė parodė, kad ferulio rūgštis buvo pagrindinis junginys vandeniniuose ir acetoniniuose ekstraktuose, o rosmarinų rūgštis – metanoliniuose ekstraktuose. Didžiausi nustatytų junginių kiekiai buvo rasti *N. racemoca, N. nuda, N. sibirica, N. melissifolia* ekstraktuose.

Atlikus biologinius tyrimus nustatyta, kad bijūnų lapų ekstraktas po tradicinės ekstrakcijos metanoliu turėjo geriausią inhibitorinį aktyvumą prieš α-amilazę. Citotoksiškumo tyrimuose *N. melissifolia* po 48 h inkubacijos turėjo citotoksinį poveikį Caco-2 linijos ląstelėse. (IC_{50} 6,98 mg/mL), o geriausias antiproliferacinis poveikis buvo nustatytas vandeniame *N. nuda* (ED_{50} 2,75 mg/mL) ekstrakte. Taigi atlikti tyrimai parodė, kad bijūnas ir katžolės yra geras antioksidantų junginių šaltinis, todėl šie augalai gali būti perspektyvus bioaktyvių junginių šaltinis maisto ir farmacijos pramonėje.

INTRODUCTION

Food contamination (it's a result of industrialization and environmental pollution), together with life style (long-term stress, excessive consumption of highly processed food, smoking) also, chemical compounds which are present in water, air, synthetic materials and other products act as harmful environmental factors in humans. It starts with the various molecular processes in human body. These processes generates a large amount of free radicals, causing a damage, called oxidative stress. Oxidative stress is important in the development of various chronic diseases. Conducted studies showed that imbalance between the production of bodily antioxidant defense system and free radical formation may lead to neurodegenerative diseases, cardiovascular diseases, accelerated aging, inflammation and cancer [1].

High antioxidant activity is characterized by a number of vegetable raw materials. In studies with different herbal raw materials it was observed that the high antioxidant activity is characterized by plants, whose chemical composition includes various compounds such as polysaccharides, steroids, flavonoids, alkaloids, saponins, terpenoids and tannins [2]. Potential sources of phenolic compounds are most vegetables, fruits, seeds, cereals, herbaceous plant species. Phenolic compounds are mainly found in plant leaves, flowers, stems and bark. Polythenols in plants are important for growth, development processes, for self-defense against infection and other irregularities in the process [3].

A large variety of phenolic compounds in their chemical composition is characterized by plants of the genus *Nepeta* and *Paeonia*. Antioxidant and biological activities in most of *Nepeta* and *Paeonia* species are widely studied too [4, 5]. However, the data on antioxidant and biological properties, as well as phytochemical composition of various plant species and varieties used in the present study are rather scarce (except *N. cataria*).

The aim of this study: to investigate chemical composition, antioxidant and bio activity of extracts of various *Nepeta* and *Peonia* species.

The approach that has been followed to fulfill these aims is:

- Production of various types of extracts from *Nepeta* and *Paeonia* species using different extraction methods and determination of extract yields;
- Determination of the activities in order to get the most promising extracts using various *in vitro* antioxidant activity assays ($ABTS^{\bullet+}$, $DPPH^{\bullet}$, F-C);
- Determination of extracts chemical composition using novel chromatographical methods (UPLC);
- Determination of antioxidant activity of compounds using HPLC-$DPPH^{\bullet}$ on-line radical scavenging method;

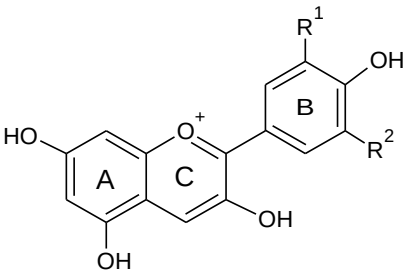
- Quantification of most active compounds in *Nepeta* species extracts using UPLC-MS method;
- Determination of antioxidant activity of selected extracts using ORAC, HORAC and HOSC methods;
- Evaluation of inhibitory properties of selected extracts using α-amylase method;
- Evaluation of cellular antioxidant activity of the selected extracts using CAA method;
- Evaluation of cytotoxic and antiproliferative activity of selected extracts using cytotoxicity and antiproliferative activity assays.

1. LITERATURE REVIEW

1.1. *Phenolic compounds*

Phenol compounds constitute the most widespread group of secondary metabolites, which due to its antioxidant properties used in the pharmaceutical, cosmetic and food industries [6]. Phenolic compounds are composed of at least one aromatic ring with one or more hydroxyl groups [7].

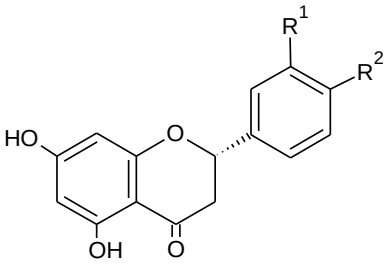
Anthocyanidin



Delphinidin: $R_1 = R_2 = OH$

Cyanidin: $R_1 = OH$, $R_2 = H$

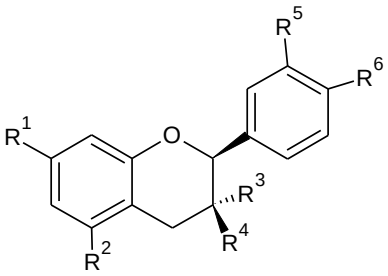
Flavanone



Hesperetin: $R_1 = OH$, $R_2 = OCH_3$

Naringenin: $R_1 = H$, $R_2 = OH$

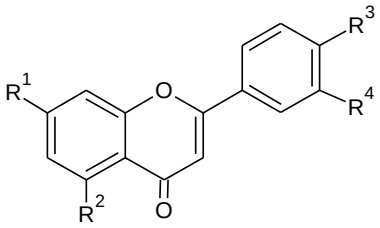
Flavanol



Catechin: $R_1 = R_2 = R_3 = R_5 = R_6 = OH$, $R_4 = H$

Epicatechin: $R_1 = R_2 = R_4 = R_5 = R_6 = OH$, $R_3 = H$

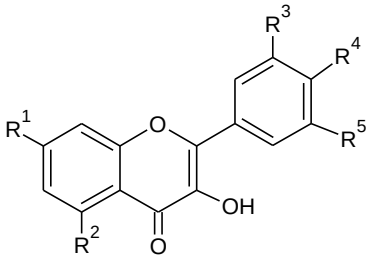
Flavone



Apigenin: $R_1 = R_2 = R_3 = OH$, $R_4 = H$

Luteolin: $R_1 = R_2 = R_3 = R_4 = OH$

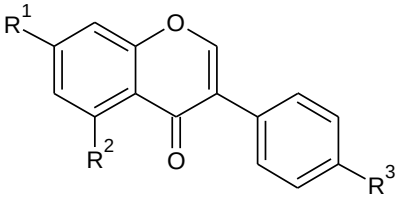
Flavonol



Quercetin: $R_1 = R_2 = R_3 = R_4 = OH$, $R_5 = H$

Myricetin: $R_1 = R_2 = R_3 = R_4 = R_5 = OH$

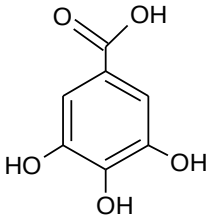
Isoflavone



Genistein: $R_1 = R_2 = R_3 = OH$

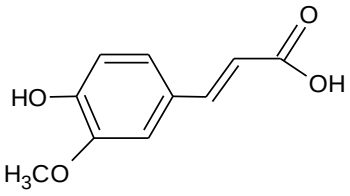
Daidzein: $R_1 = R_3 = OH$, $R_2 = H$

Hydroxybenzoic Acid

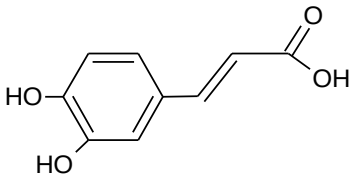


Gallic acid

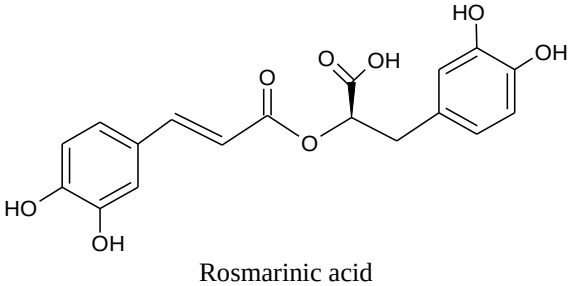
Hydroxycinnamic Acids



Ferulic acid



Caffeic acid



Rosmarinic acid

Figure 1. Chemical structures of some flavonoids and phenolic acids [9].

There are different classifications of phenol. Classification of phenolic compounds based on the number of constituent carbon atoms and basic skeleton of phenol. According to this classification there are: free phenols, phenolic acids, cinnamic acid, naftochinones, xanthones, stilbenes, flavonoids and lignans [8]. Usually in plants the most common phenols are: phenolic acids, flavonoids, tannins and the less common stilbenes and lignans [9] (Figure 1).

According to the oxidation state of the central C ring flavonoids are divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins. The structural differences in each subgroup depends on degree and pattern of hydroxylation, methoxylation or glycosylation. The most common flavonoids in plants are: myricetin, kaempferol, quercetin, rutin, hyperoside, hesperidin, apigenin, luteolin, catechin and their derivatives [9].

Phenolic acids can be divided into two groups: derivatives of benzoic acid and those which are derivatives of cinnamic acid [10]. The most common derivatives of hydroxybenzoic acids are vanillic, gallic, ellagic, protocatechuic, syringic and gentisic acid. Cinnamic acid derivatives have been represented in nature and include coumaric, caffeic, quinic, rosmarinic, ferulic, sinapic and chlorogenic acids.

1.2. *Biological activities of phenolic compounds*

One of the most frequently mentioned and the most commonly observed traits of phenolic compounds are their antioxidant activity. It is believed that the antioxidant activity is expressed as a result of phenolic compounds ability to be donors of hydrogen atoms and at the same time to remove free radicals to form less reactive phenoxyl radicals [11]. Antioxidants can inhibit, delay or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Antioxidants are important in the prevention of chronic degenerative diseases such as coronary heart disease, aging and cancer. According to Rice-Evans and Ames, phenolic compounds have strong antioxidant activity and are better antioxidants than vitamin C, E and carotenoids. It was proved in *in vitro* assays [12, 13, 14].

Flavonoids such as catechin, apigenin, quercetin and rutin, are reported for their hapatoprotective activities [15].

It is well known that plants synthesize flavonoids as a response to microbiological infection. In vitro studies were determined that flavonoids are effective antimicrobial substances against a wide range of m/o. It were reported, that plant extracts of different species which contain flavonoids have antibacterial activity [16, 17, 18, 19]. Several flavonoids such as apigenin, flavonol and flavone glycosides, flavanones, isoflavones, and chalcones have been shown to possess antibacterial activity [20]. It is believed, that their activity against m/o are based on ability

to form complex with proteins and to inactivate microbial enzymes, adhezins, transport proteins or disrupt microbial membranes [21,22]. Quercetin and apigenin are known to inhibit DNA gyrase in *Escherichia coli* [23]. Another study demonstrated inhibitory activity of robinetin, myricetin, and (−)-epigallocatechin to inhibit DNA synthesis in *Proteus vulgaris* [24].

Chemical irritation, tissue injury and microbial pathogen infection are responsible for inflammation processes in human body. Normal inflammation is self-limiting and rapid, but prolonged inflammation cause various chronic disorders [25]. It were reported, that apigenin, luteolin, quercetin and hesperidin have anti-inflammatory and analgesic effects. Anti-inflammatory properties of flavonoids may be because of inhibition of kinases such as serine-threonine protein kinases [26, 27]. Also, flavonoids are able to inhibit cyclooxygenase, lipooxygenase which are responsible for production of mediators such as prostanoids, leukotrienes cytokines, chemokines, or adhesion molecules [28]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Furthermore, some flavonoids have inhibitory effect on the production of prostaglandins, a group of powerful proinflammatory signaling molecules [29]. In vitro studies were determined that quercetin inhibit immunoglobulin secretion of IgM, IgA, and IgG [30].

Food contamination together with life style also chemical compounds which are present in water, air, synthetic materials and other products act as carcinogens and are responsible for increasing cases of cancer [31, 32, 33]. A numerous studies have shown that phenolic compounds are potential chemopreventive agents and have cancer-preventing properties [34, 35]. They can influence important molecular and cellular mechanisms associated with multiple carcinogenic steps, such as cell cycle arrest, down-regulation, inhibition and expression of various proteins and enzymes (such as heat shock, p53, IAPs proteins, tyrosine kinase etc.), which affect immune responses and metabolism of carcinogens, cell proliferation, differentiation and apoptosis [35, 36, 37]. In several human cancer cell lines (breast cancer, leukemia and colon cancer) flavonoids inhibit production of heat shock protein [38]. Quercetin can inhibit tyrosine kinase in a human phase I trial [39]. Furthermore, quercetin have growth-inhibitory effects on several cancer cell lines *in vitro*. These included HGC-27, NUGC-2, NKN-7, and MKN-28 gastric cancer cells, P-388 leukemia cells, colon cancer cells (COLON 320 DM), human squamous and gliosarcoma cells, human breast cancer cells and ovarian cancer cells [38]. Gallic acid can inhibit ribonucleotide reductase and cause apoptosis of leukemia cells. Moreover, this acid are effective against glioma cell lines (decrease cell viability, proliferation and angiogenesis). Furthermore, gallic acid proved to be effective against human lung cancer cell lines (apoptosis and activation of caspases (apoptotic enzymes), tumor inhibition *in vivo*) [40, 41, 42, 43]. Ferulic acid are effective against skin and mammary carcinomas *in vivo* murine models it prevent tumor formation [44, 45]. Rutin and apigenin are effective against human cancer cell lines (MCF-7, KB, SW-480 and A-731) [46].

Some flavones and phenolic acids are effective against human breast cancer cell lines (MCF-7 and SKBR-3) [47]. Flavonoids, simple gallate esters, gallic acid derivatives and ellagic acid derivatives are effective against human hepatocellular carcinoma cell line (Hep G2) and human lung cancer cell line (A549) [48]. Gentistic acid, luteolin, apigenin, kaempferol are effective against human colorectal carcinoma cell lines (DLD-1 and HT-29) [49]. Rosmarinic acid has anticancer activity in various cancer cell lines. These included colon cancer HT-29, HCT15, CO115 and HCT-116 [50, 51, 52]; skin cancer B16, B16F10 [53, 54]; lung cancer A549 [55]; oral cancer [56]; leukemia U937 [57]; hepatoma HepG2 [58]; breast cancer MCF7 [59]; ovarian cancer [60]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity [61]. Myricetin and ellagic acid inhibit the tumorigenicity of BP-7 cell line on mouse skin [62]. Isoflavones and other flavonoids have protection properties against prostate cancer [63]. It is known that oxidative stress initiate developing of cancer. Antioxidants can combat progression of carcinogenesis. It depends on antioxidant properties to inactivate and inhibit oxidation [70, 72].

Antivirus activity of phenolic compounds usually based on inhibition of various enzymes associated with the life cycle of virus. Flavones and flavonones were less effective than flavan-3-ols in inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections [64]. Flavonoid isolated from *Scutellaria baicalensis* (*Lamieaceae*), named baicalin inhibit HIV-1 infection and replication. Furthermore, flavonoids such as robustaflavone and hinokiflavone and baicalein have antiviral activity and inhibit reverse transcriptase in HIV-1. Another study shown inhibition effect of catechins against DNA polymerases in HIV-1 [64]. Flavonoids such as chrysin, acacetin, and apigenin can prevent HIV-1 [65]. Kaempferol and luteolin shown synergistic effect against herpes simplex virus (HSV). Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [64]. These compounds in pares exhibit synergism. It was found that flavones are less active than flavonols against HSV-1 and the activity order was as followed: galangin, kaempferol, and quercetin [65]. It was reported that rosmarinic acid have antiviral activity [66].

Antiviral activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus showed some flavonoids [64]. Quercetin was found to be effective against DENV-2 in Vero cells. Antiviral mechanisms is based on antiviral compound ability to inhibit enzymes and bind viral nucleic acids or proteins [67].

1.3. *Antioxidant mechanisms of phenolic compounds*

The antioxidant activity of the phenolic compounds is based on the direct reaction of phenol and free radicals and this process at the molecular level can be carried out in three ways [68, 69, 70, 71]:

$$R + Ar\text{-}OH \rightarrow RH + ARO^{\bullet} \quad (1)$$

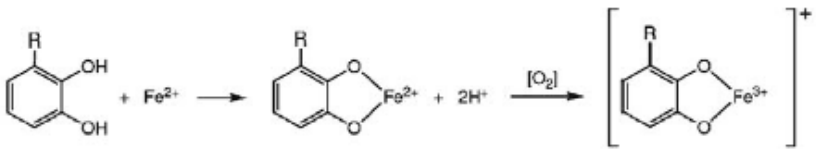
The first method of removing free radicals (R) is based on the transfer of a hydrogen atom from phenolic compound (Ar-OH) to the free radical. In this reaction formed a new phenoxy radical ($ARO^{\bullet}$). Which is less reactive, due to resonance, when the resulting phenol radicals have ability to stabilize and localises unpaired electron (1) [68, 69, 70, 71]. Moreover, the phenoxy radical can react with other free radicals and break the chain of oxidation (2) [68, 69, 70, 71]:

$$Ar\text{-}O^{\bullet} + R^{\bullet} \rightarrow Ar\text{-}OR \quad (2)$$

The second mechanism is based on the transfer of an electron from phenolic compounds, in to form of a radical cation ($ArOH^{\bullet+}$) and anion (R^{-}) (3). The resulting anion has paired electrons and their energy is stabilized. While extended conjugated aromatic system also stabilized when delocalize an unpaired electron [68, 71].

$$Ar\text{-}OH + R^{\bullet} \rightarrow ArOH^{\bullet+} + R^{-} \quad (3)$$

The third mechanism is based on the ability of phenolic compounds to bind metals and formed stable complexes, which prevents the participation of metals in reactions in which free radicals are produced (4) [72, 73, 74]. It is known, that some metals in a lower oxidation state can participate in Fenton's reaction [75] with H_2O_2, whereby a formation of $OH^{\bullet}$ radicals, which is very reactive and is one of the most harmful radicals of ROS.



(4)

1.4. *Relationships between antioxidant activity and structure of phenolic compounds*

For phenolic acids and their esters the antioxidant activity depends on the number of free hydroxyl groups in the molecule, which would be strengthened by steric hindrance [76]. Hydroxybenzoic acids were found to be less effective than their hydroxycinnamic acid counterparts, possibly due to the aryloxy-radical stabilizing effect of the –CH=CH–COOH linked to the phenyl ring by resonance [14]. The major factors that determine the radical-scavenging capability of flavonoids [77, 78] are: 3',4'-dihydroxy group in the B ring possessed the best electron donating properties, also participates in electron delocalization and confers higher stability to the radical form. The 3-OH group of the C ring is important for antioxidant activity of flavonoids. The

C2-C3 double bond conjugated with a 4-keto group in the C ring, which is responsible for electron delocalization from the B ring. The presence of both 3-hydroxy and 5-hydroxyl groups with the 4-carbonyl function and C2-C3 double bond in A and C rings increases the radical scavenging activity [79].

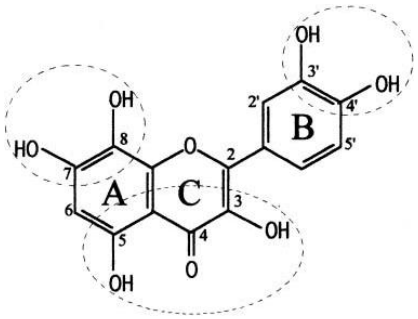


Figure 2. Structural features of flavonoids with a high radical scavenging activity [79].

The metal binding and antioxidant activity of phenolic compounds are associated with the structure, which contain hydroxy-keto group (4-*keto*, 3-hydroxy or 4-*keto*, 5-hydroxy groups in the C ring), as well as a large number of catechol/gallol groups [80, 81, 82].

Moreover, the antioxidant activity of flavonoids depends on position, structure, occurrence and number of sugar groups in flavonoids (flavonoid glycosides). Glycosides have lower antioxidant activity than their corresponding aglycones [84]. According to the Hollman (1999) in flavonol glycosides from tea the antioxidant properties declined as the number of glycosidic groups increased [83, 84]. Furthermore, procyanidins have better activity against various radical species then their degree of polymerization increases. For example, monomeric flavonoids are less effective than procyanidin dimers and trimers against superoxide anion. Moreover, trimers have lower activity against peroxynitrite and superoxide mediated oxidation than tetramers, also trimmers and tetramers demonstrate significantly lower superoxide scavenging properties than heptamers and hexamers [85].

1.5. *Reactive oxygen species (ROS) and free radicals*

The human body produce reactive oxygen species (ROS) and enzymatic antioxidants during normal physiological processes and in stressful conditions. The term does not include antioxidants as a group of compounds that are similar in a chemical structure, it is a general term for all compounds which may prevent or at least reduce the oxidation of the substrate. The imbalance created between free radicals and antioxidants leads to oxidative damage of macromolecules in cells [86, 87, 88, 89, 90], as well as peroxidation of membrane lipids, oxidative damage to nucleic acids and sugars, and oxidation of the sulfonic group in the other proteins. Any changes on the cell macromolecules lead to the emergence of many health disorders in humans [91, 92]. Free radicals are molecules having one or more unpaired electrons in their structure, which leads to their

reactivity expressed. Radicals having unpaired electron on the oxygen atom belong to the group of reactive oxygen species. These radicals are: superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), perhydroxy radical ($HO_2^{\bullet}$) and alkoxy radicals ($RO^{\bullet}$). There are not radical type oxidants that contain oxygen such as singlet oxygen ($^1O_2$) and ozone (O_3) [93]. In the absence of antioxidants that can neutralize reactive free radicals leads to many diseases [77], as well as cardiovascular diseases and cancer [94], neurodegenerative diseases, Alzheimer's disease [95] and inflammatory diseases [96]. There are a number of synthetic antioxidants which are used in the food industry, including butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) [97, 98]. It is believed that synthetic antioxidants are responsible for the occurrence of many diseases associated with liver damage and carcinogenesis [97, 98].

In recent years, great attention is paid to preventive medicine that promotes the use of antioxidants of plant origin in the diet and in therapy. The presence of nonenzymatic antioxidants such as vitamin C, vitamin E, carotenes, xanthophylls and tannins justifies the role of herbs and spices as well as powerful antioxidants. Within the natural compounds having a large, antioxidant effect especially highlights phenolic acids (gallic, caffeic, and rosmarinic acid), phenolic diterpenes (carnosol), flavonoids (quercetin, catechin), as well as the constituents of the essential oil [99, 100]. By comparing the antioxidant potential of phenolic compounds and terpenoids, phenolic compounds have proven to be better antioxidants [11, 101; 102, 103, 104, 105, 106], which is due to their characteristic to easily donate a hydrogen atom.

1.6. *Determination of antioxidant activity*

Usually plant samples contain a wide range of various phenolic compounds. The chemical composition and complexity of these compounds are difficult. Therefore to separate each phenolic antioxidant and study it individually it is costly, time consuming and inefficient. Furthermore, to evaluate antioxidant power of a whole sample often are more meaningful because of the synergistic effect and cooperation of antioxidants. Especially in health-based researches. Therefore there are different antioxidant activity screening methods for quick quantification of antioxidant effectiveness of phenolic extract samples [107].

Roughly antioxidant activity assays can be classified as electron transfer (SET) and hydrogen atom transfer (HAT) based assays [107]. HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation. Antioxidant activity or capacity measurements are based on kinetics. HAT reactions are pH and solvent independent and are usually quite rapid. The reducing agents including metals have interference for results in HAT-based assays [71, 108]. To this type of assays belong: ORAC, HORAC, HOSC, CAA and etc.

SET-based assays detect the ability of a potential antioxidant to transfer one electron to reduce any compound including carbonyls, metals and radicals [71]. SET reactions are slow and antioxidant capacity calculations are based on percent decrease in product. Contaminants and trace components (metals) interfere with SET methods. Therefore results are consistence and poor reproducible [71, 108]. To this type of assay belong: $ABTS^{\bullet+}$, $DPPH^{\bullet}$, F-C and etc.

There are a lot of assays to determine antioxidant activity of tested sample. For example:

$DPPH^{\bullet}$ scavenging activity, Hydrogen peroxide scavenging (H_2O_2) assay, Nitric oxide scavenging activity, Trolox equivalent antioxidant capacity (TEAC) method/$ABTS^{\bullet+}$ radical cation decolorization assay, Total radical-trapping antioxidant parameter (TRAP) method, Ferric reducing-antioxidant power (FRAP) assay, Hydroxyl radical scavenging activity, Hydroxyl radical averting capacity (HORAC) method, Oxygen radical absorbance capacity (ORAC) Method, Reducing power method (RP), Phosphomolybdenum method, Ferric thiocyanate (FTC) method, DMPD (*N*,*N*-dimethyl-p-phenylene diamine dihydrochloride) method, Xanthine oxidase method, Cupric ion reducing antioxidant capacity (CUPRAC) method, Metal chelating activity and etc. But a widen review will be about assays, which were used in the present research work.

1.6.1. *Folin-Ciocalteu (F – C) or Total Phenolics Assay*

The F – C assay has been used for many year to determinate total phenolic compounds in various samples. It is known that in total antioxidant capacity assays could be determined not only phenols but also metal chelators or reducing agents. Basic mechanism of this method is an oxidation/reduction reaction between F – C reagent and phenols. The experiment are performed at 765 nm wavelength. Moreover, to get more predictable data proper conditions are needed: proper volume ratio of F – C reagent and alkali; optimal reaction temperature and time for colour development and use of gallic acid as the references standard phenol [109, 110].

The advantages of the F – C method: it is simple and quick. The disadvantages of F-C method: a numerous substances have interference for the F – C method results (especially: organic acids, ascorbic acid, aromatic amines, sugars, Fe (II), sulphur dioxide, and other enediols and reductones). Furthermore, nonphenolic organic substances (cysteine, diphenylamine, EDTA, fructose, guanine, histamine, oleic acid, proteins, sucrose, thymidine and etc.) and inorganic compounds (hydrazine, iron sulfate, manganese sulfate, potassium nitrite etc.). Because of interference of these compounds phenolic concentrations in tested samples are obtained higher than real values [109, 110, 111].

1.6.2. *2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) Assay*

This assay is based on antioxidant ability to reduce $DPPH^{\bullet}$ radical. The ability can be evaluated by measuring the decrease of $DPPH^{\bullet}$ absorbance. During the measurement $DPPH^{\bullet}$

radical loses its colour after reaction with antioxidant. Reaction is monitored by a spectrometer at 515 nm wavelength. $DPPH^{\bullet}$ radical is a deep purple colour stable organic nitrogen radical. This radical does not have to be generated before the assay like $ABTS^{\bullet+}$ [113, 114].

The advantages of the $DPPH^{\bullet}$ assay. The test is quick and simple and usually needs only a UV spectrophotometer to perform. The disadvantages of the $DPPH^{\bullet}$ assay: in the case then compound spectra overlap $DPPH^{\bullet}$ at 515 nm, interpretation of results is complicated (e. g., carotenoids) [115]. $DPPH^{\bullet}$ is both antioxidant and radical probe therefore reaction is not competitive of this assay. Loss of $DPPH^{\bullet}$ colour can be via radical reaction (HAT) or reduction (SET) as well as unrelated reactions. The major determinant of the reaction is steric accessibility. Therefore it has influence for accessibility for big molecules. For example $DPPH^{\bullet}$ is stable nitrogen radical and does not have similarity to highly reactive peroxyl radical. A numerous antioxidants react quickly with peroxyl radical, while with $DPPH^{\bullet}$ they may react slowly or be inert due to steric inaccessibility. Furthermore, reducing agents as well as H transfer can decolorize $DPPH^{\bullet}$ radical, thus also have influence for interpretation of results. Therefore, it is not enough to evaluate antioxidant activity only with $DPPH^{\bullet}$ method [107, 112].

1.6.3. *$ABTS^{\bullet+}$ Assay*

This assay is based on antioxidant ability to scavenge the $ABTS^{\bullet+}$ radical [116]. Intensively coloured $ABTS^{\bullet+}$ cation is performed then peroxyl radicals or other oxidants oxidized $ABTS^{\bullet+}$. Antioxidants directly react with the $ABTS^{\bullet+}$ radical and reduce it colour. Antioxidant capacity of tested compounds are equal to their ability reduce the colour of $ABTS^{\bullet+}$ cation. Results are expressed relative to Trolox. Preparation of $ABTS^{\bullet+}$ radical requires a long time (e.g., up to 16 h) and buffer media. The reaction between the antioxidants and $ABTS^{\bullet+}$ are spectrophotometrically monitor at 415 and 734 nm wavelength [117].

The advantages of $ABTS^{\bullet+}$ method: the reactions can be automated and adapted to microplates [118], to flow injection [119]. It is simply to operate. Antioxidants reacts rapidly with $ABTS^{\bullet+}$ usually 30 min is enough. It can be used over a wide pH range. Therefore, it are able to study effects of pH on antioxidant mechanisms [120]. $ABTS^{\bullet+}$ is not affected by ionic strength, also is soluble in aqueous and organic solvents. Therefore, it can be used to determine hydrophilic and lipophilic antioxidant capacities of body fluids and extracts [121]. The disadvantages of $ABTS^{\bullet+}$ method: it takes a long time to prepare $ABTS^{\bullet+}$ cation. The $ABTS^{\bullet+}$ radical is synthetic. Thermodynamically phenolic compound which have lower redox potential than that of $ABTS^{\bullet+}$ (0.68 V) are able to react with $ABTS^{\bullet+}$. In slow reactions are needed some time to reach an endpoint and reaction with $ABTS^{\bullet+}$ may not be the same. Especially, then are used an endpoint of short

duration (4 to 6 min) or may be reading before the reaction is finished then results are lower than real TEAC values [122].

1.6.4. *Oxygen Radical Absorbance Capacity (ORAC) Assay*

The assay is based on reaction between thermally generated peroxyl radicals (it is a product of azo compounds) and antioxidant or substrate [107]. Sample antioxidants compete with fluorescein as a fluorescent probe for peroxyl radicals generated by decomposition of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The fluorescence decay kinetic curve is obtained every minute by measuring fluorescence intensity at physiological conditions (pH 7.4, 37 °C). Calculation of protective effects of an antioxidant is from the net integrated areas under the fluorescence decay curves (AUC) (subtracting the AUC of blank from that of the sample or standard e.g., Trolox). Data are expressed as micromoles of Trolox equivalents (TE) per liter or per gram of sample (μmol of TE/g or μmol of TE/L) [107, 123, 124, 125].

The advantages of the assay: method is automated, also this method can be compared with other assays to mimic better antioxidant activity of phenols in biological systems. ORAC can integrate both, the degree of activity of antioxidants and time [126]. The disadvantages of the ORAC assay: it is a time-consuming process, needs to use expensive equipment. Reproducibility of the assay can decrease if there are some small temperature differences in the external wells of the microplate [127].

1.6.5. *Hydroxyl Radical Antioxidant Capacity (HORAC) Assay*

This assay is a complement to the ORAC assay. This method is based on ability of antioxidants to utilize oxidation reaction between thermally generated hydroxyl radicals (by hydrogen peroxide H_2O_2) and fluorescein [128]. Free radicals suppress the fluorescence of fluorescein during the time. Blockage of H_2O_2 radicals reaction with the fluorescein is ended, when antioxidants in sample are completely exhausted. The fluorescence decay kinetic curve is obtained every minute by measuring fluorescence intensity at physiological conditions (pH 7.4, 37 °C). The area under the fluorescence diminishing plot is used to quantify the total hydroxyl radical antioxidant activity in a sample and is compared to a standard curve. Standard curve is made by using various concentrations of caffeic acid [129].

The advantages of this assay is that it gives a more direct measurement of antioxidant capacity for hydroxyl radicals. Unlike the ORAC which is validated for the determination of peroxyl radical absorbance capacity, the HORAC analyses the hydroxyl radical prevention capacity [129].

The disadvantages of HORAC assay: it is a time-consuming process, needs to use expensive equipment. Reproducibility of the assay can decrease if there are some small temperature differences in the external wells of the microplate [127, 129].

1.6.6. *Hydroxyl Radicals Scavenging Capacity (HOSC) Assay*

The method is based on the evaluation of hydroxyl scavenging capacity of the sample. As a probe for this method fluorescein is used and classic Fenton reaction with Fe (II) and H_2O_2 is as a source of hydroxyl radicals. In the HOSC method hydroxyl radicals react with fluorescein producing non-fluorescent product (fluorescence). Quantity of this reaction product is measured with the fluorometer. The fluorescence decay kinetic curve is obtained every minute by measuring fluorescence intensity at physiological conditions (pH 7.4, 37 °C). The area under the fluorescence decay curve (AUC) is integrated, and the net AUC, which is an index of the hydroxyl radical scavenging capacity, is calculated by subtracting the AUC of the blank from that of the antioxidant. Trolox could be used as a standard. Therefore, data are expressed as micromoles of Trolox equivalents (TE) per unit of sample [130].

The advantages of the HOSC assay: this method measure scavenging capacity against a physiologically important free radical unlike the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ methods. This method generate pure hydroxyl radicals and has been validated with ESR (electron spin resonance) technique. Similar to ORAC and HORAC assays, this HOSC method takes into account both kinetic and thermodynamic properties of the antioxidant-radical reaction. The disadvantages of HOSC assay: it is quite complicated assay system requiring skilled operators and cannot be used to measure scavenging properties of lipophilic compounds. Carbon–centered radicals can be formed in the reaction and this interfere with the assay [130].

1.6.7. *Cellular Antioxidant Activity (CAA) Aassay*

This method is based on the ability of compounds to penetrate cell membrane and prevent the oxidation of 2′7′-dichlorofluorescin ($DCFH_2$) by $ROO^{\bullet}$ radicals. This $ROO^{\bullet}$ radical is thermally generated from decomposition of the azo compound (AAPH). These radicals can be produced at the cell membrane or intracellularly. In both cases $DCFH_2$ is oxidized. In addition, $DCFH_2$ can be oxidized not only by $ROO^{\bullet}$ radical but also with other biologically produced ROS/RNS like peroxynitril or hydroxyl radical [131]. As a probe 2′7′-dichlorofluorescin ($DCFH_2$) are used, which can easily be oxidized to fluorescent dichlorofluorescein DCF. The fluorescence decay kinetic curve is obtained every 5 minutes for 1 h ($_{exc}$=485nm,$_{em}$=535nm) by measuring dichlorofluorescein DCF intensity at physiological conditions (pH 7.4, 37 °C). Quercetin is used as a standard and CAA values are expressed as μmol quercetin equivalents per unit of sample. Usually for the assay is used HepG2 and Caco2 cancer cell lines [131, 132, 133].

The working mechanism of CAA assay is presented in Figure 3. Firstly, the redox-sensitive probe is applied in the cells. As the probe is used the $DCFH_2$-DA ester. Then it permeate the membrane endogenous esterase hydrolize $DCFH_2$-DA ester to $DCFH_2$. Secondly, cells are incubated with the sample or just medium (control). Finally, AAPH are added in order to generate peroxyl radicals ($ROO^{\bullet}$). The antioxidant compounds can protect $DCFH_2$ from oxidation by different mechanisms: (1) scavenge peroxyl radicals in the membrane diminishing lipoperoxidation, (2) react with AAPH avoiding intracellular $ROO^{\bullet}$ formation, (3) compete with $DCFH_2$ for oxidants ROS/RNS, (4) react with $ROO^{\bullet}$ preventing other radicals formation, (5) inhibit a redox pathway toward formation of ROS/RNS that oxidize $DCFH_2$ [133].

The advantages of CAA method: if compound has antioxidant activity it does not mean, that it is bioactive. Using CAA method we can detect bioactive compounds because cellular assay detects only antioxidants that can penetrate cell membrane of living cell and inhibit oxidation inside the cell. Also, it gives additional information about uptake, bioavailability, metabolism and interactions with cellular components. CAA method comparing with other antioxidant methods is more realistic to the processes that occur *in vivo* [133].

The disadvantages of CAA assay: requires skilled operators, needs to use expensive equipment. Different cell lines could present different results, it depends on properties of used cell line. Differences between results may depend on the method conditions: endogenous antioxidant level, growth status of the cell line and initial ROS/RNS production [133].

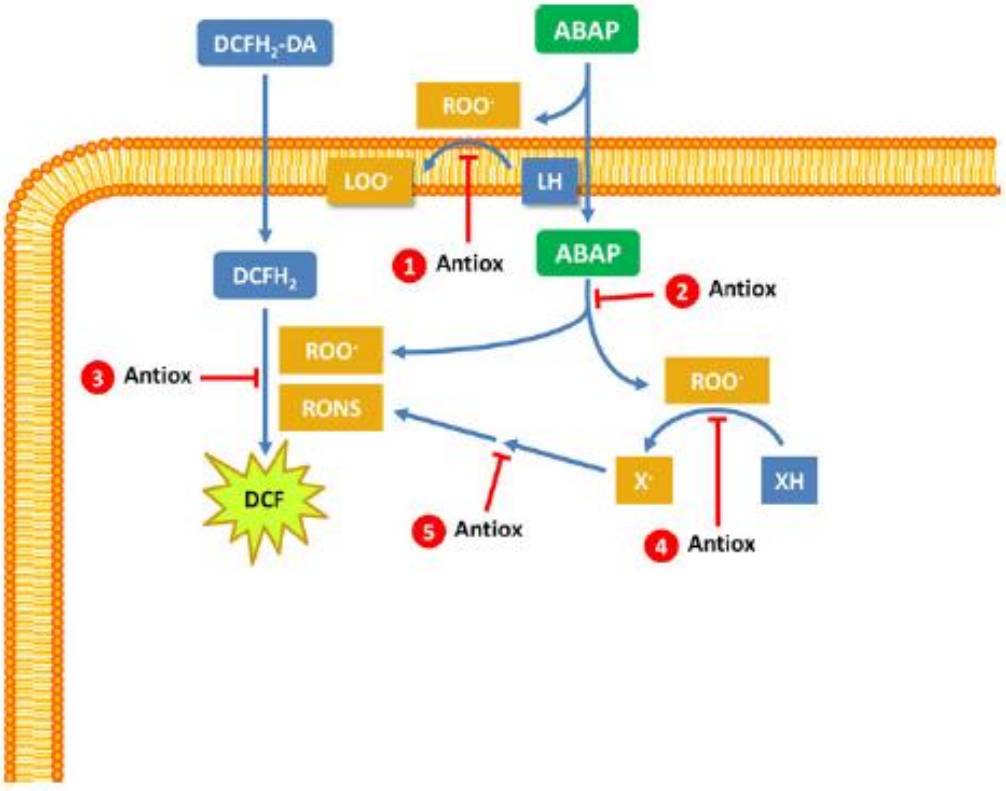


Figure 3. Working mechanism of cellular antioxidant activity (CAA) assay [133].

1.7. *General characteristics of family Lamiaceae*

Lamiaceae (syn. *Labiatae*) the mint family of flowering plants consists of more than 252 genus and 7000 species, it is the largest family of the order *Lamiales* [134]. *Lamiaceae* family is distributed nearly worldwide, especially in Mediterranean region. The family is known for their flavour, fragrance or medicinal properties, therefore plants of this family have been used since early times [135]. The aromatic essential oils are contained in leaves which are typically simple and oppositely arranged. Most plants of the family are annual or perennial with squarein shape stems. Some of the species are shrubs, subshrubs, and a few are vines or trees. The flowers are usually arranged in clusters with 5 united petals and 5 united sepals. The fruits are mostly a dry nutlets. [136, 137]. A number of widely used herbs belongs to this family such as basil, perilla, rosemary, lavender, marjoram, sage, oregano, thyme, mint, savory, betony, hyssop and catnip [138, 139, 140].

The genus *Nepeta* is one of the largest genera in the family *Lamiaceae*, belongs to *Nepetoideae* subfamily and tribe *Nepeteae* [141]. The genus includes about 300 herbaceous perennial, rarely perennial species, which are widespread in the Central and Southern Europe, Central and South Asia, the Middle East and in some areas of Africa [138]. The greatest diversity and abundance of species of the genus *Nepeta* there is in southwest Asia, especially in Turkey and Iran. Flora of Europe describes 24 species of the genus *Nepeta*, which are mainly widespread in central and southern Europe, particularly around the Mediterranean Sea [142].

- ***Nepeta Cataria***

N. cataria endemic in eastern Europe, the Middle East, central Asia, northern Europe, New Zeland, North America and parts of China. Picture of *N. cataria* is shown in Figure 4. Also, is commonly known as Catnip or Catmint because of its irresistible action on cats. *Nepeta cataria* is herbaceous perennial plant, growing 50 – 100 cm tall and wide. Leaves are green/grey coloured and heart shape with coarse-toothed edges. Also, has a squarein shape, hairy stem. Flowers grow in spikes are fragrant and showy. Furthermore, blossoms are white but can be spotted with pale purple or pink colour. The plant blooms from late spring through autumn. Catnip has been used for culinary and ornamental purposes and in folk-medicine. Medicinally it is use in the treatment of asthma, colic, diarrhea, the common cold and bronchosis [143].

- ***Nepeta racemosa***

N. racemosa is native in Caucasus, Turkey and northern Iran. It is also known as dwarf catmint or raceme catnip. Picture of *N. racemosa* is shown in Figure 4. *N. racemosa* is an herbaceous perennial plant, growing to 30 cm tall by 45 cm wide. It has leafy stems with oval, small, grey-green fragrant foliage and abundant, two-lipped, trumpet-shaped, violet or lavender-blue flowers in racemes atop square. The term *racemosa* refers to the racemes of flowers. Flowers

starts appear in early summer [145, 146]. *N. racemosa* has antibacterial activity against *E. coli* [144].

- ***Nepeta sibirica***

N. sibirica is endemic to Central Asia, Mongolia and southern Siberia [147]. Picture of *N. sibirica* is shown in Figure 4. *N. sibirica* is herbaceous perennial plant with a large number shoots up to 40 cm in length. The leaves are on short petioles, triangular, with pronounced marbling and jagged edges. The blossoms are at the peak part of the stem, sepals are triangular pointed tip and are covered with glandular hairs. The coronal slices are blue, declined in covered hairs [148]. It was determined, that methanol extract of *N. sibirica* has antimicrobial activity [149].

- ***Nepeta nuda***

N. nuda endemic in Europe and Asia (especially in Kazakhstan, Kyrgyzstan, Mongolia, Russia and Tajikistan). Picture of *N. nuda* is shown in Figure 4. *N. nuda* is perennial plant with stems 50 – 120 cm tall. Leafs shape are oblong-ovate or oblong-elliptic to lanceolate, 3.5 – 6.5 cm long, 1.5 – 2.5 wide. Leaf colour greenish, pale beneath and pubescent. Cymes numerous, axillary, many flowered, bracts linear. Calyx tubular, pubescent. Flowers are pale-violet, pink or white, 2-lipped, upper lip straight, 1.8 – 2 mm, deeply divided into 2 ovate lobes; lower lip 3-lobed. Nutlets brown, oblong. Biological activity: *N. nuda* has antiviral, antitumor and antibacterial activity [148, 150, 151].

- ***Nepeta melissifolia***

N. melissifolia is native in Crete and Aegean Islands. Picture of *N. melissiflia* is shown in Figure 4. Usually, grow amongst scrub and on rocky slopes. *N. melissifolia* is perennial plant with 40 – 60 cm long stem. Leaf are pubescent and ovate-cordate in shape, up to 3.5 cm long. Flowers are blue with red dots to 1.5 cm length. Summer is a flowering stage of *N. melissifolia*. *N. melissifolia* has antimalarian and antileishmanial activity [152, 193].

- ***Nepeta grandiflora***

Nepeta grandiflora M. Bieb. is wide spread in Central Europe and Central Asia. Picture of *N. grandiflora M. Bieb.* is shown in Figure 4. *N. grandiflora* is a perennial plant, stem erect to 40 – 80 cm. Plat is branched, glabrous to minutely pubescent. Leaves ovate, cordate at the base, crenulated [153]. A frequent casual in Europe and locally naturalized [154]. Flowers are violet-blue colour. It is used in folk medicine as a general, emollient, during anemia and as a substitute for tea [155].

1.8. *General characteristics of family Paeoniaceae*

Paeonia is the only genus in the family of *Paeoniaceae*. The genus *Paeonia* consists of three sections: *Moutan*, *Oneapia*, and *Paeonia*, which are comprised of only 35 species: The section

Moutan, with nine woody species (*P. cathayana*, *P. decomposita*, *P. delavayi*, *P. jishanensis*, *P. ludlowii*, *P. ostii*, *P. qiui*, *P. rockii*, *P. suffruticosa*) is confined to China and widely distributed in Yunnan, Xizang (Tibet), Anhui and Shanxi. The section *Onaepia*, with only two species (*P. brownie* and *P. californica*), is confined to Western North America and Mexico. Last section *Paeonia*, with 25 species, is the largest section and widely distributed throughout temperate Eurasia [156, 157, 158].

Nepeta cataria

Nepeta racemosa

Nepeta sibirica

Nepeta nuda

Nepeta melissifolia

Nepeta grandiflora M. Bieb.

Paeonia officinalis

Figure 4. Pictures of *Nepeta* and peony species.

They are shrubby or herbaceous perennial plants with round shape steams. Woody shrubs usually are from 0.25 to 3.5 metres tall, while perennial plants are from 0.25 to 1 metre tall. Leafs are divided into three lobes, each lobe being further divided into three smaller lobes. Leaves are

alternately produced all over the stems. The flowers are radially symmetrical, fragrant and large, with 5 sepals, 5 petals (sometimes 10), and with large number of stamens. Colour of flowers can ranged from white to red or yellow. Several species of peonies of southern Europe and Asia were also cultivated for food and as a medicinal herbs [158, 159].

- ***Paeonia officinalis***

Paeonia officinalis is native in south-eastern Europe and Asia. Picture of *P. officinalis* is shown in Figure 4. *P. officinalis* is an herbaceous perennial plant, with glabrous, branched, erect stem and tuberous fleshy roots. Growing to 60 – 70 cm tall and wide. Leafs have ovate lanceolate segments and are biternate or ternate. Leaves colour are green dark above and lighter below. Flowers consist from eight petals and five petal-like sepals and are white or deep pink or deep red colour, 10 – 13 cm in diameter. Blooms are fragrant. Fruits are a capsule with shiny black seeds. *Paeonia officinalis* is usually used in traditional Chinese and Indian medicine against liver dysfuncion, epilepsy, depression, migraine and etc. [160].

1.9. *Chemical composition of genus Nepeta*

Since now, 193 compounds have been identified in *Nepeta* species. Various types of chemical constituents of genus *Nepeta* have been reported such as monoterpene derivatives, sesquiterpenes, diterpenes, triterpenes, flavonoids, phenolic compounds, essential oils, and some others. The dominant constituents within the genus *Nepeta* are terpenoids and flavonoids [5].

Monoterpenes (nepetalactones and related compounds) 48 monoterpenes in total were identified from the genus *Nepeta*. The most abundant are iridoid monoterpenes, nepetalactones. It is a cyclopentanoid monoterpene with two fused rings (a cyclopentane and a lactone). For example: 4αα,7α,7αα-nepetalactone, 4αα,7α,7αβ-nepetalactone, 4αβ,7α,7αβ -nepetalactone and derivatives α-dihydronepetalactone, nepetalic acid and etc. Eight stereoisomers of nepetalactone, four diastereoisomers and their corresponding enantiomers exist. Iridoid glucosides also belongs to the group of monoterpenes. As an example could be: nepetolglucosyl ester, 1,5,9-epideoxyloganic acid, nepetariaside, velpetin, nepetanudoside A, nepetanudoside B, nepetanudoside C, 1,5,9-epideoxyloganin and etc. Futhermore, other monoterpenes are: monoterpenic g-lactones, dihydroiridodial diacetate iridodial dienol diacetate, argolic acid A and etc. Other group of compounds that consist in genus *Nepeta* is sesquiterpenes. Just two of them were identified (furoeudesmanes, nehipetol and nehipediol). One more group of terpenoids, which have been identified in *Nepeta* species are diterpenes. Abietane-type diterpenes (teideadiol and teidic acid), abietanes (coleon U 12-methyl ether and its 15,16-dehydro analogue), isopimarane-type diterpenes, (parnapimarol), isopimarane and nepetalactone derivatives, clerodane-type diterpenes and etc. The last group of terpenoids, which have been identified in *Nepeta* species, are triterpenes.

Ursolic acid, triterpenes with a lupine-type skeleton, oleananes, oleanolic acid, pentacyclic triterpenoid coupled with fatty acid moiety and triterpene-nepetalic acids belong to this group [5].

Flavonoids are the second main group of compounds identified in genus *Nepeta*. 26 flavone derivatives (apigenin, apigenin 7-O-β-D-glucuronide, luteolin, acacetin, salvigenin and etc), 9 flavonol derivatives (kaempferol, quercetin, astragalin and etc.), 1 flavanol ((-)-epicatechin), and 1 flavanone (eriodictyol), were isolated from different *Nepeta* species. It must be highlighted that the presence of flavones with a 5-hydroxy-6,7-dimethoxysubstituted A-ring (as found in cirsimaritin and salvigenin) and the unusual 5,8-dihydroxy-6,7-dimethoxy-substituted A-ring (as found in 8-hydroxycirsimaritin, 8-hydroxysalvigenin, and 8-hydroxycirsiliol, also phenolic acid rosmarinic acid appear to be a characteristic chemotaxonomic feature typical of the genus *Nepeta*. Moreover, 22 phenolic derivatives have been isolated from *Nepeta* species (ferulic acid, p-coumaric acid, caffeic acid, rosmarinic acid, chlorogenic acid and etc.) [5].

There are two chemotypes of essential oil identified in *Nepeta* species. The first one is the nepetolactone chemotype and the second one is the 1,8-cineole and/or linalool chemotype (produce an essential oil with mildy menthol-like odor). The essential oil composition in *Nepeta* species differ because of variety, growing site, climatic conditions, and analysis method [5].

Moreover, 8 other type of compounds were identified in genus *Nepeta*: (6S,9S)-roseoside, icarisides B2 and B1, one benzene derivative, a long-chain ketone, crassifone, nepetonic acid and 2 phenolic derivatives [5].

Chemical composition of investigated plant species:

It needs to be mentioned, that *Nepeta cataria* is the most studied species in *Nepeta* genus. This plant contains various types of compounds: 4αα,7α,7αα-nepetalactone, 4αα,7α,7αβ-nepetalactone, 4αβ,7α,7αα-nepetalactone, 4αα,7β,7αα-nepetalactone, 4α-dihydronepetalactone, 4β-dihydronepetalactone, 5,9-dehydronepetalactone, nepetalic acid, iridomyrmecin, isoiridomyrmecin, 1,5,9-epideoxyloganic acid, nepetariaside, nepetaside, ursolic acid, oleanolic acid, 7-O-methylapigenin (genkwanin), luteolin, 6-methoxy-7-methylapigenin (cirsimaritin), salvigenin, isothymusin, leucanthogenin, (-)-epicatechin, eriodictyol, ferulic acid, caffeic acid, gallic acid, caffeoyltartronic acid, nepetoidin A, nepetoidin B, vanillic acid, syringic acid, cinnamic acid, myricetin, hesperidin, hyperoside, sinapic acid, quercetin, kaempferol, rosmarinic acid, syringic acid O-hexoside, hydroxybenzoic acid O-hexoside, luteolin, apigenin, acacetin, chrysin [5, 161, 162].

Chemical compositon of *N. racemosa*: 4αα,7α,7αα-nepetalactone, 4αα,7α,7αβ-nepetalactone, 4αα,7α,7αβ-nepetalactone, nepetaracemoside A, nepetaracemoside B, syringic acid, syringic acid O-hexoside, protocatechuic acid, hydroxybenzoic acid O-hexoside, vanillic

acid, aesculin, cinnamic acid, caffeic acid, rosmarinic acid, ferulic acid, luteolin, apigenin, acacetin, rutin, quercetin [5, 161].

Chemical composition of *N. sibirica*: 4αα,7α,7αα-nepetalactone, 4αα,7α,7αβ-nepetalactone, 7-O-methylapigenin (genkwanin), 6-methoxy-7-methylapigenin (cirsimaritin), isothymusin, syringic acid, syringic acid O-hexoside, protocatechuic acid, hydroxybenzoic acid O-hexoside, vanillic acid, aesculin, cinnamic acid, caffeic acid, rosmarinic acid, ferulic acid, luteolin, apigenin, acacetin, rutin, quercetin, kaempferol [5, 161].

Chemical composition of *N. nuda*: 4αα,7α,7αα-nepetalactone, 4αα,7α,7αβ-nepetalactone, 4αβ,7α,7αβ-nepetalactone, 4αβ,7α,7αα-nepetalactone, 4αα,7β,7αβ-nepetalactone, iridomyrmecin, isoiridomyrmecin, genkwanin, cirsimaritin, isothymusin, 8-hydroxysalvigenin, rosmarinic acid, luteolin, apigenin, acacetin, quercetin, syringic acid, syringic acid O-hexoside, hydroxybenzoic acid O-hexoside, vanillic acid, caffeoylquinic acid, aesculin, cinnamic acid, caffeic acid, ferulic acid, protocatechuic acid [5, 161].

Chemical composition of *N. melissifolia*: gallic acid, gentisic acid, caffeic acid, p-coumaric acid, vanillic acid, syringic acid, ferulic acid, p-hydroxybenzoic acid, quercetin, apigenin, myricetin, rutin, (+) catechin (hydrated) [163].

Chemical composition of *N. grandiflora*: 4αα,7α,7αβ-nepetalactone, 4αβ,7α,7αα-nepetalactone, 1,5,9-epideoxyloganic acid, 1,5,9-epideoxyloganin, 6'-O-(acetoacetyl)-1,5,9-epideoxyloganic acid, 2'-O-methyl-1,5,9-epideoxyloganic acid, 4'-O-methyl-1,5,9-epideoxyloganic acid, 6'-O-methyl-1,5,9-epideoxyloganic acid, ursolic acid, cirsimaritin, isothymusin, 8-hydroxysalvigenin, leucanthogenin, quercetin, rutin, syringic acid, syringic acid O-hexoside, hydroxybenzoic acid O-hexoside, vanillic acid, caffeoylquinic acid, aesculin, cinnamic acid, caffeic acid, rosmarinic acid, ferulic acid, luteolin, apigenin, acacetin, kaempferol [5, 161].

1.10. *Chemical composition of Paeoniaceae family*

According to the literature, over the past six decades 262 compounds were identified in *Paeoniaceae* family, including all parts of the plant (roots, stems, leaves, seeds and flowers). These compounds can be assigned to seven different groups: monoterpenoid glucosides, flavonoids, tannins, stilbenes, triterpenoids and steroids, paeonols, and phenols [4].

To date, 69 monoterpenoid glucosides were identified from *Paeonia* plants. For example, peaoniflorin and its derivatives, paeoniflorigenone, nepetalactone, paeonilide, paeonisuffrone and etc. There are 42 flavonoid compounds, which have been isolated from the plants of *Paeoniaceae*. These compounds there identified mainly from flowers and can be divided into: anthocyanidins (pelargonidin, cyanidin 3-glucoside), flavones (apigenin, luteolin), flavonols (kaempferol,

quercetin), chalcones (isosalipurposide, chalcone), flavanone (liquiritin apioside) and flavan-3-ol (catechin). Tannins were isolated mostly from the fruits. For example, gallotannins (mudanoside B, 4-O-galloylquinic acid, 1,2,6-tri-O-galloyl-β-D-glucose, tannic acid) with and without glucose moieties, ellagitannins (paeonianin B, pedunculagin) and condense tannin ((-)-epigallocatechin gallate). The nine stilbene derivatives ((E)-resveratrol, gnetin H and etc.) were identified only in *Paeonia* plants seeds. In different parts (roots, rhizomes, leaves, flowers, root cortex and callus tissues) of *Paeonia* plants 33 triterpenes were identified (two of them are tetracyclic (palbinone) and the rest of them are pentacyclic (mudanpinoic acid, paeonenoide A) triterpenoids) and four sterols (campesterol, b-sitosterol and etc.). Ten paeonols were isolated from *Paeonia* plant. For example: suffruticoside A – E, paeonolide, paeonol, paeonoside, apiopaeonoside and 3-hydroxypaeonol. Furthermore, 39 phenols and their analogues were detected in this family. These compounds were found mostly in the roots or root cortex. Some of them are distributed widely in *Paeonia* plants, such as gallic and benzoic acids, 2-phenylethanol. Moreover, 29 other kind of compounds such as fatty alcohols, sugars, benzamide, fatty acids, anthraquinone and etc., were isolated from this family. It could be highlighted, that paeoniflorin is chemotaxonomic marker of family *Paeoniaceae* [4].

Compounds isolated from *Paeonia officinalis* can be divided in two main groups: compounds isolated from root oil (saponifiable and unsaponifiable lipids) and compound isolated apart from that the root (stems, leafs, flowers etc.). Saponifiable lipid has been reported to contain stearic, octanoic, palmitic, decanoic, lauric, linoleic, palmitoleic, myristic, myristoleic, and oleic acid. While unsaponifiable lipid contain: campesterol, sitosterol, butyrospermol, C14-33n-alkanes, cycloartenol, lupeol, 24-methylenecycloartanol, and cholesterol. Compounds founded in other parts of the plant contains: triterpenoids, benzoic acid, asparagine, tannic acid, flavonoids, protoanemonin, paeonin, paeonol, paeoniflorin and volatile oil [160].

1.11. *Biological activity of genus Nepeta*

Species of the genus *Nepeta* have a number of pharmacological and biological activities mainly attributed to nepetalactone. People have used them for decades in folk medicine to treat headaches, fever, toothache, but also in cooking. Alcoholic extracts of some *Nepeta* species are used in rheumatic, gastrointestinal and respiratory illnesses (colic, diarrhea, asthma, bronchitis) [5].

Antinociceptive, Analgesic, and Anti-Inflammatory Activities. The essential oil of *N. caesarea* works as analgesic, by blocking the binding of naloxone for opioid receptors. This biological activity of essential oils *N. caesarea* can be attributed to 4αα, 7α, 4αα-nepetalactone, which represents the main constituent (95%) of the essential oil of the species. In addition, the oil

rich in 1,8-cineole (47.02%) and a-pinene (9.99%) of *N. italic* also, shown analgesic activity by blocking the binding of naloxone for kappa opioid receptors. Furthermore, was reported that extracts of *N. sibirica* and *N. grandiflora* possess antinociceptive activity. The methanol extract of *N. sibthorpii*, that contains ursolic acid and a polyphenol fraction consisting of chlorogenic acid and the flavonoids rutin, luteolin-7-O-glucoside and a luteolin derivative shown anti-inflammatory activity against carrageenan-induced oedema. Another study of *N. sibthorpii* shown that essential oil rich in 4αα,7αα,7αβ-nepetalactone and methanol extract, which contained rutin, ursolic acid epinepetalactone, luteoline derivatives and chlorogenic acid showed anti-inflammatory activity, together to neuropharmacological effects. Finally, the essential oil of *N. glomerata* showed anti-inflammatory activity against inflammation mediators such as NO [5].

Cytotoxic Activity. Cytotoxicity of *N. glomerata* essential oil was studied against C32 (C32 amelanotic melanoma) and ACHN (renal adenocarcinoma) cancer cell lines. The oil was more active against ACHN, then C32. The human bronchial epithelial and keratinocyte cell lines were used to test cytotoxic activity of *N. cataria var. citriodora* and *N. cataria* essential oils. Was determined, that oils were cytotoxic to both cell lines [5].

Anti-Atherosclerotic Activity. A very important pharmacological effect of some species of the genus *Nepeta* reflected in the prevention of atherosclerosis, reducing the amount of lipid and lipoproteins. There are commercial pharmacological supplements that contain essential oil and aqueous extract of the flower *N. Hindostan*, which act to reduce cholesterol and triglycerides [5].

Antibacterial, Antifungal, and Antiviral Activities. The essential oil of some species of the genus *Nepeta* have been shown antimicrobial activity. Intensively studied species of this genus *N. cataria* shown expressed antioxidant potential. Methanol and chloroform extracts of *N. nuda* were tested *in vivo* and *in vitro* systems, and it possessed antiviral activity against HSV type 1 and type 2 in MDBK cells [157].Water extracts of *N. Nepetalla, N. coerules* and *N. tuberosa* have antiviral activity on the two different types of DNA and RNA viruses, and demonstrate antiviral effect on viruses such as Herpes simplex type I (HSV-1. In addition, the aqueous extract *N. nepettela* and N. *coerulea* and *N. tuberosa* have effect on Vesicular stomatitis virus. Was determined that the essential oil (with 4αα,7α,7αα-nepetalactone, 4αα,7α,7αβ-nepetalactone, and b-caryophyllene as main components) of *N. cataria* was active against seven fungi and five bacteria. Furthermore, the essential oils of *N. cataria var. citriodora, N. sibirica* and *N. transcaucasica* were showed to possess strong antimicrobiological activity. In another study of antimicrobial activity was determined that essential oil (of *N. cataria)* that consist 4αα,7α,7αβ-nepetalactone, 4αα,7α,7αα-nepetalactone, and 4αα,7β,7αα-nepetalactone as main components, showed exhibited activity against 12 fungi, 11 bacteria and a yeast. In the same study also was tested and MeOH extract of *N. cataria*. MeOH extract shown activity just against 5 bacteria and 7 fungi [5].

Activity on central nervous system. It is shown, that nepetalactone has effect on the central nervous system in mice and rats. Extract containing *N. cataria* nepetalactone, nepetalic acid, thymol, geraniol and citronellol causes drowsiness, lowering blood pressure and affects memory. Essential oil of *N. sibthorpii* leads to shift the usual patterns of behaviour which rodents, while the hydroalcoholic extract of *N. persica* showed the anxiolytic effects of the less sedating and mesmerizing effect from diazepam. The compounds of *N. cataria* causing a unique pattern of behaviour in many species family *Felideae* [5].

Feline-Attractant and Insect-Repellent Activities. Earlier studies of the biological activities of the genus *Nepeta* and nepetalactone including a repellent activity against mosquitoes, cockroaches, flies, worms and ticks. Trans, cis-stereoisomer nepetalactone showed a very toxic effect and stronger repellency to cockroaches of the interaction of cis, trans-stereoisomer, while both stereoisomers nepetalactone showed activity against mosquito repellent. Trans, cis and cis, trans-nepetalactone the pheromones of bees but more the fact exerts trans, cis-nepetalactone [5].

Phytotoxic Activity. The essential oil of some species of the genus *Nepeta* has been shown phytotoxic activity at large the number of weed species and crops, but the mechanism of action has not known. It is shown, that essential oils of genus *Nepeta* have expressed an inhibitory effect on the growth of the aerial parts and roots of seedlings *Lepidium sativum*. It was recently reported that the essential oil of *N. meyeri* leads to oxidative stress in seedlings of some weed species [5].

Other activities. The most studied specie of this genus *N. cataria*, which is also known as catnip, operates as a sex attractant in cats. It has shown, that the behaviour in cats affected by intense cis, trans stereoisomer nepetalactone (4αα, 7α, 4αα) with respect to the trans, cis-nepetalactone (4αα, 7α, 4αβ). Also, was showed myorelaxant and spasmolytic activities of *N. cataria*. It inhibit calcium channels and phosphodiesterase, which may explain its traditional use in diarrhea, asthma colic, and cough [5].

1.12. *Biological activity of genus Paeonia*

Antioxidant Activities. Significant enzyme-inhibition activities against α-chymotrypsin and urease showed ethanol extracts isolated from aerial parts of *P. emodi*. Furthermore, *Paeoniae radix* and Cortex Mountain (CM) extracts can scavegning chemically generated superoxide and hydroxyl radicals. Also, can suppress phenylhydroquinone, which is responsible for Puc18 DNA cleavage. In another study was determined that ethanol extract of Cortex Mountain can suppress oxidative stress in PC12 cell, induced by ROS. In addition, 1,2,3,4,6-penta-O-galloyl-β-D-glucose and galloylpaeoniflorin possess higher antioxidant activity comparing with other compounds, which were isolated from CM and *P. radix* plants. Significant antigenotoxic effect showed ethanol

extract (rich in gallic acid and methyl gallate), which was produced from root of peony. Finally, was reported that *P.lactiflora* can be used as a treatment against neuronal diseases [4].

Antitumor Activities. *Paeoniae Radix* water extract had growth-inhibitory effect on Hep3B- and HepG2-cell lines. 1,2,3,4,6-Penta-O-galloyl-β-D-glucose have inhibitory properties on SK-HEP-1 (hepatocellular carcinoma) cells. Paeonol had properties to enhance the cytotoxicity of chemotherapeutic agents. Moreover, had inhibitory effect on HepG2 (human hepatoma) cell line [158]. Resveratrol (including its dimers and trimmers) isolated from *P. lactiflora* seeds can induced DNA damage and suppressed HL-60 cell proliferation. Also, resveratrol can induced apoptosis in cells and suppressed cytochrome P450 (CYP) 1B1 gene [4].

Antipathogenic Activities against Microorganisms. Methanol extract obtained from *P. suffruticosa* leafs had inhibitory activity against Herpes simplex virus and HIV-1 integrase. In addition, this extract had nematicidal effect. Water extract from the root of *P. lactiflora* showed inhibition properties against *T. rubrum* IFO 5467 and *Trichophyton mentagrophytes* IFO 6202 proliferation. Compounds such as benzoic acid, monoterpenoids and paeonol isolated from root bark of *Paeonia* have ability to control *Tyrophagus putrescentiae*. They also shown activities against acaricides. *P. veitchii* aqueous extracts showed activities against enteroviruses, such as Echo virus 29, Echo virus 9, Polio virus I, Coxsackie virus B3 and Coxsackie virus B5. 1,2,3,4,6-Penta-O-galloyl-β-D-glucose extracted from the root of *P. lactiflora* showed activity against hepatitis B virus. Furthermore, essential oil extracted from *P. mascula* shown antibacterial activity against *Bacillus cereus* and *Yersinia pseudotuberculosis* [4].

Immune-System-Modulation Activities. Total glucosides of peony have influence for humoral immunity, cell immunity, and inflammation in autoimmune diseases (*rheumatoid arthritis* and systemic *Lupus erythematodes).* Cortex Mountain is useful for treatment allergy-related diseases such as atopic dermatitis. Methyl Gallate has anti-inflammatory activity against cyclooxygenase-2/5-lipoxygenase. Paeoniflorin have inhibitory properties against abnormal proliferation of synoviocytes, and the production of GM-CSF, IL-1, VEGF, IL-6, and PGE2 by synoviocytes. Total glucosides of peony have therapeutic effect on arthritis. Because of their ability to ameliorate the metabolism and secretion of synoviocytes. Paeonol had anti-anaphylactic activity due to its ability regulate TNF-a and histamine, also can be used to treat colitis. Furthermore, (-)-epigallocatechin gallate, 1,2,3,4,6-penta-O-galloyl-β-D-glucose and gallacetophenone, had ability to inhibit NO production in macrophages [4].

Cardiovascular-System-Protective Activities. P. anomala extracts shown anticoagulant activity in rats. Cortex Mountain water extract had inhibition properties against platelet thromboxane B2 formation and platelet aggregation. The gallic acid and its esters have anti-cholesterol effect. Cortex Mountain has vasorelaxant properties and its vasorelaxant effect can be

due to presence of pinane glycosides and galloylglucoses. Cortex Mountain also had a significant cardioprotective effect against myocardial ischemia. In the dose-depended manner was showed that total peony glycosides have a protective effect against myocardial injury induced by isoprenaline. Paeonol and paeoniflorin inhibit cardiac-cell apoptosis, thus reducing myocardial damage in rats. Gallotannin extracted from the roots of *P. lactiflora* had endothelium-dependent vasodilator effect on isolated rat aorta. Paeoniflorin can significantly reduce triglyceride, total cholesterol and low-density lipoprotein levels [4].

Central-Nervous-System Activity. Chronic cerebral hypoperfusion induce brain damages, thus could be treat by using paeoniflorin. Furthermore, aging induced cognitive dysfunction and senile dementia can be treat by paeoniflorin. Paeonol possessed an anxiolytic-like effect in mice and can reduced cerebral infarct and neuro-deficit in rats. Paeoniflorin has an analgesic effect against hypersensitivity and nociception. Also, can reduce the infarct volume. In addition, paeoniflorin reduced the 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine induced toxicity, and could be used to treat Parkinsons disease. *P. peregrine* methanol extract from the leaves had antiserotonin and anticholinergic activity. In a dose-dependent manner extract from aerial part of *P. emodi* showed spasmolytic activity. While *P. mascula* ssp. *hellenica*, *P. clusii* ssp. *clusii* and *P. parnassica* root extracts showed anticonvulsant activity. In addition, albiflorin and gallotannin are responsible for anticonvulsant activity of peony roots [4].

Other Activities. Root extract of *P. lactiflora* can be used for inhibition of postprandial hyperglycemia. *P. su*ffruticosa extracts strongly inhibited maltase and sucrose enzymes. Paeonol has inhibitory properties against osteoclastogenesis. 6’-O-β-d-glucopyranosylalbiflorin extracted from *Paeonia lactiflora,* had effect on bone formation in vitro. Paeoniflorin has protective effect against optic nerve crush [4].

2. EXPERIMENTAL PROCEDURE

2.1. *Plant material*

Nepeta cataria L., *Nepeta racemosa* L., *Nepeta sibirica* L., *Nepeta nuda* L., *Nepeta melissifolia* L., *Nepeta grandiflora* M. Bieb. *and Peonia officinalis* were collected from Kaunas Botanical Garden of Vytautas Magnus University (Lithuania). Herbs were harvested by hand during flowering stage dried at room temperature in shade and stored in glass containers in the dark. Herbs were collected:

- *N. cataria* – 2014-07-02;
- *N. racemosa* – 2014-07-09;
- *N. sibirica* – 2014-07-01;
- *N. nuda* – 2014-06-04;
- *N. melissifolia* – 2014-07-01;
- *N. grandiflora* – 2014-07-05;
- *P. officinalis* (leafs) – 2015-05-18;
- *P. officinalis* (roots) – 2013-04-17.

The leaves and roots (only for *Peony*) were grounded in a laboratory mill Vitek (An-Der, Austria) into 0.5 mm particle size and were kept in a cold, dark and dry environment until further analyses.

2.2. *Chemicals and reagents*

Organic solvents used for different extraction methodologies were: n-hexane, acetone and methanol (99.8 %) from Sigma Aldrich (Darmstadt, Germany).

For the *Folin-Ciocalteu* reagent assay: sodium carbonate (Na_2CO_3) was acquired from Sigma-Aldrich (St. Quentin Fallavier, France), *Folin-Ciocalteu* reagent and gallic acid were purchased from Sigma Chemical Co (Darmstadt, Germany).

Chemicals used for antioxidant activity assays were: 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$, 98 %), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$, 98 %), potassium persulfate ($K_2S_2O_8$), 2',2'-Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), picolinic acid ($C_6H_5NO_2$), hydrogen peroxide (H_2O_2), caffeic acid ($C_9H_8O_4$), cobalt (II) fluoride tetrahydrate (CoF_2) and reagent used for sodium phosphate buffer solution (SPB) and phosphate buffer saline solution (PBS) preparation included monopotassium phosphate (KH_2PO_4), potassium chloride (KCl), sodium chloride (NaCl) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \times H_2O$) all from Sigma-

Aldrich (Darmstadt, Germany). Microcrystalline cellulose (20 μm) from Sigma-Aldrich (Steinheim, Germany). Iron chloride ($FeCl_3$) and sodium phosphate dibasic dehydrate ($Na_2HPO_4 \times 2H_2O$) were obtained from Riedel-de-Haen (Seelze, Germany). FL (disodium fluorescein) was from TCI Europe (Antwerpen, Belgium).

Chemicals used for cell-based assays were: 2',7'-dichlorofluorescin diacetate (DCFH-DA), quercetin (95 %) from Sigma-Aldrich (St. Quentin Fallavier, France) and EtOH (99 %) from Scharlau (Barcelona, Spain). All cell culture media and supplements: namely glutamine, trypsin, RPMI 1640, PS (penicillin streptomycin) and fetal bovine serum (FBS) were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK) and PBS used for cells was acquired from Sigma-Aldrich (St. Louis, USA).

Reagents used for chromatography analysis were: formic acid, also HPLC grade and LS-MS grade acetonitrile were obtained from Sigma Aldrich (Darmstadt, Germany). Analytical grade methanol and acetone were purchased from StanLab (Lublin, Poland). Chlorogenic acid was from Sigma-Aldrich (Darmstadt, Germany). Rosmarinic acid from Linegal Chemicals (Warsaw, Poland).

For α-amylase assay were used: potassium iodine (KI) from Riedel-de-Haen (Seelze, Germany), iodine (I_2) and α – amylase, type VI-B: from porcine pancreas from Sigma-Aldrich (St. Quentin Fallavier, France), starch from potatoes was obtained from Fluka (Buchs, Switzerland), and acarbose from Bayer Pharma AG (Leverkusen, Germany).

2.3. *Structure of the research work*

Research work consist of two main parts: preparation of plant extracts and investigation of their chemical composition, as well as antioxidant and bio activities. The principle scheme of the research work is presented in Figure 5. Furthermore, methods used in the research work also can be divided in three main parts:

- assays used to determine antioxidant activity ($ABTS^{\bullet+}$, F-C, $DPPH^{\bullet}$, ORAC, HORAC, HOSC, QUENCHER, HPLC-$DPPH^{\bullet}$);
- assays used to evaluate chemical composition (UPLC);
- assays used to test bioactivity of selected extracts (α-amylase, CAA, antripoliferative activity and cytotoxicity assays).

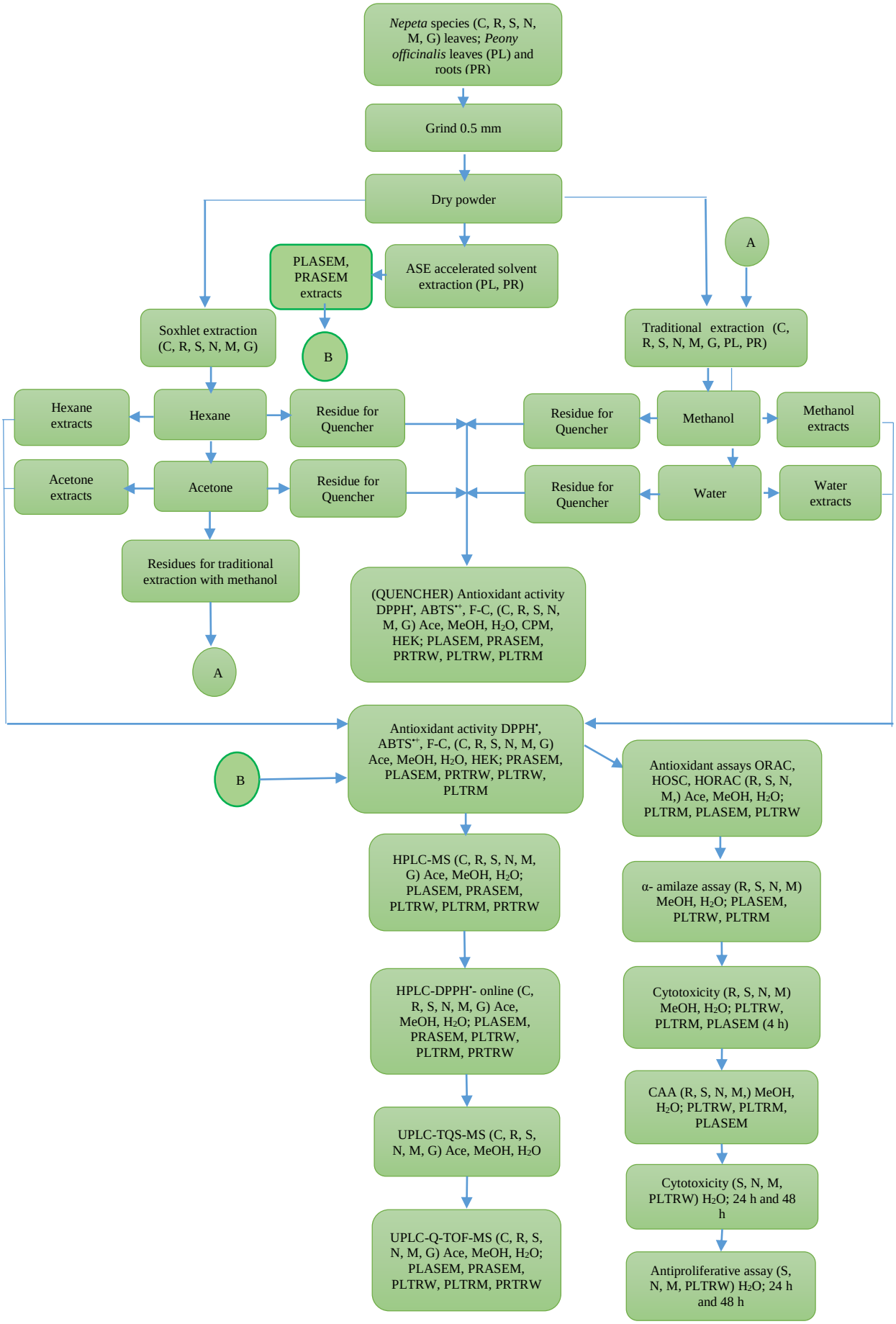


Figure 5. Scheme of the research work.

2.4. *Equipment used in the research work*

Brand, name	*Company, city, country*
ZM 200 laboratory mill	Restsch, Haan, Germany
KERN 770 analytical balance	Goti, Kern&Sohn gmbh., Alkstad, Germany
Dionex, ASE 350 accelerated solvent extractor	Sunnyvale, USA
„Red Rotor“ vortex	Hoefer Pharmcia Biotech Ins., USA
Ultrasonics ultrasound bath	Astra-Son^{TM}, USA
Biuchi R-U4 rotary evaporator	Biuchi labortechnic AG, Konztanz, Switzerland
Automatic pipets: 20-200 µL, 100-1000 µL	CAPP, Denmark
Automatic pipet 1000 µL	Hirshmann Laborgerate, Hirshmann Technocolor, Germany
GENESYS 8, 10 UV spectrophotometer	Spetronic instruments, Rocester, USA
FL800 microplate fluorescent reader	Bio-Tek Instruments, USA
Epoch Microplate spectrophotometer	Bio-Tek, Instruments, Winooski, VT, USA
Lyophilyzer	MAXI DRY LYO, Denmark
Microsyringe	SGE, Australia
Binary pump Waters 1525	Waters, Hilford, MA
Rheodyne 7725i injector	Rheodyne, Rohnert Park, CA
UV detector Waters 996	Waters, Hilford, MA
Water-Micromass ZQ 2000 mass detector	Water, Arlington, MA
Soxhlet apparatus	Behr Labor-Technik, Dusseldorf, Germany
Buchner funnel	-
FLUOstar Omega spectrophotometer	BMG LABTECH GmbH, Ortenberg, Germany
Water aquity system	Waters, Milford, MA, USA
Quadrupole mass detector Maxis 4G	Bruker Daltonic, Bremen, Germany
Microplates	Anicrin, Scorze, Italy

2.5. *Preparation of extracts*

- Soxhlet Extraction

Hexane and acetone extracts of *Nepeta* species were prepared using multistep soxhlet extraction method by Raaman [164]. Grounded leaf material (8 g) in triplicate was extracted with organic solvents such as n-hexane and acetone for 3 h at 70 ºC. After first extraction step thimble with plant material was dried and 1 g of material were taken off and used for quencher approach. The same was done after acetone extraction, respectively. All hexane and acetone extracts were concentrated in a rotary evaporator Rotavapor R-114 (Buchi, Flavin, Switzerland) in vacuum (0.06 MPa) at 40 ºC. Dried extracts were weighed and stored at -18 ºC in freezer until further use.

- Accelerated solvent extraction (ASE)

Solvent extraction of peony leafs was carried out using Dionex-ASE 350 extractor. 10 g of sample and 4 g of diatomaceous earth mixture were placed in an 66 mL stainless steel extraction cell. Samples were heated 5 min at 60 ºC. Two circular filters (30 mm, Thermo Scientific): the first one was placing in the capped end and the second one was placed before capping the cell in order to prevent suspended particles from entering the collection vials. Both extraction cells were placed into ASE carousel for extraction process. Extraction were performed using methanol. Briefly, all extractions were carried out at 6.8 MPa at 60 ºC, with a 15 min static time and a 90 s purge time for each extraction cycle. Solutions after extraction were filtered using a 0.3 µm filter (Filtrac, Niederschlag, Germany) and evaporated to dryness around 40 ºC at reduced pressure (0.06 MPa) using a rotary evaporator Rotavapor R-114 (Buchi, Flavin, Switzerland). And finally dried in a flow of nitrogen (20 min); weighted and stored in freezer at -18 ºC. All experiments were carried out in triplicates.

- Traditional extraction with methanol

Grounded plant material of *Nepeta* species (5.5 g) and 10 g of peony were taken in a conical flasks and extracted with methanol (200 mL) in a mechanical shaker (Sklo Union LT, Teplice, Czech Republic) at room temperature at constant shaking at 170 rpm. It was left for 3 h for *Nepeta* species and 24 h for peony. Before extraction from flasks were removed oxygen by a flow of nitrogen for 5 min to prevent oxidation of the compounds. Solids were filtered using a 0.3 µm filter (Filtrac, Niederschlag, Germany). After filtration the filtrates were then evaporated to dryness at 40 ºC at reduced pressure (0.06 MPa) using a rotary evaporator Rotavapor R-114 (Buchi, Flavin, Switzerland). Finally, dried in a flow of nitrogen (20 min); weighted and stored in freezer at -18 ºC. The extractions were performed in triplicates for *Nepeta* species and in five replicates for peony plant. Solid residues after extraction were collected and used for quencher approach.

- Traditional extraction with water

2 g of grounded plant material of *Nepeta* and 5 g of leafs and 2 g of roots of peony species were suspended in 50 mL of distilled water and were extracted three times; each lasting for 15 min, with continuous stirring (400 rpm) at 80 ºC in two separate days. After extraction all solutions were filtered three times through 0.3 µm filter (Filtrac, Niederschlag, Germany). After filtration extracts were then freezed with liquid nitrogen and lyophilized with freeze dryer Maxi Dry Lyo (Hetto-Holton AIS, Allerod, Denmark). Dried extracts were weighted and stored at -18 ºC in

freezer until further use. The extraction were performed in triplicates. Solid residues after extraction were collected and used for quencher approach.

2.6. *Folin-Ciocalteu Reagent Assay (F-C)*

The total polyphenol content was analysed using the *Folin-Ciocalteu* method of Singleton et al. [165] with some modifications. A volume of 30 µL of the extract sample (1:2, 1:4, 1:8, 1:10, 1:100 (depending on extract)) was mixed with 150 µL 10-fold diluted (v/v) *Folin-Ciocalteu* reagent, and 120 µL of 7 % Na_2CO_3 in a micro-plate well. Micro-plate was placed in to FLUOstar Omega reader (BMG Labtech, Offenburg, Germany) and shaken for 10 s. The absorbance was recorded every minute, totally 30 min at 765 nm. All measurements were performed in twelve replicates. Gallic acid was used as a standard for the calibration curve in the concentration range of 0 – 250 µg/mL. Polyphenol content was expressed in mg of gallic acid equivalents (GAE) in g of extract. F-C was calculated by the following formula:

$$C = \frac{c \times V}{m}; \quad (5)$$

where: C is concentration of the total phenolics, in GAE (mg/g); c is the concentration of gallic acid, determined from the calibration curve (mg/mL); V is the volume of plant extract (mL); m is the weight of pure plant extract (g).

2.7. *DPPH• Scavenging Activity Assay*

The stable 2,2-diphenyl-2picrylhydrazyl (DPPH•**)** radical was used to measure the free radical scavenging activity with some modifications [113]. Stock solutions were prepared for each extract sample (10 mg of extract was diluted in 1 mL of methanol). From stock was prepared series samples at various concentrations (1:1.25, 1:2, 1:3.3, 1:5, 1:10 (v/v)) in MeOH/H_2O (1:4). Diluted extract sample 8 µL and 292 µL of 0.06 mM methanolic DPPH• solution were pipetted in to 96 well FLUOstar Omega micro-plate reader (BMG Labtech, Offenburg, Germany) set at 515 nm for 60 min. The absorbance was measured every min until the end point (60 min). Measurements were conducted in quadruple. Trolox solutions in the concentration range 299 – 699 µM/L were used for the calibration curve. The final results were expressed in µM Trolox equivalents/g of extract. Radical scavenging capacity was calculated by using the following formula:

$$I = \frac{A_B - A_A}{A_B} \times 100; \quad (6)$$

where: I is DPPH• inhibition, %; A_B is the absorbance of a blank sample (t=0 min); A_A is the absorbance of extract solution (t=60 min).

2.8. *$ABTS^{\bullet+}$ Decolourization Assay*

$ABTS^{\bullet+}$ radical cation assay by Re, et al. (1999) was also used to test the radical scavenging ability of extracts [166]. Briefly, in 1 L of distilled water were dissolved 1.78 g Na_2HPO_4 x $2H_2O$, 0.27 g KH_2PO_4, 8.18 g NaCl and 0.15 g KCl in order to prepared phosphate buffered saline (PBS) solution; if solution pH was lower or higher than 7.5 it was adjusted with NaOH or HCl, respectively. The radical cation was produced by reacting 50 mL of $ABTS^{\bullet+}$ stock solution with 200 µL of potassium persulfate and leaving the mixture to stand in the dark at room temperature for 14 – 16 h before use. FLUOstar Omega micro-plate reader (BMG Labtech, Offenburg, Germany) was used for measuring the absorbance of working solution. To obtain the absorbance of 0.800 ± 0.020 at 734 nm working solution of $ABTS^{\bullet+}$ was diluted with PBS and 294 µL of it were pipetted with 6 µL methanolic solution of extract (1:1, 1:2, 1:5, 1:10 v/v depending on extract) in to the micro-plate wells. As a blank was used PBS solution. The absorbance was read every min, totally 30 min. The calibration curve was prepared by using a series of Trolox solutions in the concentration range 399-1198 µM/L. The results were expressed as Trolox TEAC value (µM TE/g of extract). The results were performed as a mean of six replicates and calculated by using the following formula:

$$I = \frac{A_D - A_C}{A_D} \times 100; \quad (7)$$

where: I is $ABTS^{\bullet+}$ inhibition, %; A_D is the absorbance of a blank sample (t=0 min); A_C is the absorbance of extract solution (t=30 min).

2.9. *Oxygen Radical Absorbance Capacity (ORAC) Assay*

ORAC assay was performed essentially as described by Huang *et al.* (2002) by using fluorescein as a fluorescent probe [167]. The experiment was based on ability of the antioxidant species in the sample to inhibit the oxidation of disodium fluorescein catalysed by peroxyl radicals, which were generated from AAPH.

Briefly, 25 µL of sample/Trolox standards and 150 µL of fluorescein solution ($2x10^{-7}$ mM) were pipetted to the black 96-well microplate. Afterward, the microplate was incubated in fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 37 ºC for 10 min. After incubation 25 µL of AAPH (153 mM) were added to each of the well containing standards/samples and blank (PBS) automatically through the injector coupled with microplate reader. Measurements were recorded every 1 min at 37 ºC, totally 60 min. Emission and excitation wavelength were 530 ± 25 nm and 485 ± 20 nm, respectively. The extract sample were diluted: first in respective organic solvents and the following dilution also AAPH and fluorescein solutions

were prepared daily in PBS (75 mM, pH 7.4). Trolox concentrations prepared in PBS in rage of 5 – 40 µM were used for the calibration curve. ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the curve (AUC). The results were presented as Trolox equivalent antioxidant capacity TEAC (µM Trolox equivalents/g of extract) and were expressed as a mean of four replicates.

The AUC and Net AUC were calculated using the following formulas:

$$AUC = 0.5 + \frac{f_1}{f_0} + \cdots + \frac{f_i}{f_0} + \cdots + \frac{f_{39}}{f_0} + 0.5\ (\frac{f_{40}}{f_0}) \quad (8)$$

$$Net\ AUT = AUC_{sample} - AUC_{blank} \quad (9)$$

where: f_0 initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

2.10. *Hydroxyl Radical Adverting Capacity Assay (HORAC)*

The hydroxyl radical adverting capacity method was performed as described by Ou et al. (2002) [129]. This assay is using fluorescein as a probe to evaluate the hydroxyl radical prevention capacity of the sample.

Briefly, 30 µL of appropriate sample dilutions/trolox standards/blank and 150 µL of fluorescein solution (9.28×10^{-8} M) were added to a black 96-well microplate. Then, 40 µL of H_2O_2 (0.1990 M) were pipetted to each 60 wells of the micropate and to the rest 36 wells were added distillate water to prevent sample evaporation. Then, 60 µL of CoF_2 (3.43 mM) was added to start the reaction. Finally, microplate was placed in fluorescein microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 37 ºC using excitation (485 ± 20 nm) and emission (530 ± 25 nm) fluorescence filters. Data was recorded every 1 min during 60 minutes. The calibration curve was prepared by using caffeic acid standard dilutions (50, 100, 150, 200 and 250 µM) in acetone:Milli-Q water (50:50 v/v). Also, as a blank and for the sample preparation were used acetone:Milli-Q water (50:50 v/v). H_2O_2 and fluorescein solutions were prepared in sodium phosphate buffer (SPB), 75mM, pH 7.4. CoF_2 solution was prepared in Milli-Q water. The HORAC units were expressed as µM of caffeic acid equivalents antioxidant capacity per g of extract (µM CAE/g of extract), and were expressed as a mean of four replicates. The AUC was calculated as:

$$AUC = 0.5 + \frac{f_1}{f_0} + \cdots + \frac{f_i}{f_0} + \cdots + \frac{f_{59}}{f_0} + 0.5\ (\frac{f_{60}}{f_0}) \quad (10)$$

$$HORAC\ value = \left[\frac{AUC_{sample} - AUC_{blank}}{AUC_{cofeic\ acid} - AUC_{blank}}\right] \times (\frac{molarity\ of\ coffeic\ acid}{concentration\ of\ sample}) \quad (11)$$

where: f_0 initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

2.11. *Hydroxyl Radicals Scavenging Capacity (HOSC) Assay*

The HOSC assay was performed according to Moore, Yin, and Yu (2006) using the FL800 microplate fluorescence reader [130]. In this method as a probe was used fluorescein and as a source of hydroxyl radicals – classic Fenton reaction with Fe (II) and H_2O_2. This method evaluate the hydroxyl scavenging capacity of the sample.

Briefly, 170 µL of fluorescein solution (9.28×10^{-8} M), 30 µL of appropriate sample dilutions/trolox standard/blank and 40 µL of H_2O_2 (0.1990 M) were pipetted to a 96-well microplate. In outer of the microplate wells were added water to prevent evaporation of the sample. Finally, 60 µL of $FeCl_3$, 3.43 mM, were added rapidly to the wells of the microplate to start the reaction by using multichannel pipette. Afterward, microplate was placed in fluorescein microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at 37 ºC by using excitation (485 ± 20 nm) and emission (530 ± 25 nm) fluorescence filters. Data was recorded every 1 min during 60 minutes. SPB (75 mM, pH = 7.4) was used to prepare the solution of fluorescein, while hydrogen peroxyde (H_2O_2) and $FeCl_3$ solutions were prepared with Milli-Q water. To create the calibration curve Trolox standard dilutions (5, 10, 15, 20, 30 µL) in acetone:Milli-Q water (50:50 v/v) were used. As a blank and prepared sample dilutions, solution of acetone:MilliQ water (50:50 v/v) was used. Data was expressed as µM of Trolox equivalents per g of extract (µM TE/g of extract). The results were performed as a mean in four replicates.

2.12. *Antioxidant activity evaluation of solid material (QUENCHER procedure)*

Then samples have strong antioxidant activity, solid fraction are mixing with microcrystal cellulose. For investigation was prepared several concentration solid material of plant and cellulose mixtures in relation to (1:5, 1:10, 1:20, 1:40, 1:50, 1:80, 1:100), it depends on searching system.

For $ABTS^{\bullet+}$ method was weighted 5 mg of sample (plant material/cellulose), and mixed with 40 µL of PBS. To start the reaction 1.96 mL of the $ABTS^{\bullet+}$ solution was added. Then, mixture was vortexed for 1 min, and shacked for 30 min (250 rpm) in the dark, then centrifugated at 4800 rpm for 3 min, and 300 µL transparent solution were placed to the microplate. $DPPH^{\bullet}$ scavenging method has been done similar to $ABTS^{\bullet+}$ method. Data of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ were expressed mmol/g of dry plat weight.

F-C assay, 5 mg of sample was diluted in 150 µL of MeOH/H2O (1:4), 750 µL of *Folin-Ciocalteu* reagent and 600 µL Na_2CO_3. Prepared mixture was mixed 15 s and shacked for 3 h (250 rpm) in the dark, then centrifugated at 4500 rpm for 10 min. After all, 300 µL of transparent solution was added to the 96-well microplate. Data was expressed mg/g of dry plant material.

2.13. *α-amylase Inhibitory Activity by Iodine-starch Assay*

This assay was performed using iodine-starch procedure of Al-Dabbas et al. (2006) [168]. In this method α-amylase was incubated with substrate (starch solution) and sample/α-amylase inhibitor. The capacity of the sample to inhibit the breakdown of starch was determined by the addition of iodine solution, which reacts with the starch producing a purple-black colour.

Briefly, 60 µL of PBS (negative control)/appropriate sample dilutions/acarbose (positive control, 0,02 mg/mL) and 200 µL of starch solution (400 µg/mL) were added to 6 eppendorfs, and incubated at 37 ºC during 5 min. Then, reaction was initiated by 20 µL of α-amylase (50 µL/mL) which was added to 3 eppendorfs and to the rest of 3 – 20 µL of phosphate buffer (pH=7.4) as a control of the sample. Before last incubation at 37 ºC for 7.5 min to all eppendorfs 20 µL of phosphate buffered saline (pH 7.4) solution were pipetted. After incubation, to determine degradation of the starch, 200 µL of iodine solution was added. The reaction mixture was diluted with 1 mL of distillated water and the absorbance was measured at 660 nm using Thermo Scientific GENESYS 10 Spectrophotometer (Thermo Scientific, USA). To evaluate influence of solvent instead of negative control (PBS) a control with solvent (methanol) was used. Stock solution (5 mg/mL) of acarbose in PBS was used to made dilutions for the calibration curve in order to determinate IC_{50} of the acarbose. Experiments were performed in triplicates Calculations were performed using the following formulas:

Subtract the absorbance value of the blank/sample with enzyme to the absorbance value of the blank/ sample without enzyme:

$$\Delta_{Abs} = Abs_{without\ enzyme} - Abs_{with\ Enzyme}; \quad (12)$$

Percentage inhibition (%) was calculated by the expression:

$$\%\ Inhibition = \left[\frac{Abs_{control} - Abs_{extracts}}{Abs_{control}}\right] \times 100; \quad (13)$$

Concentration of extracts resulting 50 % inhibition of enzyme activity (IC_{50}) were determined graphically.

2.14. *Cell culture*

Human colon cancer cell lines, HT29 and Caco2, were obtained from ATCC (USA) and DSMZ (Braunschweig, Germany), respectively. Both cell lines were grown in RPMI 1640 medium supplemented with 10 % FBS, 2 mM glutamine and 1 % Penicillin-Streptomycin (PS) for Caco-2 and 0.5 % for HT29. Stock of cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37 ºC with 5 % CO_2 in a humidified atmosphere.

2.15. *Cytotoxicity Assay*

The assay was performed as described by Sambuy et al., (2005) [169]. This method was accomplished using confluent Caco2 cells, which are a good model of the intestinal barrier [169].

Briefly, Caco2 cells were seeded at a density 2×10^4 cells/well in transparent 96 well plates and the medium (RPMI + 10 % FBS + 1 % PS) was changed every 48 hours. The experiment were carried out after one week, while cells formed monolayer. In the day of the assay, the medium was removed and differentiated Caco2 cells were incubated 4, 24 and 48 hours with 100 µL series dilutions of extracts (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128). After 4, 24 or 48 hours of incubation extracts were removed and cells were washed with PBS. To determine viability of the cells 100 µL of MTS (preparation of MTS: first dilution is 1:10 in RPMI + 0.5 % FBS. Then 16 % of that was pipetted to a final solution of RPMI + 0.5 % FBS) reagent to each well with cells was added, and incubated for 2 h 30 min at 37 ºC in 5 % CO_2 atmosphere. Afterward, plates were read with Epoch Microplate Spectrophotometer (Bio-Tek, Instruments, Winooski, VT, USA) at 490 nm wavelength. Sample dilutions and MTS reagent were prepared in (RPMI + 0.5 % fetal bovine serum) medium. Controls were performed in RPMI medium (RPMI medium + solvent (water or ethanol). Stock solution of the extracts were prepared in water and ethanol (EtOH). Data was expressed in terms of percentage of cellular viability relative to control (%). Experiments were performed in triplicates.

2.16. *Cellular Antioxidant Activity (CAA) Assay*

This method evaluates the antioxidant capacity of a sample/compound at a cellular level using dichlorofluorescein (DCFH-DA) as a probe. Cells are pretreated with antioxidant compounds or natural extracts and DCFH-DA (non -fluorescent compound). The antioxidant bond to the cell membrane and/or pass through the membrane to enter the cell. DCFH-DA diffuses into the cell where cellular esterases cleaved the diacetate moiety to form more polar DCFH (non -fluorescent compound), which trapped within the cell. Cells are treated with AAPH, which is able to diffuse into cells and spontaneously decomposed to form peroxyl radicals. These peroxyl radicals attack the cell membrane to produce more radicals and oxidized the intracellular DCFH to the fluorescent DCF. Antioxidants prevent oxidation of DCFH and membrane lipids and reduce the formation of DCF [170].

Briefly, Caco2 cells were harvested from the cell culture flask and inoculated in a 96-well plates at a density 2×10^4 cells/well and the medium was changed every 48 h. CAA experiments were performed in confluent of Caco2 monolayers (after one week). In the day of the experiment medium was removed and cells were washed with pre-wormed PBS (10 mM, pH=7.4, ~37 ºC)

twice. Then, to each inner 60 wells were added 50 µL of PBS/ quercetin standards (2.5, 5, 10, 15, 20 µM)/sample dilutions (prepared in PBS) and 50 µL of DCFH-DA solution (50 µM). Then microplate was pre-incubated at 37 ºC, 5 % of CO_2 humidified incubator for 1 hour, after which 100 µL of AAPH (12 mM) solution was added to the wells containing PBS/quercetin standards/samples. For blank wells were added 100 µL of PBS. Fluorescence intensities were measured (excitation filter 485/20 nm; emission filter 528/20 nm) every 5 min for one hour using a FL800 mocroplate fluorescent reader (Bio-Tek Instruments, USA). Data acquisition was performed by Gen5 software. CAA of samples were quantified according to Wolfe and Liu (2008) [173]. CAA value at each concentration of the sample was integrated the area under the curve for fluorescence versus time. The results were calculated by the following formula:

$$CAAunit = 1 - \left(\frac{\int SA}{\int CA}\right) \quad (14)$$

$\int$SA is the integrated area under the sample fluorescence versus time curve and $\int$CA is the integrated area of the control curve. The median effective concentration (ED_{50}) was determined for the sample from the median effect plot of log (fa/fu) versus log (dose), where fa is the fraction affected (CAA unit) and fu is the fraction unaffected (1-CAA unit) by the treatment. ED_{50} were converted to CAA values, expressed as µM QE/g of sample, using the mean ED_{50} value for quercetin in triplicates from three independent experiments.

2.17. *Antiproliferation Assay*

Antiproliferative assay were performed as previously described by Serra, Duarte, et al. (2011) [171]. Briefly, cells were seeded in 96-well microplates at a density of 1×10^4 cells/well and incubated for 24 hour at 37 ºC in 5 % CO_2 atmosphere. After incubation time the medium of each well was removed and cells were incubated with peony and *Nepeta* samples diluted in medium (RPMI + 0.5 % FBS). After 24 and 48 h the extracts were removed; washed with PBS and the cell viability was determined using the kits described in 2.15 subsection. The results were expressed in terms of percentage of cellular viability relative to control (%) and ED_{50} (effective dose), was calculated too. Experiments were performed in triplicates.

2.18. *HPLC-UV- DPPH• online - MS analysis*

Analysis were performed using an on-line method, when DPPH• free radical reacts with radical scavenging components at 515 nm absorbance due to reaction were recorded as negative peaks. Figure 6 shows DPPH• online principle scheme.

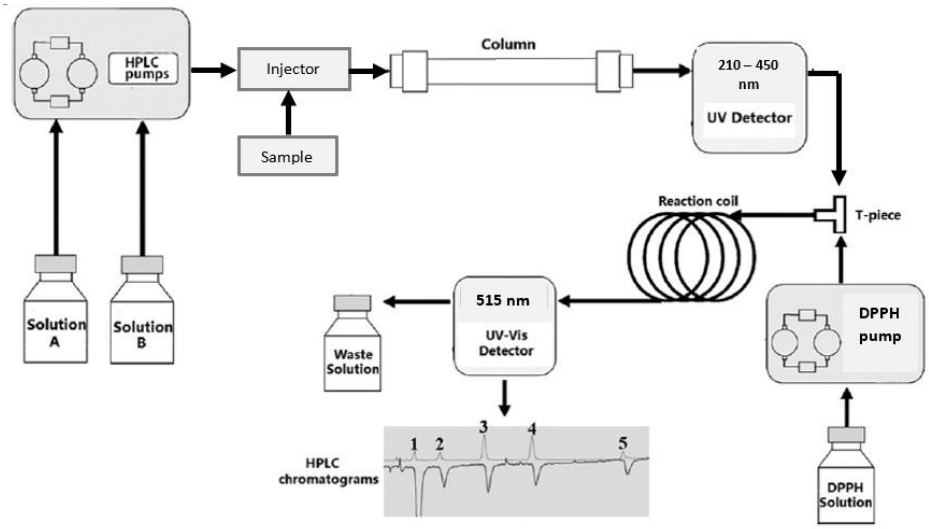


Figure 6. Principle scheme of the HPLC – UV – DPPH• online detection [185].

Experiment was performed using Waters 1525 binary pump (Milford, USA), Rheodyne 7125 manual injector (Rheodyne, RohnertPark, CA) with 20 µL injection loop and Hypersil ODS C_{18} (250 × 4.6 mm, 5 µm) column (Thermo scientific, USA). Column temperature was kept at 40 ºC. Waters 996 photodiode array detector (Milford, USA) was used to detect compounds eluted from the column in the range 210 – 450 nm. A binary pump Aligent 1100 series (Aligent Technologies, Inc. Santa Clara, CA, USA) was used to mix and introduce DPPH• (5×10^{-6} M) solution into a 15 m (0.25 mm ID) reaction coil, which was made of PEEK (polyetheretherketone) tubing (Interchim, Frankfurt, Germany). Finally, decrease of absorbance was detected photometricaly with variable-wavelength Shimadzu SPD-20A UV detector (Shimadzu Corporation, Kyoto, Japan). DPPH• solution was prepared in methanol and kept protected from light. Separations were carried out at a flow rate of 0.8 mL/min for the mobile phase, and 0.6 mL/min for the DPPH• solution. Mixture of two eluents: A (0.4 % for *Nepeta spp*. and 1 % for peony formic acid in water) and B (100 % methanol) was used as the mobile phase. The following eluent gradient is shown in table 1. Extracts were dissolved in methanol water (50:50, v/v) solution and used directly before analysis. For the identification of phenolic compounds, the HPLC system was coupled to a quadrupole mass detector Micromass ZQ (Waters, Milford, USA) scanning from 100 to 1200 *m/z* and operating in ESI negative mode at capillary voltage of + 3000 V. Other parameters were: desolvation gas flow 310 L/h, desolvation temperature 300 ºC, cone gas flow 80 L/h, cone voltage 30 V, source temperature 120 ºC. The injection volume and the flow rate were 20 µL and 1 mL/min, respectively. Standard solutions of rosmarinic and chlorogenic acids were prepared 1 mg/mL and 1.25 mg/mL in methanol, respectively.

Table 1. Gradient of the mobile phase for *Nepeta* and *Paeonia spp.* extracts

Gradient for all *Nepeta* species			Gradient for *Paeonia* officinalis		
Time, min	**Eluent A, %**	**Eluent B, %**	**Time, min**	**Eluent A, %**	**Eluent B, %**
1	**2**	**3**	**4**	**5**	**6**
0	95	5	0	95	5
2	85	15	2	90	10
40	75	25	40	78	22
50	0	100	50	0	100
55	0	100	55	0	100
60	95	5	60	95	5

2.19. *UPLC/ESI-QTOF-MS analysis*

Analysis was carried out using Waters Acquity UPLC system (Milford, MA) with: sample manager, binary solvent manager, PDA detector and MaXis QTOF MS (the quadrupole - time of flight mass spectrometer) (Bruker Daltonic, Bremen, Germany), which was equipped with an electro spray ionization mode and controlled by HyStar software (Bruker Daltonic, Bremen, Germany). For the analysis Acquity C_{18} column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, USA) was used and the separation temperature was 25 ºC.

Table 2. Gradient of the mobile phase for *Nepeta spp.* and *Paeonia* extracts.

Gradient for all *Nepeta* species			Gradient for *Paeonia* officinalis		
Time, min	**Eluent A, %**	**Eluent B, %**	**Time, min**	**Eluent A, %**	**Eluent B, %**
1	**2**	**3**	**4**	**5**	**6**
0	95	5	0.5	100	0
9	80	20	0.7	85	15
12	50	50	1.7	80	20
14	0	100	9	0	100
15	0	100	10	0	100
16	5	95	11	100	0
17	5	95	12	100	0

The mobile phase was consisted of eluent A (1 % and 0.4 % formic acid in ultrapure water for peony and Nepeta, respectively) and eluent B (100 % acetonitrile). The gradient elution used for the analysis is shown in table 2. The flow rate was 0.4 mL/min for Nepeta and 0.45 mL/min for peony extracts, the injection volume was 2 µL (10 mg of extract was dissolved in 1 mL of methanol and diluted 1:10 in methanol before injection). The negative ion mode was performed at +4.0 kV capillary voltage, with the end plate offset at - 500 V, collision cell energy 35 eV. The

absorbance wavelength was in the range of 220 – 500 nm. Other parameters were: nitrogen gas pressure 2.5 bar and flow rate 10.0 L/min. For the drying and nebulizing nitrogen was used. Masses for the detection were in range 79 – 1200 m/z and 79 – 2400 m/z for Nepeta and peony extracts, respectively. Eluted compounds were identified based on their peak retention time, compared with corresponding reference compounds, MS fragmentation patterns and accurate masses in public databases.

2.20. *Quantitative UPLC-MS analysis*

Analysis was performed using UPLC system with Xevo TQ-MS triple quadrupole mass detector (Waters, Corp., Milford, MA, USA) equipped with an electrospray ionization source (ESI) and Acquity C_{18} column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, USA). Also, Waters Acquity UPLC H class autosampler (which maintained at 10 ºC) and quaternary pump were used.

Table 3. Gradient of the mobile phase for *Nepeta spp*. extracts

Time, min	Eluent A, %	Eluent B, %
1	**2**	**3**
0	85	15
5	80	20
7	50	50
8	0	100
8.5	0	100
9	85	15
10	85	15

In this study, the mobile phase consisted of eluent A (1 % formic acid in ultrapure water) and eluent B (100 % acetonitrile). Gradient elution were shown in table 3. The flow rate was 0.4 mL/min, the injection volume was 1 µL. Capillary and cone voltages were set at 0.7 kV and 9 V, respectively. Nitrogen was used as both cone gas (150 L/h) and desolvation gas (900 L/h). Desolvation temperature was set to 500 ºC; nebuliser gas pressure 7.0 bar, the source off set 50 V. The eluting constituents were identified by comparing their retention times and MS spectra with reference compounds. Quantitative analysis was performed by using external standards. Calibration curves were drawn using standard solutions in a 0.0625-5 µg/mL concentration range (for rosmarinic acid, $y = 61.5098\ x$; $R^2 = 0.989$; for chlorogenic acid, $y = 109.048\ x$; $R^2 = 0.990$ and for ferulic acid $y=1111.96 \cdot x$, $R^2 = 0.991$). The concentrations of compounds were calculated by linear regression plots constructed by TargetLynx software and expressed in mg/100 g of plant DW.

2.21. *Statistical treatment of data*

Statistical analysis for α-amylase assay was performed using GraphPad Prism 5 statistical package (GraphPad Software, USA).

All other experimental results were presented as means ± SD (standard deviations) and statistically analysed using MS Excel software. Individual experiments were performed at least in triplicates.

3. RESULTS AND DISCUSIONS

3.1 Extract yields of Nepeta and P. officinalis plants extracts

In plants biologically active compounds are usually distributed in low concentrations. Different solvents and an extraction techniques are used to extract out specific compounds from plant matrix and to obtain high yields with minimal changes to the functional properties of the extract [172]. Polar solvents such as water or methanol are used for the extraction of phenolic compounds while non-polar solvents such as hexane is usually used for the extraction of non-polar compounds like terpenes, lipids, carotenes and chlorophylls [173].

In the present study leaves and roots (only for peony) of *Nepeta* and peony species were extracted with four solvents (*n*-hexane, acetone, methanol and water) using three extraction methods: traditional extraction (TR), Soxhlet extraction (SE) and accelerated solvent extraction ASE (at 68.9 bar and 60 ºC). Yields were defined for each extraction method as g extract/100 g of dry plant material.

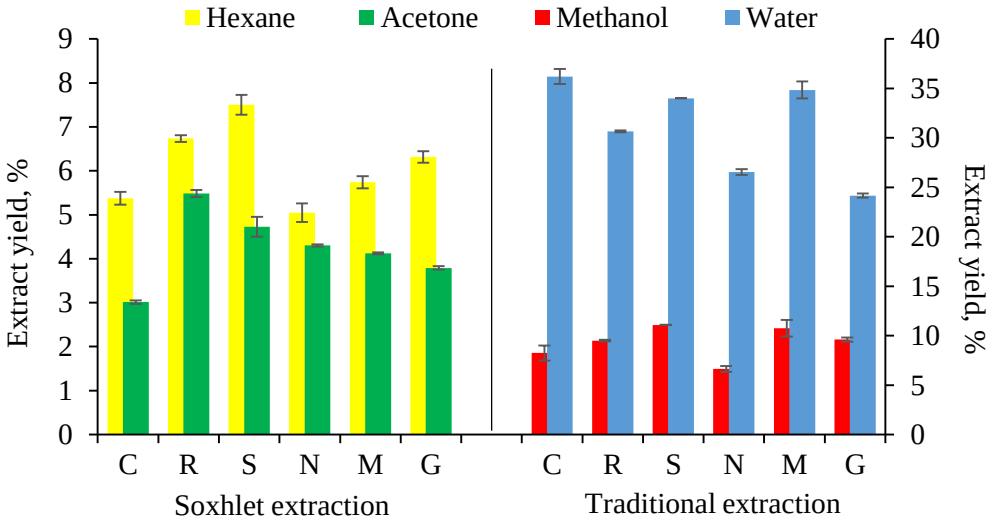


Figure 7. Yields after soxhlet and traditional extraction of *Nepeta* species leafs using different solvents.

Hexane and acetone were tested as solvents for the SE of all *Nepeta* species. In order to extract broad range of compounds (non-polar to polar) these two solvents were chosen because of their different properties. Also, hexane was used in order to clean extracts from lipid fraction due to get cleaner extracts with phenolic compounds. Yields of hexane extracts were found to be the highest in all tested *Nepeta* species compared with acetone extracts (Figure 7). After SE with hexane *N. sibirica* extract (S) gave the highest yield (7.5 %) while *N. nuda* (N) extract gave the lowest yield (5.05 %). After SE with acetone *N. racemosa* (R) gave the highest yield (5.48 %) while *N. cataria* (C) gave the lowest yield (3.01 %).

The most effective solvent for traditional extraction in terms of yield for all *Nepeta* species was water (45 min, at 80 ºC) followed by methanol (at room temperature, 24 h (Figure 7)). *N.*

cataria (C) water extract and *N. sibirica* (S) methanol extract resulted highest yields (36.21 % and 11.09 %, respectively) while *N. grandiflora* (G) water extracts and *N. nuda* (N) methanol extract showed lowest extraction yields (24.17 % and 6.64 %, respectively).

Comparing the three extraction methods, TR with water gave the highest yield of all extracts from tested *Nepeta* species. Yields of water extracts were ~ 3.4, ~ 5.2 and ~ 7.6 times higher than methanol, hexane and acetone extracts, respectively. The variability of the results could depend on several factors. Firstly, the polarity of solvent has a great influence on the yield of extract, this has been confirmed by several studies [173, 174, 175]. In addition, the use of higher temperatures, increase the ability of solvent to solubilize the analyte and decrease the viscosity of solvent. This allows better penetration of the solvent into the plant matrix [173]. Furthermore, different methodological approaches: extraction time (SE: ~ 3 h; TR (with methanol): ~ 24 h; TR (with water): ~ 45 min), different extract-collection steps after extraction (SE: continuous; TR: discontinuous, requires filtration after extraction), also cause the variation of results.

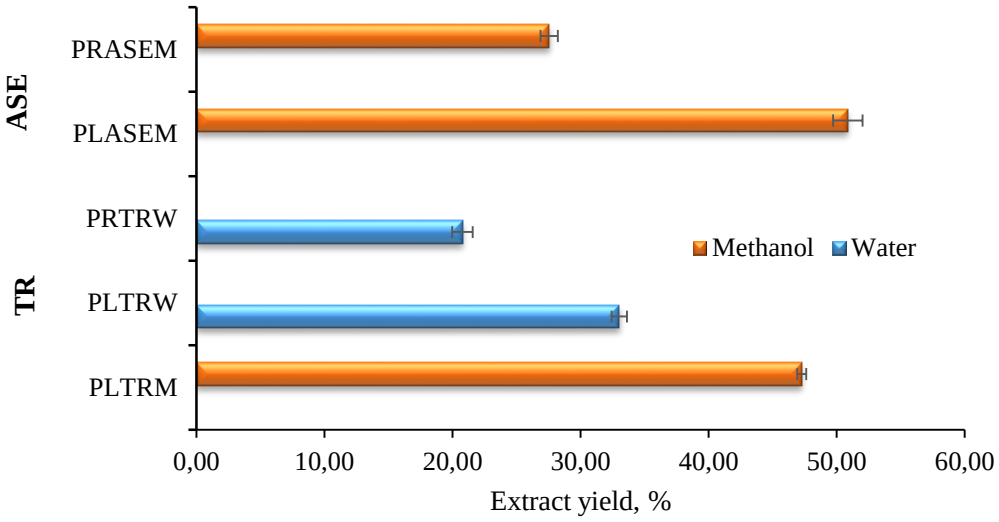


Figure 8. Yields after ASE and traditional extraction of peony leafs and roots using different solvents.

As can be seen from Figure 8 ASE using methanol as solvent was found to be a more efficient extraction method with very high yields (50.88 % and 27.55 %) for peony leafs and roots, respectively. Methanol and water extracts yields obtained with TR of peony leafs comparing with ASE were lower ~ 7 % and ~ 35 %, respectively. The water TR provided an extraction yield of *P. officinalis* roots and leafs equal to 20.77 % and 33.02 %, respectively. In this case, methanol was better solvent than water. Probably this could be because of differences between extraction conditions (for the TR with methanol room temperature was used; with water 80 ºC, while for ASE 60 ºC and 68.9 bar pressure).

3.2 Nepeta and peony antioxidant activity in TPC, ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ model systems

In order to choose the most promising extracts for further analysis a preliminary screening of the antioxidant activity and the total content of phenolic compounds in the methanolic, water and acetonic extracts of *Nepeta* species (*Nepeta cataria*, *Nepeta racemosa*, *Nepeta sibirica*, *Nepeta melissifolia*, *Nepeta grandiflora*, *Nepeta nuda*) and also methanolic, water extracts of *Paeonia officinalis* were performed.

The antioxidant properties of obtained extracts depend on phytochemical composition, structure and the amount of bioactive compounds especially polyphenolic compounds such as flavonoids and phenolic acids and sometimes to synergistic effects between them. In order to save the time the antioxidant capacity was carried out of the whole sample.

The amounts of TPC's and antioxidant activities with two stable synthetic radicals ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ of obtained *Nepeta* and peony extracts are presented in Table 4.

As can be seen the amount of TPC's of *Nepeta* species ranged from 669.38 to 443.05 mg GAE/g of extract in water, from 234.11 to 105.91 mg GAE/g of extract in methanol and from 94.61 to 51.38 mg GAE/g of extract in acetone extracts. The highest amount was found in water extract after traditional extraction of *N. melissifolia* (669.38 mg GAE/g of extract), followed by methanol and acetone extracts of *N. nuda* (234.11 and 94.61 mg GAE/g, respectively) after soxhlet extraction. The amount of total content of phenols of *Nepeta* species decreased in the following order: M>C>S>N>R>G in water, N>C>R>S>M>G in methanol and N>S>C>M>R>G in acetone extracts. The highest values were obtained in water compared with acetone and methanol extracts, that's why water extracts could be a good source of antioxidant compounds.

The established TPC's content in the tested extracts of peony decreases in the following order: PLTRM>PLASEM>PLTRW>PRASEM>PRTRW. The highest content of phenolic compounds showed peony leaf extract after traditional extraction with methanol (965.65 mg GAE/g of extract), while the lowest amount was determined in peony roots extract after traditional extraction with water (399.63 mg GAE/g of extract). It could be concluded, that peony leafs compared with peony roots including both extraction assays (ASE, TR) and solvents (methanol, water) were better source of TPC's.

In the DPPH$^{\bullet}$ scavenging system the highest values expressed in trolox equivalents (μM TE/g DW) were determined in water 972.77 extract of *N. melissifolia*, followed by methanol 379.91 and acetone 94.09 extracts of *N. nuda*. DPPH$^{\bullet}$ activity of *Nepeta* species extracts ranged from 379.91 to 95.39 μM TE/g DW in methanol, from 94.09 to 31.98 μM TE/g DW in acetone and from 972.77 to 402.57 μM TE/g DW in water extracts. The effectiveness of the *Nepeta* species of AA increases in the following order: C<R<G<S<M<N in acetone, G<C<S<M<R<N in

methanol and G<C<S<N<R<M in water extracts. The lowest activity possessed acetone extracts, while the highest activity have water extracts, followed by methanol extracts.

The $DPPH^{\bullet}$ scavenging results represented in peony extracts were in range from 4636.13 to 394.32 μM TE/g DW. The antioxidant activity of peony extracts increases in the following order: PRTRW<PRASEM<PLTRW<PLASEM<PLTRM. The highest TEAC-value 4636.13 μM TE/g DW gave PLTRM extract, while the lowest one gave PRTRW exstract 394.32 μM TE/g DW extract.

In $ABTS^{\bullet+}$ decolorisation system water extracts showed highest activities, while acetone and methanol extracts were worse radical scavengers. $ABTS^{\bullet+}$ scavenging activity of *Nepeta* species extracts ranged from 580.59 to 138.72 μM TE/g DW in methanol, from 239.49 to 130.97 μM TE/g DW in acetone and from 1347.22 to 374.09 μM TE/g DW in water extracts. The antioxidant activity of *Nepeta* species decreased in the following order: N>C>S>R>M>G in acetone, R>N>S>C>M>G in methanol and R>M>N>C>S>G in water extracts. The highest AA's were determined in water 1347.22 μM TE/g DW and methanol 580.59 μM TE/g DW extracts of *N. racemosa* and in acetone extract 239.49 μM TE/g DW of *N. nuda*.

The results for scavenging of $ABTS^{\bullet+}$ radical of peony extracts were in range from 4628.35 to 887.61 μM TE/g DW. The antioxidant activities of peony extracts decreased in the following order: PLTRM>PLASEM>PLTRW>PRASEM>PRTRW. Peony leafs extract after tradition extraction with methanol showed the best $ABTS^{\bullet+}$ scavenging activity with the highest value 4628.35 μM TE/g DW, while peony root extract after traditional extraction with water had the lowest one 887.61 μM TE/g DW.

The higher amount of phenolic compounds and the stronger scavenging activity of *Nepeta* species were in the traditional extraction (TR) obtained extracts with water and methanol than that after soxhlet extraction (SE) obtained extracts with acetone. Probably it was due to higher concentration of antioxidant compounds in the extracts. Also it can be seen, that higher amount of TPCs was extracted with more polar solvents and their content increased in the following order: acetone<methanol<water. So, this means that in these plants there are more polar compounds than non-polar. While for peony extracts highiest amounts and stronger antioxidant activities were obtained with methanol using TR for leafs and ASE for roots. In addition, peony extracts showed better AA's and had higher amounts of TPCs than all *Nepeta* species in all used assays. It could be because of different extraction techniques used in this work.

In general, it could be concluded that methanol as solvent for peony and water for *Nepeta* species were more efficient by application of TR than these of ASE and SE methods, respectively.

Table 4. AA's and TPC's of *Nepeta* and *P. officinalis spp*. extracts.

Extracts	Antioxidant assays		
	TPC[a] ± SD	**DPPH·[b] ± SD**	**ABTS·+[b] ± SD**
1	**2**	**3**	**4**
	Methanol extracts		
C	232.05 ± 0.004	190.44 ± 8.05	409.51 ± 9.38
R	228.07 ± 0,009	303.13 ± 9.76	580.59 ± 6.70
S	206.17 ± 0.007	260.04 ± 8.55	447.20 ± 6.20
N	234.11 ± 0.012	379.91 ± 7.24	466.75 ± 6.68
M	185.11 ± 0.005	293.18 ± 6.33	293.39 ± 8.42
G	105.91 ± 0.004	95.39 ± 3.56	138.72 ± 2.79
PRASEM	436.05 ± 0.052	601.84 ± 8,17	935.70 ± 4.50
PLASEM	882.39 ± 0.099	4512.56 ± 7,02	4504.76 ± 7.43
PLTRM	965.65 ± 0.009	4636.13 ± 5.09	4628.35 ± 4.49
	Acetone extracts		
C	80.86 ± 0.006	31.98 ± 0.13	187.34 ± 7.55
R	56.52 ± 0.006	43.74 ± 1.21	169.05 ± 1.88
S	83.48 ± 0.004	45.44 ± 1.38	179.10 ± 0.37
N	94.61 ± 0.007	94.09 ± 2.12	239.49 ± 5.44
M	70.34 ± 0.003	53.29 ± 1.42	157.19 ± 6.31
G	51.38 ± 0.009	44.18 ± 1.65	130.97 ± 4.48
	Water extracts		
C	616.83 ± 0.016	569.24 ± 7.53	1252.51 ± 4.92
R	537.54 ± 0.012	695.67 ± 5.95	1347.22 ± 2.68
S	572.79 ± 0,019	666.49 ± 9.28	952.37 ± 6.04
N	572.69 ± 0,015	678.94 ± 7.51	1253.80 ± 4.03
M	669.38 ± 0.014	972.77 ± 1.10	1277.22 ± 4.89
G	443.05 ± 0.008	402.57 ± 3.30	374.09 ± 6.29
PRTRW	399.63 ± 0.011	394.32 ± 5.72	887.61 ± 6.97
PLTRW	715.76 ± 0.013	4306.35 ± 6.37	4298.12 ± 5.10

[a] – mg GAE/g of extract; [b] - μM TE/g DW.

Differences in contents of activity may be affected by the type of extraction, polarity of solvents, which was used for extraction, and differences between scavenging methods. Also it might be influenced by environmental factors (such as light, temperature, soil nutrients), which may influence phenylpropanoid metabolism and concentrations of bioactive compounds in plants [176].

The total content of phenolic compounds presented in plants in not a very informative of their antioxidant activity. Also, the antioxidant activity of various phenolic compounds can differ

significantly, because of nature of bioactive compound. In order to obtain more precise results the structures of individual compounds need to be determined.

The obtained extracts possessed strong antioxidant activity, therefore it was interesting to evaluate the antioxidant activity of solid residue after each extraction step and crude plant material before the extraction. It is known that some bioactive compounds could have strong bonds with other in the plant matrix presented compounds. These compounds using conventional extraction techniques, does not extractable with organic solvents and water. Antioxidant activity of these compounds were evaluated employing the QUENCHER approach for the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and *Folin–Ciocalteu's* assays. It's based on a redox reaction between the surface of soluble free radicals and bound active compounds. Results are presented in Table 5.

After evaluation of antioxidant activity of solid residues using the QUENCHER approach method it was determined that residues after and before extraction had a small amount of active compounds. Depending on the solvent used for the extraction TPC's in residues of *Nepeta* species ranged from 9.88 to 6.35 mg GAE/g of dry plant matter, from 8.46 to 5.49 and from 4.59 to 1.55 after acetone, methanol and water extraction, respectively. While activity of solid residue in $DPPH^{\bullet}$ scavenging system ranged from 24.76 to 10.89, from 18.70 to 10.86 and from 8.19 to 4.15 µM TE/g of dry plant matter after acetone, methanol and water extraction, respectively. In the $ABTS^{\bullet+}$ decolourization system highest activities showed residues after methanol extraction. It ranged between 63.55 – 38.05, while acetone and water residues ranged between 55.59 – 41.48 and 12.14 – 10.02 µM TE/g of dray plant matter, respectively. TPC's in crude plant matter of *Nepeta* species ranged from 9.83 to 4.30 mg GAE/g of dry plant matter. The highest amount of polyphenols was determined in *N. melissifolia*, while the lowest value was in crude plant material of *N. cataria*. The highest $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activities showed crude plant materials of *N. sibirica* and *N. melissifolia*, respectively. Residues after water extraction had low AA comparing to residues after acetone and methanol extraction. Probably it could be because water was better solvent for extracting phenolic compounds from *Nepeta* plants then acetone and methanol.

Table 5. Total phenolics and antioxidant activities of solid residues and crude plant materials of *Nepeta* and peony species.

Solid residues	Antioxidant assays		
	TPC[a] ± SD	**DPPH˙[a] ± SD**	**ABTS˙+[b] ± SD**
1	**2**	**3**	**4**
	Residues after methanol extraction		
C	6.14 ± 0.03	14.56 ± 0.34	43.85 ± 3.96
R	5.92 ± 0.01	15.30 ± 0.77	45.09 ± 3.92
S	8.22 ± 0.02	18.70 ± 1.30	56.15 ± 0.98
N	5.49 ± 0.05	10.86 ± 0.60	38.05 ± 4.25
M	8.46 ± 0.03	16.76 ± 2.36	63.55 ± 4.92
G	5.94 ± 0.03	15.56 ± 0.36	49.40 ± 8.53
PRASEM	0.82 ± 0.11	9.72 ± 0.17	13.33 ± 2.31
PLASEM	13.30 ± 0.03	53.46 ± 0.96	86.22 ± 9.85
PLTRM	18.56 ± 0.13	75.81 ± 0.29	101.36 ± 1.89
	Residues after acetone extraction		
C	6.35 ± 0.02	13.06 ± 0.90	44.10 ± 7.84
R	7.21 ± 0.03	15.75 ± 1.30	47.42 ± 4.36
S	9.88 ± 0.02	19.91 ± 0.97	55.39 ± 4.63
N	7.36 ± 0.02	10.89 ± 1.44	41.48 ± 7.98
M	9.79 ± 0.02	24.76 ± 1.25	55.59 ± 4.01
G	7.93 ± 0.08	15.46 ± 1.42	44.21 ± 3.07
	Residues after water extraction		
C	1.55 ± 0.02	4.15 ± 0.18	11.03 ± 0.79
R	2.68 ± 0.20	6.96 ± 0.19	10.73 ± 0.10
S	3.17 ± 0.02	8.19 ± 0.32	11.13 ± 0.16
N	1.71 ± 0.02	6.12 ± 0.21	11.90 ± 1.95
M	4.59 ± 0.31	8.14 ± 0.24	10.02 ± 0.73
G	2.05 ± 0.02	5.34 ± 0.09	12.14 ± 0.09
PRTRW	0.27 ± 0.01	6.32 ± 0.11	8.56 ± 0.07
PLTRW	12.63 ± 0.02	49.54 ± 0.34	73.12 ± 5.10
	Crude plant materials		
C	4.30 ± 0.02	10.07 ± 0.61	36.69 ± 6.64
R	6.14 ± 0.05	16.27 ± 1.43	36.16 ± 4.76
S	8.07 ± 0.05	18.24 ± 2.03	51.34 ± 3.08
N	4.69 ± 0.02	11.17 ± 1.02	29.24 ± 5.44
M	9.83 ± 0.11	20.91 ± 0.91	48.55 ± 4.55
G	5.96 ± 0.03	11.46 ± 1.51	32.36 ± 8.15
PL	20 56 ± 0.23	80.87 ± 0.31	120.56 ± 3.81
PR	1.51 ± 0.10	35.13 ± 0.11	47.87 ± 2.13

[a] – mg GAE/g of dry plant matter; [b] - μM TE/g of dry plant matter.

Relatively high values of phenolic compounds and radical scavenging activity was determined in residues after methanol and water extraction in peony leafs comparing with those determined in peony roots. The highest value of polyphenols was in residue of peony leafs after TR with methanol, while the lowest value was in residue of peony roots after TR with water. Also, higher values of polythenols and antioxidant activity was determined in crude peony leafs (PL) than in peony roots (PR).

The results of this study suggest that residue fractions before and after extractions of peony have better AA and TPC values than *Nepeta* species. Probably that some antioxidants are securely bonded in plant matrix and are insoluble in used solvents.

3.3 Identification of phytochemicals in Nepeta and Paeonia plants extracts

In the present work a qualitative analysis of the phenolic composition from the methanol and water extracts of *Nepeta* and *P. officinalis* species have been carried out using UPLC–QTOF–MS in negative ionisation mode. The method was used to detect and characterise 54 (29 of *Nepeta* and 25 of *P. officinalis*) phytochemical compounds, of which 45 were tentatively characterised by MS data, together with those found in the literature, and 9 were confirmed by comparing chromatographic retention times, accurate masses and formulas, UV and MS spectra with available standards in UPLC–MS analysis. In the identification process ChemSpider public database was used.

Table 6 shows the list of 29 compounds identified in *Nepeta* species by UPLC–Q-TOF-ESI-MS experiments along with their retention times (t_R), detected accurate masses (ionisation mode negative), molecular formulas of each phytochemical, as well as the authentic and the bibliographic references used in the characterisation process. UPLC-Q-TOF-MS base peak chromatograms are presented in Figure 9.

Compound **1** had a molecular ion at *m/z* 195.0510, which corresponded to molecular formula $C_6H_{12}O_7$. It was tentatively characterised as galactonic acid, which is in agreement with literature data [177]. Two compounds **2** and **7** showed a molecular ion $[M-H]^-$ at *m/z* 191.0196, ($C_6H_8O_7$). Based on the MS data and literature [178], these compounds were identified as citric acid isomers. Compound **3** with *m/z* 341.1090 corresponded to $C_{12}H_{22}O_{11}$ formula and was assigned to dihexose. Compound **4** with *m/z* 179.0561 corresponded to molecul $C_6H_{12}O_6$ and was identified as hexose. Compounds **5**, **10**, **11**, **13**, **14**, **26**, **28** and **29** were identified as malic acid, syringic acid, chlorogenic acid, caffeic acid, ferulic acid, apigenin, umbelliferone and rosmarinic acid, respectively. These compounds were identified by comparison of the retention times with a commercial standards. A precursor ion of *m/z* 175.0248 at retention time 1.0 min, gave molecular formula $C_6H_8O_6$, and tentatively was identified as ascorbate **6**. Compound **8** ($[M-H]^-$ at *m/z*

177.0404) has been tentatively assigned as galactonic acid lactone. The compound **12** (t_R 1.6 min) with the molecural formula $C_5H_8O_5$ and having the precursor ion at *m/z* 147.0298 in the ESI^- mode, has been tentatively identified as ribonolactone. Compounds **9** and **15** gave molecular ions *m/z* = 263.0409 and *m/z* = 281.0303, which corresponded to $C_9H_{12}O_9$ and $C_{12}H_{10}O_8$ formulas, respectively. Unfortunately, these compounds have not been identified. Molecular ion of compound **16** was detected at *m/z* 637.1033 fitting molecular formula $C_{27}H_{26}O_{18}$, tentatively it was identified as luteolin-7-O-diglucuronide, which is in agreement with literature data [179]. Compounds **17** and **24** gave molecular ion at *m/z* 285.0402 matching molecular formula $C_{15}H_{10}O_6$. These compounds were tentatively identified as derivatives of luteolin. Compound **18** at 2.0 min exhibited molecular ion at *m/z* 351.0568 and was tentatively assigned to diglucuronide. Compounds **19** and **20** displayed a molecular ions $[M-H]^-$, *m/z* = 387.1658 and *m/z* 135.0448 matching molecular formulas $C_{18}H_{28}O_9$ and $C_8H_8O_2$, respectively. According to the literature, these compounds tentatively were characterised as tuberonic acid glucoside and benzoic acid methyl ester [180, 181]. Compound **21** tentatively was identified as ferulic acid derivative.

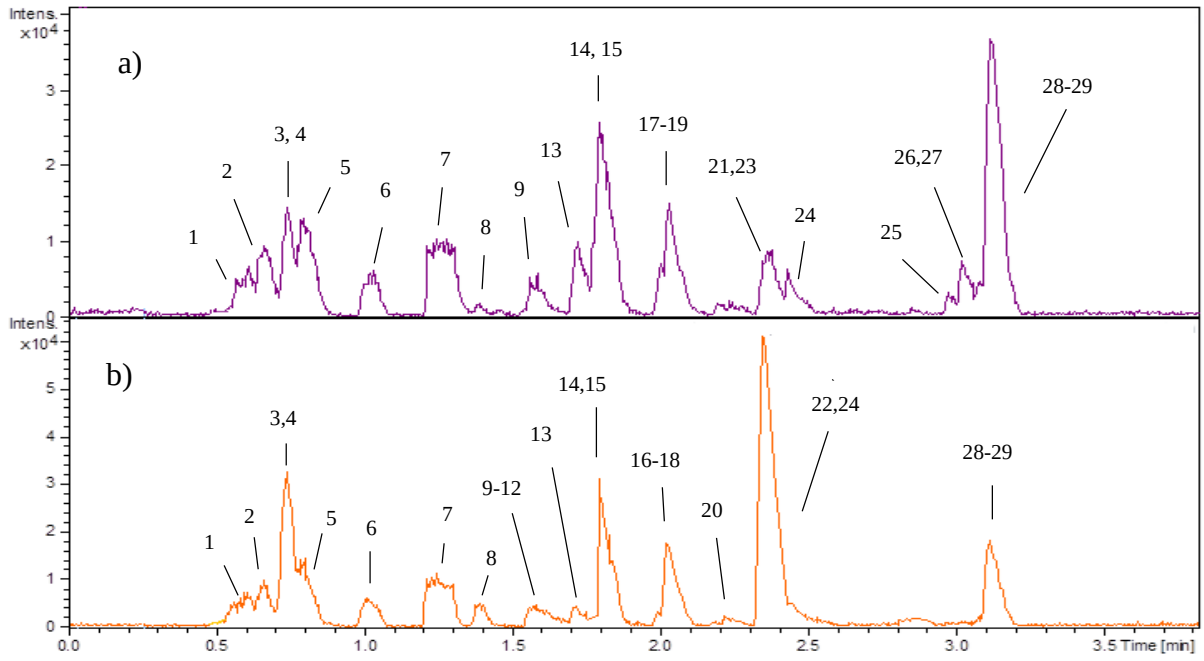


Figure 9. Chromatograms of *N. sibirica* and *N. nuda* extracts obtained with UPLC-Q-TOF; a) *N. nuda* extract after traditional extraction with water; b) *N. sibirica* extract after traditional extraction with water.

Compound **22** was tentatively identified as 7-deoxyloganic acid. This compound presented a fragment at *m/z* 359.1347, which corresponds to molecular formula $C_{16}H_{24}O_9$. Compound **23** with *m/z* 295.0460 corresponded to molecular formula of $C_{13}H_{12}O_8$ was identified as caffeoylmalic acid. A precursor ion of *m/z* 431.0982 at retention time 3.0 min, gave molecular formula $C_{21}H_{20}O_{10}$, and tentatively was identified as apigenin-O-glucoside **25**. Compound **27** gave molecular ion peak at *m/z* 445.0772, which was assigned to molecular ion formula $C_{21}H_{18}O_{11}$, tentatively this compound identified as apigenin-7-O-glucuronide, because MS/MS fragmentation

gave molecular ion at *m/z* 269.0457, which corresponds to molecular formula similar to apigenin ($C_{15}H_{10}O_5$), while loss of *m/z* 176.0552 unit indicates glycopyranose unit ($C_6H_8O_6$).

Table 6. Characterization of phenolic compounds in extracts from *Nepeta* species.

No.	*Compound*	*Molecular formula*	*RT (UPLC)*	*m/z, [M – H]⁻*
1	Galactonic acid[b,c]	$C_6H_{12}O_7$	0.6	195.0510
2	Citric acid izomer[b,c]	$C_6H_8O_7$	0.7	191.0196
3	Dihexose[b,c]	$C_{12}H_{22}O_{11}$	0.7	341.1090
4	Hexose[b,c]	$C_6H_{12}O_6$	0.7	179.0561
5	Malic acid[a]	$C_4H_6O_5$	0.8	133.0142
6	Ascorbate[b,c]	$C_6H_8O_6$	1.0	175.0248
7	Citric acid isomer[b,c]	$C_6H_8O_7$	1.3	191.0197
8	Galactonic acid lactone[b,c]	$C_6H_{10}O_6$	1.4	177.0404
9	ni	$C_9H_{12}O_9$	1.6	263.0409
10	Syringic acid[a]	$C_9H_{10}O_5$	1.6	197.0454
11	Chlorogenic acid[a]	$C_{16}H_{18}O_9$	1.6	353.0951
12	Ribonolactone[b,c]	$C_5H_8O_5$	1.6	147.0298
13	Caffeic acid[a]	$C_9H_8O_4$	1.7	179.0347
14	Ferulic acid[a]	$C_{10}H_{10}O_4$	1.8	193.0507
15	ni	$C_{12}H_{10}O_8$	1.8	281.0303
16	Luteolin-7-O-diglucuronide[b,c]	$C_{27}H_{26}O_{18}$	2.0	637.1033
17	Luteolin derivative[b,c]	$C_{15}H_{10}O_6$	2.0	285.0402
18	Diglucuronide[b,c]	$C_{12}H_{16}O_{12}$	2.0	351.0568
19	Tuberonic acid glycoside[b,c]	$C_{18}H_{28}O_9$	2.0	387.1658
20	Benzoic acid methyl ester[b,c]	$C_8H_8O_2$	2.2	135.0448
21	Ferulic acid derivative[b,c]	$C_{10}H_{10}O_4$	2.2	193.0507
22	7-deoxyloganic acid[b,c]	$C_{16}H_{24}O_9$	2.3	359.1347
23	Caffeoylmalic acid[b,c]	$C_{13}H_{12}O_8$	2.4	295.0460
24	Luteolin derivative[b,c]	$C_{15}H_{10}O_6$	2.4	285.0404
25	Apigenin-O-glucoside[b,c]	$C_{21}H_{20}O_{10}$	3.0	431.0982
26	Apigenin[a]	$C_{15}H_{10}O_5$	3.0	269.0457
27	Apigenin-7-O-glucuronide[b,c]	$C_{21}H_{18}O_{11}$	3.0	445.0772
28	Umbelliferone[a]	$C_9H_6O_3$	3.1	161.0243
29	Rosmarinic acid[a]	$C_{18}H_{16}O_8$	3.1	359.0771

a Confirmed by a standard.
b Confirmed by a reference.
c Confirmed by parent ion mass using free chemical database (Chemspider).
ni, not identified.

Table 7 shows the list of 25 compounds identified in *P. officinalis* specie through UPLC–Q-TOF-ESI-MS experiments along with their retention times (t_R), detected accurate mass (ionisation mode negative), molecular formula of each phytochemical, as well as the authentic and the bibliographic references used in the characterisation process. UPLC-Q-TOF-MS base peak chromatogram is presented in Figure 10.

Compound **30** had a molecular ion at *m/z* 191.0563, which corresponded to the molecular formula $C_7H_{12}O_6$ was tentatively characterised as quinic acid, which is in agreement with literature data [182]. Compound **31** with *m/z* 331.0671 corresponded to molecular formula $C_{13}H_{16}O_{10}$ and was identified as 1-O-galloylhexose. Compound **32** with *m/z* 421.1357 corresponded to formula

$C_{17}H_{26}O_{12}$ and was assigned to 1,2-dihydroxypropanyl-tetraacetylhexosyde. Compounds **33**, **42**, **46**, **49**, **53** and **54** have not been identified. Compound **34** was identified as gallic acid by comparison of the retention time with a commercial standard. Compound **35** was tentatively identified as digallic acid, which is in agreement with literature data [182]. Compound **36** with *m/z* 183.0302 corresponded to molecular formula $C_8H_8O_5$ and was identified as methyl gallate [182]. Compound **37** was tentatively identified as tri-galloyl-hexose. This compound presented a fragment at *m/z* 635.0889, which corresponded to molecular formula $C_{27}H_{24}O_{18}$. Two compounds **39** and **48** showed a molecular ions $[M-H]^-$ at *m/z* 525.1617, ($C_{24}H_{30}O_{13}$). Based on the MS and literature data [183], these compounds were identified as derivatives of mudanpioside. A precursor ion of *m/z* 491.1768 at retention time 4.5 min, gave molecular formula $C_{21}H_{32}O_{13}$, and tentatively was identified as quercetin derivative **38**. Compound **40** was identified as quercetin dihexoside. Molecular ion of compound **41** was detected at *m/z* 615.0993 fitting molecular formula $C_{28}H_{24}O_{16}$, tentatively it was identified as myricitrin-O-gallate, which is in agreement with literature data [182]. Compounds **43** and **44** displayed a molecular ions $[M-H]^-$, at *m/z* = 433.0779 and at m/z 335.0410 and were matching molecular formulas $C_{20}H_{18}O_{11}$ and $C_{15}H_{12}O_9$, respectively. According to the literature, these compounds tentatively were characterised as quercetin pentoside and methyl digallate [182]. Compound **45** with *m/z* = 939.1122 corresponded to molecular formula $C_{41}H_{32}O_{26}$ was identified as penta-O-galloyl-hexoside. Compound **47**, **50**, **51** with *m/z* = 545.0582, fitting $C_{24}H_{18}O_{15}$ molecular formula were tentatively identified as derivatives of dihytroxybenzoic acetate-digallate. Compound **52** gave molecular ion peak at *m/z* 1091.1236, which was assigned to molecular ion formula $C_{48}H_{36}O_{30}$, tentatively this compound identified as hexagalloyl hexoside.

Compounds **32**, **33**, **42**, **48** have not been shown in Figure 10, because these compounds were identified just in PLTRW extract (data not shown).

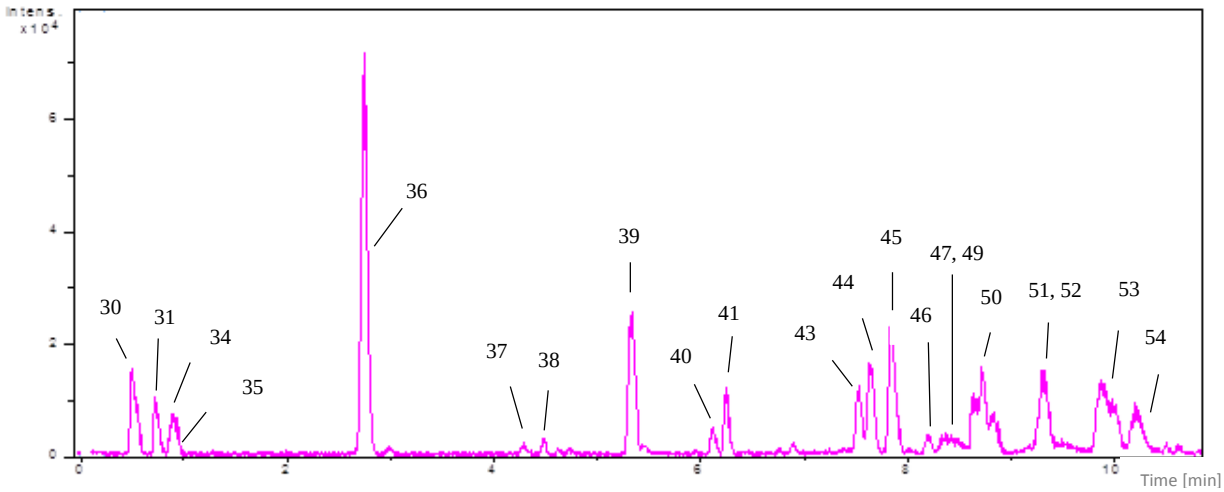


Figure 10. Chromatogram of *P. officinalis* extract (PLASEM) obtained with UPLC-Q-TOF.

Table 7. Characterization of phenolic compounds in extracts from *P. officinalis*.

No.	*Compound*	*Molecular formula*	*RT (UPLC)*	*m/z, [M – H]⁻*
30	Quinic acid[b,c]	$C_7H_{12}O_6$	0.5	191.0563
31	1-O-galloylhexose	$C_{13}H_{16}O_{10}$	0.7	331.0671
32	1,2-dihydroxypropanyl-tetraacetylhexosyde[b,c]	$C_{17}H_{26}O_{12}$	0.7	421.1357
33	ni	$C_{19}H_{26}O_{15}$	0.7	497.1199
34	Gallic acid[a]	$C_7H_6O_5$	0.9	169.0144
35	Digallic acid[b,c]	$C_{14}H_{10}O_9$	1.9	321.0253
36	Methyl galate[b,c]	$C_8H_8O_5$	2.8	183.0302
37	Tri-galloyl-hexose[b,c]	$C_{27}H_{24}O_{18}$	4.3	635.0889
38	Quercetin derivative[b,c]	$C_{21}H_{32}O_{13}$	4.5	491.1768
39	Mudanpioside derivative[b,c]	$C_{24}H_{30}O_{13}$	5.3	525.1617
40	Quercetin dihexoside [c]	$C_{27}H_{30}O_{16}$	6.1	609.1460
41	Myricitrin-O-gallate[b,c]	$C_{28}H_{24}O_{16}$	6.1	615.0993
42	ni	$C_{21}H_{32}O_{12}$	6.1	475.1826
43	Quercetin pentoside[b,c]	$C_{20}H_{18}O_{11}$	7.5	433.0779
44	Methyl digallate[b,c]	$C_{15}H_{12}O_9$	7.6	335.0410
45	Penta-O-galloyl-hexose[b,c]	$C_{41}H_{32}O_{26}$	8.1	939.1122
46	ni	$C_{29}H_{26}O_{16}$	8.2	629.1149
47	Dihydroxybenzoic acetate-digallate[b,c]	$C_{24}H_{18}O_{15}$	8.4	545.0580
48	Mudanpioside derivative[b,c]	$C_{24}H_{30}O_{13}$	8.4	525.1615
49	ni	$C_9H_{15}O_4$	8.4	187.0977
50	Dihydroxybenzoic acetate-digallate[b,c]	$C_{24}H_{18}O_{15}$	8.7	545.0581
51	Dihydroxybenzoic acetate-digallate[b,c]	$C_{24}H_{18}O_{15}$	9.3	545.0582
52	Hexagalloyl hexoside[b,c]	$C_{48}H_{36}O_{30}$	9.3	1091.1236
53	ni	$C_{15}H_{26}O_{26}$	9.9	621.0637
54	ni	$C_{31}H_{22}O_{19}$	10.2	697.0695

a Confirmed by a standard.
b Confirmed by a reference.
c Confirmed by parent ion mass using free chemical database (Chemspider).
ni, not identified.

Chemical compositions of *Nepeta* and *P. officinalis* species extracts were studied using UPLC-QTOF-MS, while radical scavenging of separated compounds were detected by the HPLC-DPPH$^{\bullet}$ on-line method. Combined UV (positive signals) and DPPH$^{\bullet}$ quenching (negative signals) chromatograms of water extract of *N. sibirica* and peony extract after accelerated solvent extraction with methanol are presented in Figure 11. Syringic acid **10**, chlorogenic acid **11**, caffeic acid **13**, ferulic acid **14**, luteolin derivatives (**16**, **17**, **24)**, umbelliferone **28** and rosmarinic acid **29** were determined to be the most active radical scavengers in the investigated extracts of *Nepeta spp.* (a). While quinic acid **30**, gallic acid derivatives (**34**, **35**, **36**, **37**, **41**, **44**, **45**, **47**, **50**, **51, 52**), mudanpioside derivative **39**, quercetin dihexoside **40**, unknown compounds (**46**, **53**, **54**) were determined to be the most active radical scavengers in extracts of peony.

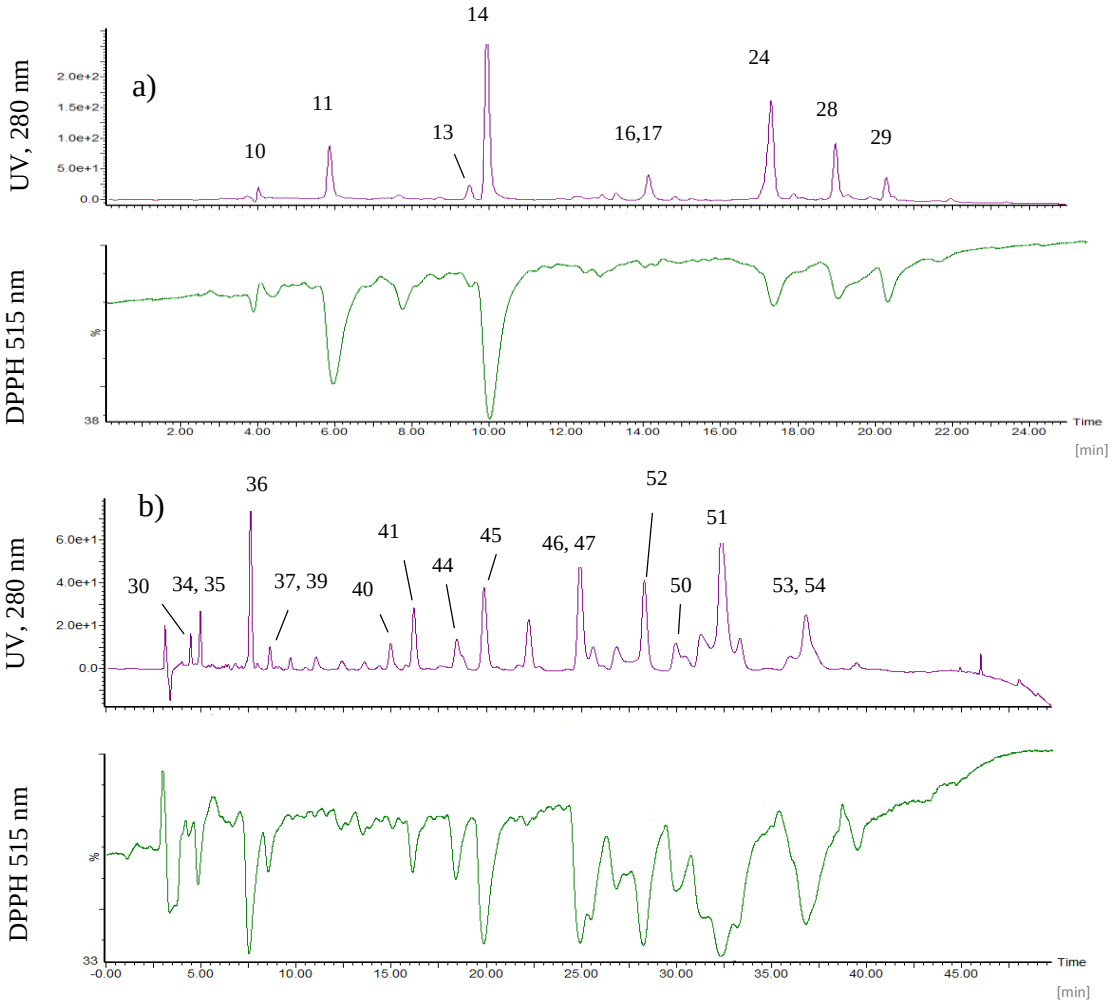


Figure 11. HPLC-DPPH˙ profiles of *N. sibirica* and *P. officinalis* extracts. a) N. sibirica water extract; b) P. officinalis methanol extract (PLASEM). DPPH˙ scavenging at 515 nm.

UPLC-MS analysis of *Nepeta* species extracts resulted in several quantitatively important peaks: three of them were identified as rosmarinic acid, chlorogenic acid and ferulic acid. Identification of these antioxidants were based on comparison of their retention times, UV and MS spectra compared with that of authentic standards. The contents of individual compounds are presented in Figure 12.

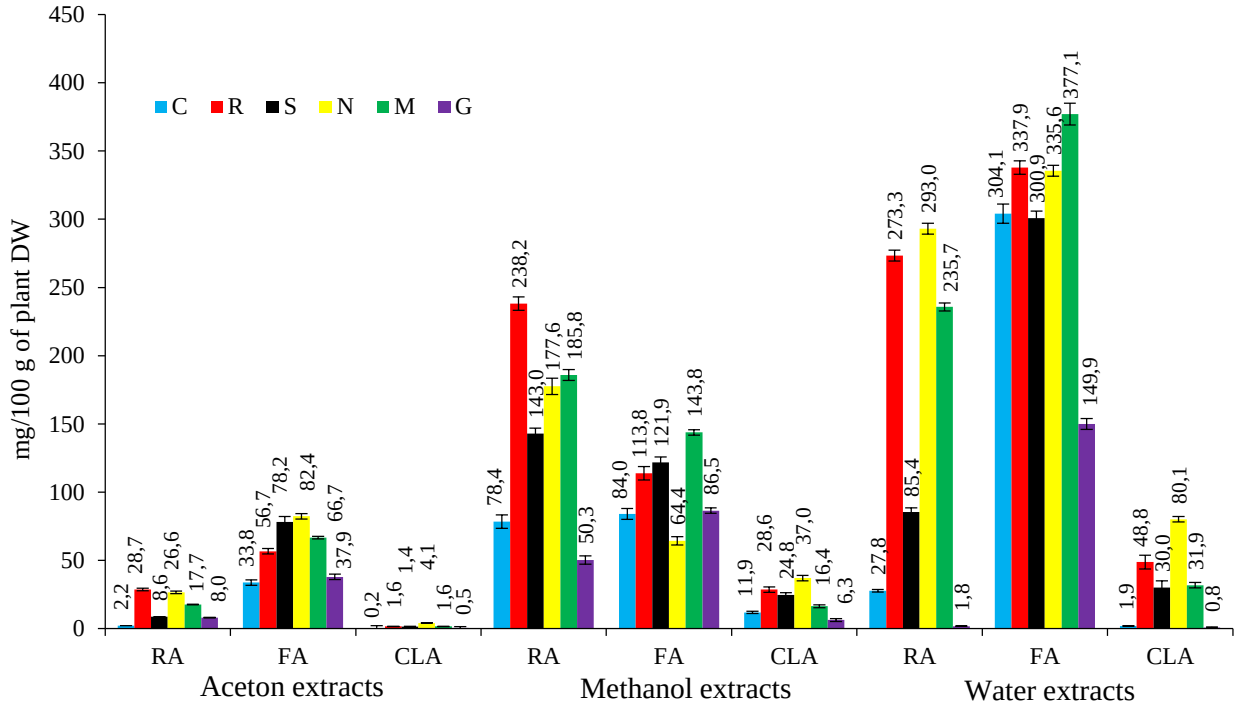


Figure 12. Total amounts of rosmarinic acid, chlorogenic acid and ferulic acid detected in *Nepeta* species. RA – rosmarinic acid; CLA – chlorogenic acid, FA – ferulic acid.

The highest concentration of identified compounds was found in methanol and water extracts obtained from all analysed *Nepeta* varieties. Ferulic acid (FA) was the major compound found in water and acetone extracts, while rosmarinic acid (RA) was major in methanol extracts. Values of FA ranged from 82.4 to 33.8 mg/100 g of plant DW in acetone, from 377.1 to 149.9 mg/100 g of plant DW in water and 143.8 to 64.4 mg/100 g of plant DW in methanol extracts. Highest concentrations of ferulic acid were found in *N. Nuda* (82.4 mg/100 g of plant DW) acetone extract and in *N. melissifolia* (377.1 and 143.8 mg/100 g of plant DW) water and methanol extracts. While lowest values were in *N. cataria*, *N. nuda* and *N. grandiflora* acetone, methanol and water extracts, respectively. Values of RA ranged from 238.2 to 50.3 in methanol, from 28.7 to 2.2 in acetone and from 293.0 to 1.8 mg/100 g of plant DW in water extracts. Highest concentrations of rosmarinic acid were found in *N. nuda* (293.0 mg/100 g of plant DW) water extract and in *N. racemosa* (28.7 mg/100 g of plant DW) acetone and (238.2 mg/100 g of plant DW) methanol extracts. While lowest concentrations of RA were found in acetone extract of *N. cataria* and in methanol and water extracts of *N. grandiflora*. Values of CLA ranged from 4.1 to 0.2 mg/100 g of plant DW in acetone, from 37.0 to 6.3 mg/100 g of plant DW in methanol and from 80.1 to 0.8 mg/100 g of plant DW in water extracts. Highest concentrations of chlorogenic acid (CLA) were found in methanol and water extracts of *N. nuda* (37.0 and 80.1 mg/100 g of plant DW, respectively). Lowest concentrations of CLA were found in acetone extracts. As can be seen *N. racemosa*, *N. sibirica*, *N. nuda* and *N. melissifolia* extracts possess highest amounts of RA, CLA and FA of all tested samples. Through the future perspective these plant extracts may will show good bio activities. It

can be concluded, that higher amount of reported compounds found in water depends on polarity of solvent. Usually to obtain phenolic acids such RA, FA and CLA better to use water and my results showed that water was effective solvent for these acids.

Mihaylova et al., [163] reported what RA, FA and CLA amounts in water extract of *N. cataria* were 630.9, 160.1 and 119.2 µg/g DW, respectively. While amounts of RA, FA, and CLA set out in this work were 27.8, 304.1 and 1.9 mg/100 g of plant DW, respectively. According to Kraujalis et al., [184] amounts of RA in acetone, methanol and water extracts of *N. cataria* were 487.8, 1743.8 and 193.3 mg/100 g of extract, respectively. These results are very different from that determined in this and in Mihaylova et al., [163] study. Differences in contents of RA, FA and CLA may be affected of type of extraction, also it might influence environmental factors (such as light, temperature, soil nutrients) which may influence phenylpropanoid metabolism and concentrations of bioactive compounds in plants [176].

3.4 Radical scavenging activity of different Nepeta and Paeonia leafs extracts

Collected evidence shows that reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide ion ($O_2^{-\bullet}$), hydroxyl radicals ($HO^\bullet$) and peroxyl radicals ($ROO^\bullet$), are involved in the pathophysiology of aging and a lot of diseases, such as atherosclerosis, cardiovascular diseases and cancer [186, 187]. In order to investigate the antioxidant activity (Oxygen Radical Absorbance Capacity (ORAC), Hydroxyl Radical Adverting Capacity (HORAC) and Hydroxyl Radical Scavenging Capacity (HOSC)) against ROS species 12 extracts of catnip and 3 extracts of peony were selected. The ORAC, HOSC and HORAC results were expressed as µmol TE/g of extract and µmol CAE/g of extract, respectively.

The results for antioxidant capacity of ORAC are presented in Figure 13. The ORAC values of the different extracts of *Nepeta* species were in a range from 2476 ± 0.8 to 331 ± 0.9 µmol TE/g of extract. Water extract of *N. melissifolia* (M) also methanol and acetone extracts of *N. nuda* (N) showed highest ORAC-values, while methanol and acetone extracts of *N. melissifolia* (M) and water extract of *N. racemosa* (R) gave lowest ORAC-values. ORAC-values of water extracts were 1.5 - 3.8 and 5 - 7 (depends on extract) times higher than methanol and acetone extracts, respectively.

The results for antioxidant capacity of HOSC are presented in Figure 13, they were in a range from 2618 ± 1.1 to 313 ± 0.9 µmol TE/g of extract. Water extract of *N. sibirica* showed the highest HOSC hydroxyl radical scavenging activity with the highest value 2618 ± 1.1 µmol TE/g of extract from all tested catnip extracts, followed by the methanol extract of *N. nuda* (2317 ± 0.8 µmol TE/g of extract) and that obtained with acetone (481 ± 0.5 µmol TE/g of extract) of *N. sibirica*. The water extract of *N. racemosa*, methanol extract of *N. sibirica* and acetone extract of *N. melissifolia*

showed lowest antioxidant activities 1965 ± 1.1, 840 ± 1.0 and 313 ± 0.9 µmol TE/g of extract, respectively.

The HORAC values for different extracts of *Nepeta* species were in range from 1351 ± 0.8 to 243 ± 0.9 µmol CAE/g of extract (Figure 13.). Water extract of *N. sibirica* had the strongest adverting capacity of hydroxyl radicals of all examined extracts (1351 ± 0.8 µmol CAE/g of extract), while the lowest HORAC-value was determined for acetone extract of *N. sibirica* (243 ± 0.9 µmol CAE/g of extract).

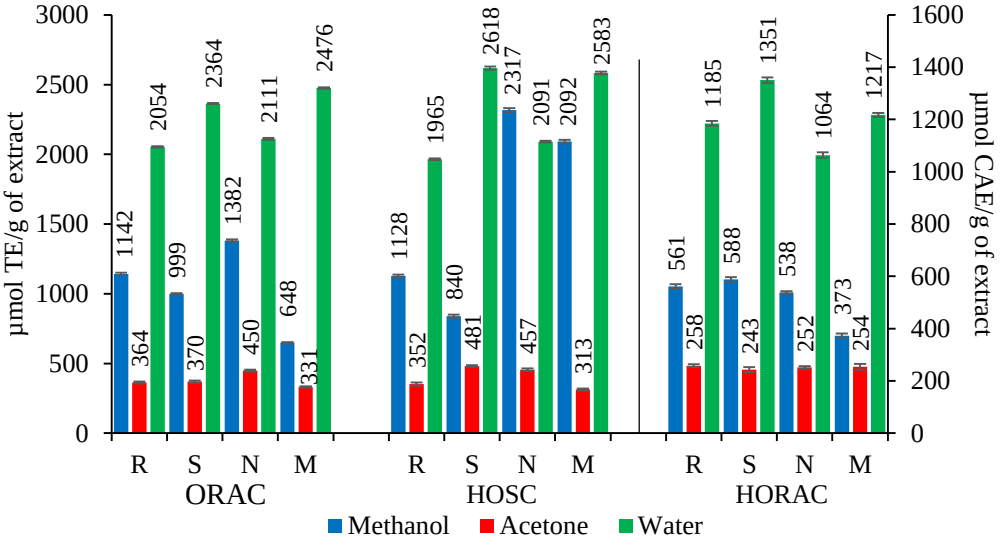


Figure 13. Antioxidant activity of *Nepeta* species extracts evaluated by the ORAC, HORAC and HOSC methods.

In all three antioxidant activity (ORAC, HORAC and HOSC) assays the lowest antioxidant activities had acetone extracts. Therefore, these extracts were not used in further analysis for α-amylase and cell based assays.

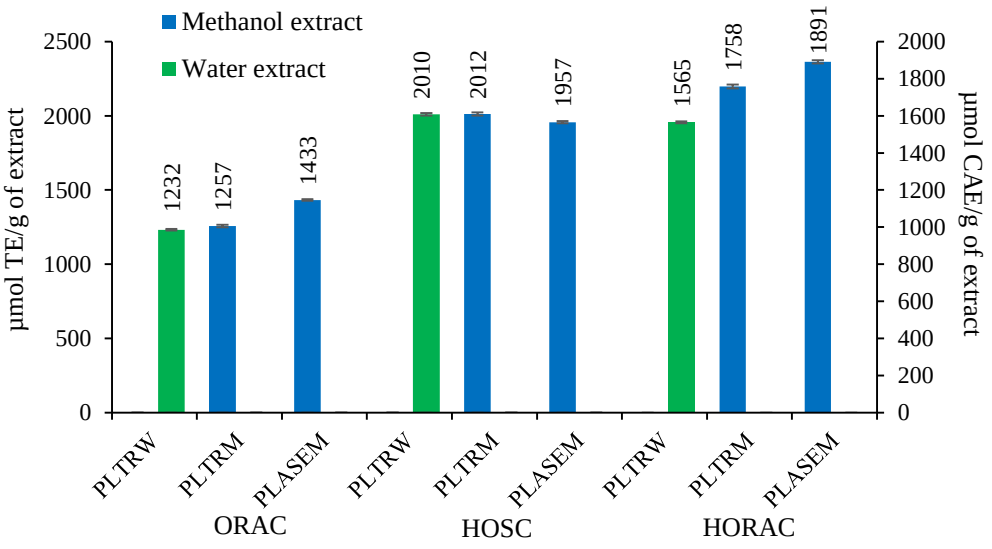


Figure 14. Antioxidant activity of peony extracts evaluated by the ORAC, HORAC and HOSC methods.

Antioxidant activities of peony extracts investigated in this study had a values varying from 1433 ± 0.67 to 1232 ± 0.5 μmol TE/g of extract, from 1891 ± 0.8 to 1565 ± 0.5 μmol CAE/g of extract and from 2012 ± 1.0 to 1957 ± 0.7 μmol TE/g of extract for ORAC, HORAC and HOSC assays, respectively (Figure 14). Antioxidant activity measured by HOSC showed the same relationships as did HORAC assay but ORAC values were lowest. The highest ORAC-value was measured for PLASEM extract, followed by PLTRM and PLTRW extracts. The highest HOSC-value showed PLTRM extract, followed by the PLTRW and PLASEM extracts. Finally, PLASEM extract showed the highest HORAC-value, followed by the PLTRM and PLTRW extracts. It could be concluded, that in all ORAC, HORAC and HOSC assays all peony extracts showed strong antioxidant activity. Because of that all peony extracts were used for further analysis. The differences in the antioxidant capacities found with the three methods were due to the different nature of the three assays.

3.5 In vitro α-amylase inhibitory properties of selected extracts

Type 2 diabetes mellitus is a progressive metabolic disorder of glucose metabolism which could be treated by decreasing the postprandial glucose levels. The α-amylase is enzyme responsible for the breakdown of complex polysaccharides into disaccharides. The inhibition of this enzyme could prolong overall carbohydrate digestion time causing reduction of glucose level in postprandial plasma [188].

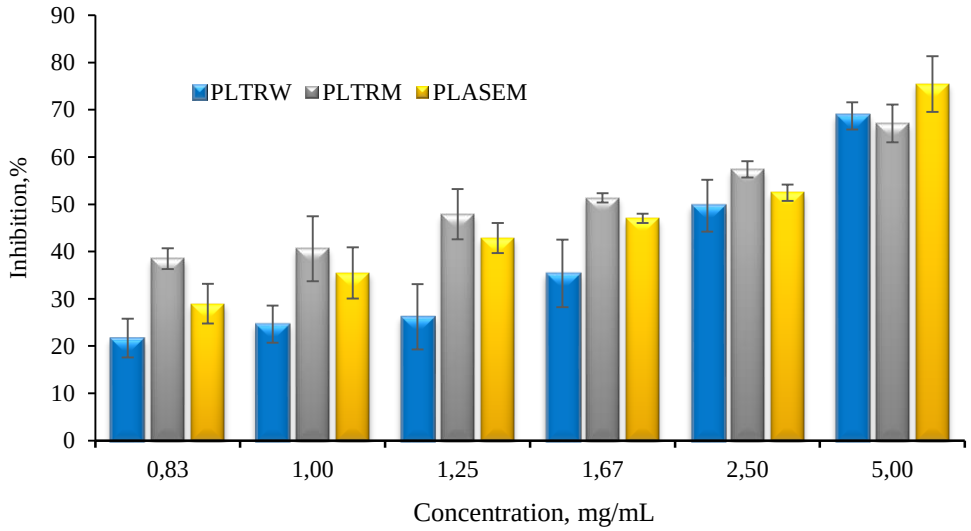


Figure 15. Inhibitory potency of *P. officinalis* methanol and water extracts against porcine α–amylase activity. The values are expressed as mean ± SD of triplicate tests.

In *vitro* α-amylase inhibition test was performed in order to investigate the inhibitory effect of selected extracts, 11 at all (8 extracts of *Nepeta* and 3 of peony). All 8 extracts of *Nepeta* possessed a weak α-amylase activity (inhibition do not reach 20 %. Data not shown). PLTRW showed a maximum percentage inhibition of 68.70 % at 5 mg/mL (Figure 15.). The inhibition

ranged from 68.70 to 21.68 %. Concentrations between 0.83 and 2.5 mg/mL failed to produce any detectable inhibition. PLASEM produced a maximum inhibition of 75.43 % at 5 mg/mL. At the lowest concentration of 0.83 mg/mL there was 28.97 % inhibition. The PLTRM extract produced a maximum inhibition of 67.10 % at a concentration of 5 mg/mL. The percentage inhibition ranged from 67.10 to 38.53 %.

The inhibition of α-amylase by PLTRM extract at lower concentrations (0.83 – 2.50 mg/mL) was higher by all the extracts, but at the highest concentration (5.0 mg/mL) the inhibitory potential of PLTRM extract was 2.33 % and 11.04 % lower than PLTRW and PLASEM extracts, respectively. The dose-response curve to determine the IC_{50} values of α-amylase inhibition for the *P. officinalis* extracts was used (Table 8). The IC_{50} values for PLTRM, PLTRW and PLASEM extracts are 1.67, 2.52 and 2.34 mg/mL, respectively. As can be seen all peony extracts showed lower inhibition comparing with acarbose (0.3 mg/mL).

Table 8. IC_{50} values for α-amylase inhibitory potential of *P. officinalis* leafs extracts.

Extracts	IC_{50} (mg/mL) α-amylase
1	2
PLTRW	2.52 ± 0.32
PLTRM	1.67 ± 0.17
PLASEM	2.34 ± 0.18
AC	0.30 ± 0.12

Values were expressed as mean ± SD of triplicate tests. AC – acarbose.

3.6 In vitro cytotoxic activity of obtained extracts

Cytotoxicity is characteristic of being toxic to cells. The response of cells then they are exposed to a cytotoxic compound can be in a number of ways. Firstly, they can stop dividing and growing. Secondly, cells can lose membrane integrity and die rapidly as a result of cell lysis or they can activate apoptosis, a genetically controlled cell death. The main concern is the toxicity of the extracts. Natural origin does not automatically imply that they are safe. Therefore, toxicological analysis of these extracts has to be done before further analysis with cells.

There are no reports on the cytotoxicity of selected methanolic and water extracts of *Nepeta* and peony species. Therefore, this research was initiated to screen the cytotoxic activity induced by *Nepeta* and peony methanol and water extracts in human epithelial colorectal adenocarcinoma (Caco-2) cell line. The results are presented in Figure 16. As can be seen that each *Nepeta* and peony extract does not show cytotoxic effect in the range from 12.5 to 0.097 mg/mL at four hours of treatment in a concentration dependent manner. It can be concluded that all extracts can be used in further analysis.

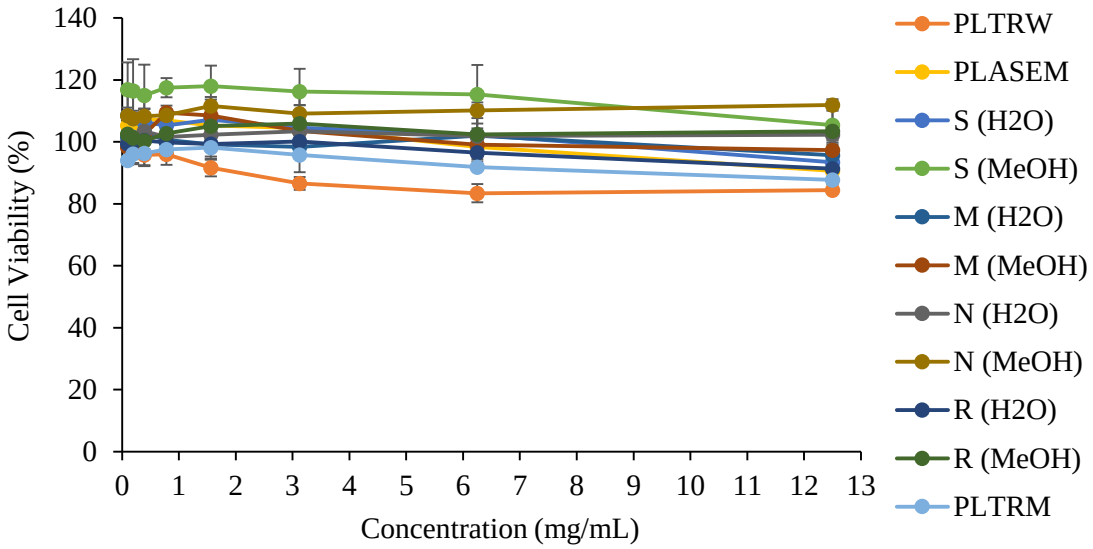


Figure 16. Cell cytotoxic analysis in Caco-2 cells treated with different concentrations of *Nepeta* and peony extracts. Cell cytotoxicity was determined for 4 h by using colorimetric MTS cell cytotoxicity assay. All data are showed as mean ± SD of three independent experiments each done in triplicate.

There are very few reports in the literature regarding the cytotoxicity of *Nepeta* species. In one of the studies, the chloroform leaf extract of *Nepeta deflersiana* was fractionated and 15 fractions were collected. All of them (except fraction 1) were found to be cytotoxic at 500 µg/mL concentration and above it against both tested cell lines MCF-7 and A-549 [189]. In another study was found that the methanolic leafs extract of *Nepeta deflersiana* had cytotoxicity with IC_{50} >50 [190]. Cytotoxicity of different extracts of *Nepeta juncea* was analysed using brine shrimp bioassay. Water fraction exhibited lower LD_{50} values (88.1253 mg/mL) as compared to the other fractions [191]. Essential oils of *N. cataria* var. citriodora and *Nepeta cataria* were evaluated for they cytotoxic properties on two human cancer cell lines (bronchial epithelial and keratinocyte). Oils were cytotoxic to both cells at IC_{50} values from 0.0012 to 0.015 % (v/v) [192].

3.7 Evaluation of the cellular antioxidant activity in Nepeta and peony leafs extracts

ORAC, HORAC and HOSC are useful assays to see if the compounds had antioxidant activity in general, but do not give any information about bioavailability in cellular physiological conditions after absorption and metabolism. For example, compound which has strong antioxidant activity *in vitro* is not necessarily biologically active *in vivo*. Therefore, CAA assay is used to get complimentary information about bioavailability of selected compounds in a cellular environment, in order to measure the antioxidant power inside human cells. In addition, ORAC and CAA assays used a fluorescent probe in order to measure the antioxidant power to prevent oxidation, also peroxyl radicals, the most common free radical in the human body [194, 195]. This assay was also performed in order to select the most effective extracts for further analysis with cells. The results are presented in Figure 17.

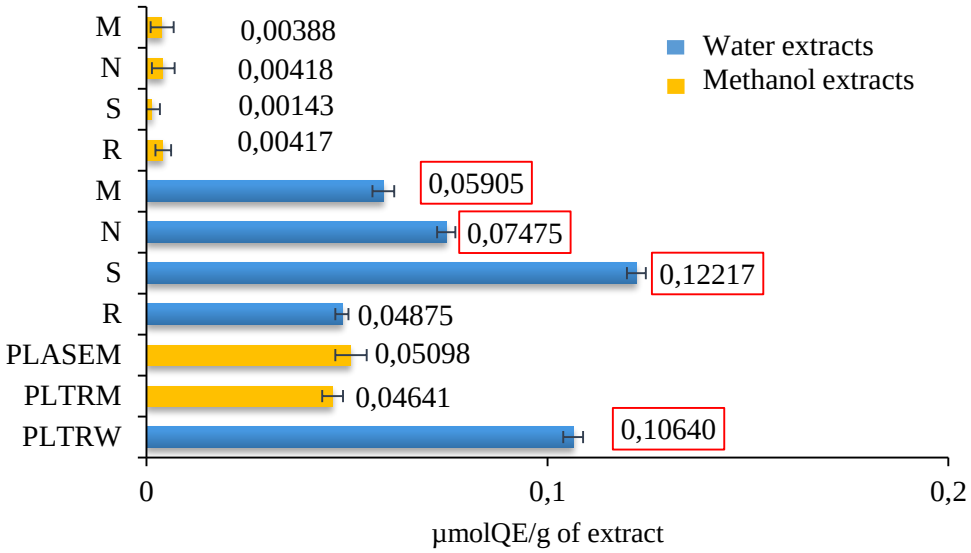


Figure 17. Cellular antioxidant activity of *Nepeta* and peony leafs extracts.

CAA-values in methanol extracts ranged from 0.00418 to 0.00143 µmol QE/mg of extract. Cellular antioxidant activity in methanol extracts decreases in the following order: N>R>M>S. In water extracts values were between 0.12217 – 0.04875 µmol QE/mg of extract and CAA decreases in the following order: S>N>M>R. The highest CAA-value was determined in water extract of *N. sibirica* (0.12217 µmol QE/mg of extract) it means higher CAA, while the lowest CAA-value was in methanol extract of *N. sibirica* (0.00143 µmol QE/mg of extract), it means lower CAA. As can be seen, better CAA properties showed water than methanol extracts (included all *Nepeta* variaties). Probably it could be due to higher amounts of rosmarinic and ferulic acids present in water extracts. These compounds have been associated with anticancer activity, which means that they share a significant antioxidant activity of extracts and it can be considered as one of the major contributors for the bioactivity of water extracts in *Nepeta* plants [196]. Peony leafs extract after TR with water have the highest CAA-value 0.10640 µmol QE/mg of extract, followed by peony leafs extract after ASE with methanol 0.05098 µmol QE/mg of extract and peony leafs extract after TR with methanol have the lowest CAA-value (0.04641 µmol QE/mg of extract). It is mean that cellular antioxidant activity of peony extracts increases as followed PLTRM<PLASEM<PLTRW. It also can be concluded, that extract of *N. sibirica* obtained after TR with water has the best antioxidant activity of all tested extracts (included and peony extracts). According to previous results of ORAC, HORAC and HOSC a clear relationship between CAA and AA was not found. It could be because of antioxidant's ability to cross cell membrane and survive metabolism by many enzymes and degradation processes in live cell, also it depends on nature of compound and quantity of antioxidants in represented extracts. Because of possessed higher cellular antioxidant activity it was decided that for further analysis three water extracts of *Nepeta* (*N. sibirica*, *N. melissifolia* and *N. nuda*) and one extract of peony (PLTRW) will be used.

3.8 Antiproliferative activity of selected extracts against human colon carcinoma HT29 line cells

Cancer is a lethal disease. According WHO (World Health Organisation) 8.2 million people each year die from cancer. In addition, colorectal cancer is one of top five cancers that usually diagnosed among women and men [197]. To treat the disease clinical attention is needed. Despite the advantages of the combinatorial and synthetic chemistry as well as molecular modeling, there is growing interest in chemotherapeutic application of natural substances. For this purpose a huge number of plant species is screened and bio-assayed worldwide. In several studies were reported that polyphenols which are presented in plants, fruits and vegetables have been pointed to be powerful anticancer ingredients [198, 199, 200].

In accordance to this worldwide trend four water extracts of *N. sibirica*, *N. nuda*, *N. melissifolia* and *P. officinalis* were evaluated for their anti-proliferative activity in human colon carcinoma cell line (HT29). The cells were cultured in RPMI 1640 medium and incubated for 24 and 48 hours.

In order to investigate cytotoxicity of the extracts after 24 and 48 h of incubation, parallel to antiproliferative assay, cytotoxicity of the extracts at the same concentration range were tested. Peony extract was contaminated with m/o, therefore to determned cytoxicity and antiproliferative activity was impossible. Because m/o have interfarence with the results. The results suggested that all extracts were not cytotoxic in Caco-2 cell model after 24 h of incubation. Results are presented in Figure 18.

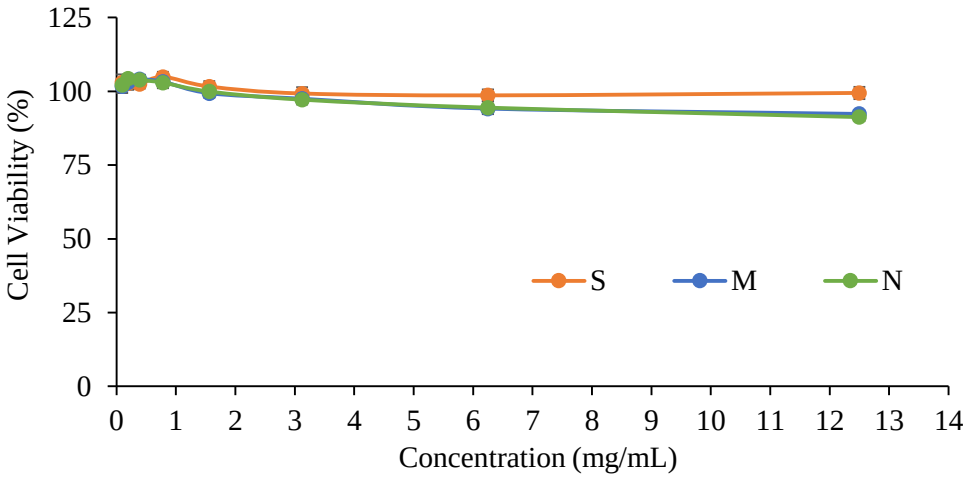


Figure 18. Cytotoxicity assessments of water extracts in Caco-2 cells by MTS assay. The cell there treated at various concentrations (12.5 to 0.097 mg/mL) for 24 h. Values are as mean ± SD of three independent experiments each done in triplicate.

Extracts after 48 h incubation were not cytotoxic in Caco-2 model cell line, with the exception of N. melissifolia water extract that was cytotoxic (cell viability < 50 %) at concentration above 6.98 mg/mL. Results are presented in Figure 19.

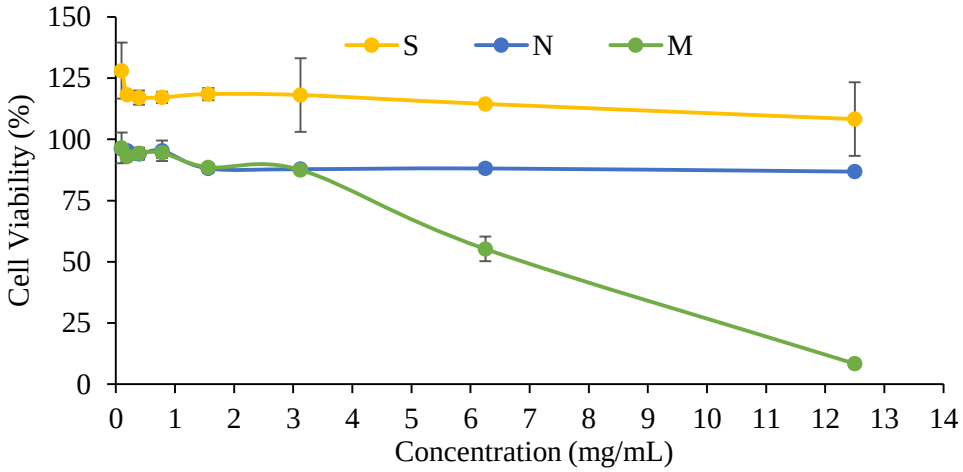


Figure 19. Cytotoxicity assessments of water extracts in Caco-2 cells by MTS assay. The cell there treated at various concentrations (12.5 to 0.097 mg/mL) for 48 h. Values are as mean ± SD of three independent experiments each done in triplicate.

Extracts of *N. sibirica*, *N. nuda* and *N. melissifolia* were applied at concentration that range from 12.5 to 0.097 mg/mL. As can be seen just two extracts of three inhibited cancer cell growth (Figure 20.). Better antiprolliferative activity after 24 h of incubation showed *N. nuda*. The effective dose (then inhibited 50 % of cells growth) was 7.5 mg of extract/mL. While the estimated effective dose of *N. melissifolia* was 1.13 times higher, it is mean 1.13 times less effective than *N. nuda*. While *N. sibirica* after 24 h of incubation do not reach 50 % of inhibition (data not shown).

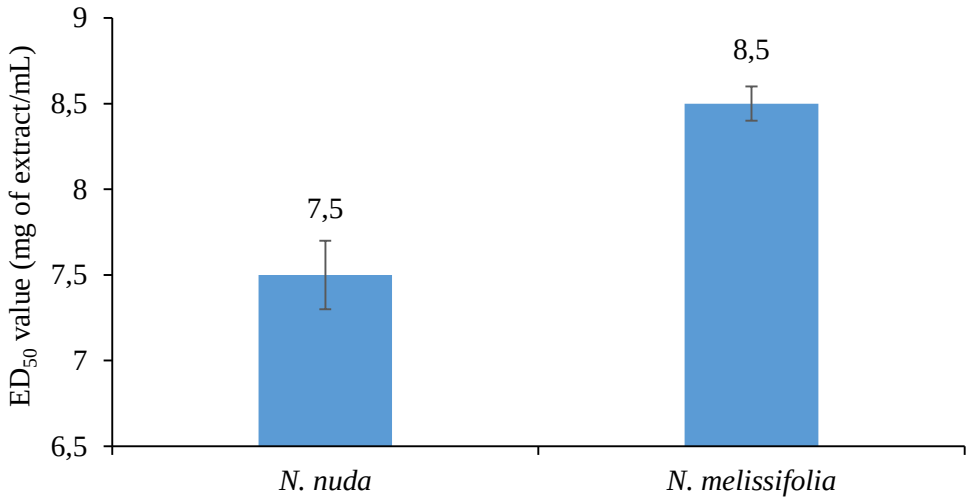


Figure 20. Antiproliferative activity of *Nepeta spp*. water extracts in HT29 cells after 24 h of incubation. Results present the ED_{50} (concentration then inhibited 50 % of cell growth) after 24 h of incubation. Results are the average and ± SD of three separate experiments done in triplicates.

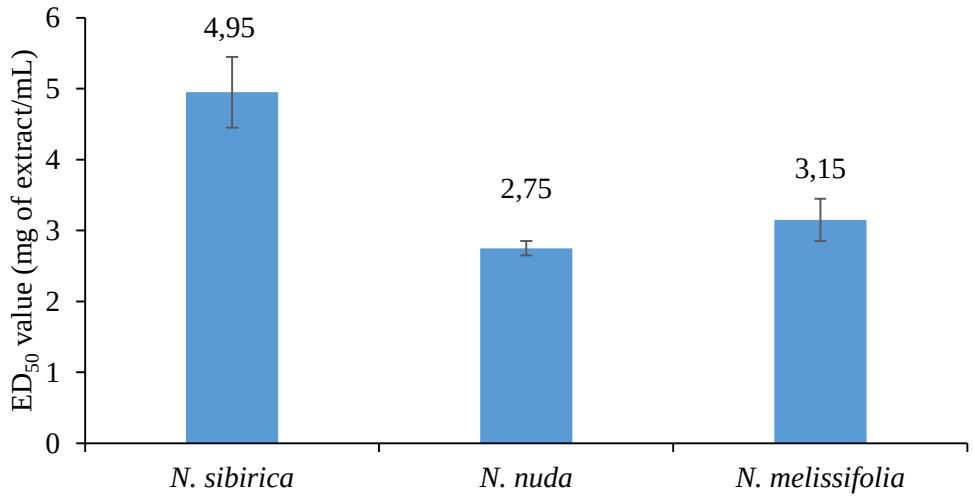


Figure 21. Antiproliferative activity of *Nepeta spp*. water extracts in HT29 cells after 48 h of incubation. Results present the ED_{50} (concentration then inhibited 50 % of cell growth) after 48 h of incubation. Results are the average and ± SD of three separate experiments done in triplicates.

The antiproliferative activity of tested extracts after 48 h of incubation are demonstrated in Figure 21. The results showed that at the same concentration range (from 12.5 to 0.097 mg/mL) three extracts inhibited cancer cell growth. The antiproliferative activity increases as followed: *N. sibirica*<*N. melissifolia*<*N. nuda*. After 48 h of incubation the highest antiproliferative effect showed water extract of *N. nuda* (2.75 ml of extract/mL) while the lowest effect possessed water extract of *N. sibirica* (4.95 ml of extract/mL).

It could be concluded that better antiproliferative effect in the same concentration range (from 12.5 to 0.097 mg/mL) after 24 and 48 h of incubation showed extracts after 48 h of incubation. Probably it could be because of longer cancer cell interaction time with extracts. *N. nuda* exhibited the highest antiproliferative effect of all tested extracts. It seemed that the bioactive effect of *N. nuda* can be related to higher TPC's content and composition in phenolic acids.

In general, after literature review was determined that this study for the first time describes antiproliferative effect of *N. sibirica*, *N. nuda* and *N. melissifolia* plant extracts on human colorectal cancer cell line HT29. The results showed that water extract of *N. nuda* is a good source of antiproliferative compounds, namely RA and FA. These compounds could be used for development for bioactive ingredients with promising application in pharmaceutical and food industries.

CONCLUSIONS

1. After extraction with different polarity solvents it was determined that extract yields depended on solvent polarity that was used for the extraction. The highest yields for peony and *Nepeta spp*. were obtained with methanol and water, respectively. Extraction of peony leafs and roots with methanol using accelerated solvent extraction gave yields of 50.88 % and 27.55 %, respectively. While highest yields of *Nepeta spp.* using different solvents, were obtained from *N. sibirica* (7.5 %), *N. racemosa* (5.48 %), *N. sibirica* (11.09 %) and *N. cataria* (36.21 %) using hexane, acetone, methanol and water, respectively.
2. After evaluation of extracts with various antioxidant methods it was determined that $DPPH^{\bullet}$ activity of tested extracts ranged from 4636.13 to 31.98 μM TE/g DW. The highest $DPPH^{\bullet}$ scavenging activity was found in *N. Mellisifolia* water extract - 972.77 μM TE/g DW and in peony extract - 4636.13 μM TE/g DW after traditional extraction with methanol. $ABTS^{\bullet+}$ activity of all tested extracts ranged from 4628.35 to. 130.97 μM TE/g DW. The highest activity was in *N. Racemosa* water extract (1347.22 μM TE) and in methanol extract of peony (4628.35 μM TE/g DW) obtained by traditional extraction. Total phenolics ranged from 965.65 mg GAE/g of extract to 51.38 mg GAE/g of extract. The highest values were determined in *N. melissifolia* (669.38 mg GAE/g of extract) water extract, while for peony (965.65 mg GAE/g of extract) in extract obtained by traditional extraction with methanol. After evaluation of antioxidant activity of solid residues using the QUENCHER approach method it was determined that in peony solid fractions there are higher amounts of active compounds comparing with *Nepeta* species. Therefore, peony solid materials could still be interesting source of bioactive compounds.
3. In total 54 compounds were detected in investigated extracts and 46 of them were identified. 29 compounds in *Nepeta* species and 25 compounds in peony were detected. The most common compounds found in *Nepeta s*pecies were phenolic acids (ferulic acid, chlorogenic acid, rosmarinic acid, caffeic acid and etc.), while in peony gallic acid derivatives (gallic acid, digallic acid, methyl gallate, methyl digallate, tri-galloyl-hexose and etc.).
4. Syringic acid, chlorogenic acid, caffeic acid, ferulic acid, luteolin derivatives, umbelliferone and rosmarinic acid in *Nepeta* species extracts and quinic acid, gallic acid derivatives, mudanpioside derivative, quercetin dihexoside, unknown compounds in peony extracts had the highest influence on $DPPH^{\bullet}$ radical scavenging of all separated compounds in investigated extracts.
5. After quantitative analysis of rosmarinic, ferulic and chlorogenic acids in *Nepeta* species it was found that ferulic acid was a major compound in water and acetone extracts, while

rosmarinic acid was dominant in methanol extracts. Quantities of chlorogenic acid were found to be lowest in all tested extracts. The highest amounts of reported acids were in *N. nuda*, *N. melissifolia*, *N. sibirica* and *N. racemosa*.

6. Due to their higher activities extracts of 5 species were selected for testing in ORAC, HORAC and HOSC modell systems. Among them water extracts of *N. sibirica* and *N. melissifolia* possessed highest activities in all used assays, while highest activities of peony extracts were shown by peony leafs extract after accelerated solvent extraction with methanol in ORAC and HORAC and peony leafs extract after traditional extraction with methanol in HOSC method.
7. 11 extracts, having highest radical scavenging activities, were selected for testing in α-amylase assay. After evaluation was determined that the best IC_{50} value had peony leafs extract after traditional extraction with methanol (1.67 mg/mL).
8. Cellular antioxidant activities of tested samples were higher in water extracts. The highest activity had water extract of *N. sibirica* (0.12217 μmol QE/mg of extract).
9. The results suggested that all extracts were not cytotoxic in Caco-2 cell model after 4 h and 24 h of incubation in the concentration range 12.5 to 0.097 mg/mL, while after 48 h of incubation only *N. melissifolia* water extract was cytotoxic (cell viability < 50 %) at concentration above 6.98 mg/mL. Best antiproliferative activity of selected extracts after 24 and 48 h of incubation was shown by water extract of *N. nuda* (7.5 mg/mL and 2.75 mg/mL, respectvely).
10. Tested plants of *Nepeta* and peony are a good source of the bioactive compounds, therefore these plants, except *N. melissifolia* water extract, which has some cytotoxic activity, could be used for development for bioactive ingredients with promising application in pharmaceutical and food industries.

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