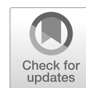
Chapter 14
Halophyte Species as a Source of Secondary Metabolites with Antioxidant Activity



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Abstract As naturally salt-tolerant plants, halophytes can grow in a variety of saline habitats due to the development of special adaptations, particularly secondary metabolites with antioxidant properties. Since, in order to overcome harsh environmental conditions, halophytes have the ability to produce phenolic molecules with powerful biological capacities, this interesting ecological group of plants gets more attention in recent years because of a rapid increase in demand for natural bioactive substances. Having in mind that specific conditions of saline habitats cause specific responses of biochemical pathways of plant metabolites, which is related to their biological activities, the developmental stage and yield of individual plant species together with environmental factors must be considered in further studies. In this paper, halophyte secondary metabolites with antioxidant properties were reviewed in terms of their contributions to ecophysiological adaptations. Additionally, a complete experimental screening – from plant sampling through the methodological procedure to the presentation of the obtained results – was displayed in order to

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enable the selection of appropriate screening method together with the proper methods of extractions and applications of obtained results.

Keywords Salt-tolerant plants · Halophytes · Phenolic acids · Flavonoids · Antioxidant activity · Screening · Extraction

14.1 Introduction

Salinity is common phenomena for the world arid and semiarid regions. Saline habitats and conditions of increased salinity cause development of special adaptations and different adaptive mechanisms of the salt-tolerant plants. These mechanisms are not yet clearly understood but include osmotic adjustment and succulence, ion transport and uptake regulation, ion compartmentation, salt exclusion and excretion, maintenance of redox and energetic status, regulation of Na^+/K^+ selectivity, as well as a range of biochemical and signaling pathways under complex genetic control (Hasegawa et al. 2000; Dajic 2006; Hasanuzzaman et al. 2013). As naturally salt-tolerant plants, halophytes can grow in a variety of saline habitats and extreme locations characterized with high temperature and salinity conditions such as coastal sand dunes, salt marshes, salt flats, and steppes (Ksouri et al. 2010). In the case of halophytes occurring in arid and semiarid regions, combined stresses (salinity and drought) resulting in the appearance of the reactive oxygen molecules, such as singlet oxygen ($^1O_2$), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•) (Xiong et al. 2002). The scavenging of reactive oxygen species (ROS) in plants exposed to salt and drought stress is associated with activity of the different enzymes involved in antioxidant defense mechanisms (Sekmen et al. 2012; Jakovljević et al. 2017), osmoprotectant compounds (Xiong et al. 2002), as well as nonenzymatic antioxidant compounds and secondary metabolites (Xiong and Zhu 2002; Ksouri et al. 2007).

Biosynthesis and accumulation of polyphenols as well as other secondary metabolites in plants is evolutionary response of biochemical pathways under environmental influences (biotic/abiotic constraints), including increased salinity and drought (Navarro et al. 2006; Meot-Duros et al. 2008; Selmar and Kleinwachter 2013). Consequently, to overcome harsh environmental conditions, halophytes have a particularity which lies in their ability to produce secondary compound, mainly phenolic molecules, with powerful biological capacities (Trabelsi et al. 2012). Those molecules originated from halophytes, including vitamins, phenolics, polysaccharides, and glycosides, displaying a wide variety of biological activities, including antioxidant, antimicrobial, and anti-inflammatory, and may be crucial for the prevention of several human diseases, e.g., inflammation, cancer, and cardiovascular disorders (Ksouri et al. 2012; Rodrigues et al. 2015). Since halophyte plant species have been evaluated as natural sources of phenolic compounds, as well as other secondary metabolites with biological properties in adaptive responses to stress conditions (Ksouri et al. 2007; Benhammou et al. 2009), and because of a rapid increase in demand for natural bioactive substances on the world market, there is a

need for further phytochemical investigation of species from this specific ecological group. Additionally, potential of some halophytic species for antioxidant activity and demonstration of antimicrobial, antiviral, anticancer, and other biological effects have been recently reported (Meot-Duros et al. 2008; Trabelsi et al. 2010; Lee et al. 2011; Oueslati et al. 2012).

This chapter aims to provide short insight into the salinity and environmental problems in saline habitats together with main characteristics of a halophytes as a specific ecological group adapted to these conditions. Plant secondary metabolism and plant secondary metabolites in these plants were discussed in the terms of their physiological adaptations. Having in mind that specific conditions of saline habitats cause specific responses of plant metabolism, which is related to their biological activities, halophytes were considered as a source of secondary metabolites with antioxidant activity. Finally, eight halophyte species were selected to serve as a model to display a complete experimental screening – from plant sampling through the methodological procedure to the presentation of the obtained results.

14.2 Plant Secondary Metabolites

Unfavorable growth conditions are a complex process leading to coordinated changes at different levels of plant metabolism. Plant secondary metabolites, the products of plant secondary metabolism which is a resumption of the essential primary metabolism, have a main role in their interaction in the environment. As per definition given by Verpoorte (2000) "secondary metabolites are compounds with a restricted occurrence in taxonomic groups, that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its ecosystem."

Major roles in the adaptation of plants to their environment belong to the products of secondary metabolism, and plant secondary compounds are usually classified according to their biosynthetic pathways (Bourgaud et al. 2001). Quantitative and qualitative composition of secondary metabolites in plant tissues and organs depends on numerous abiotic and biotic ecological factors (Oh et al. 2009). Secondary metabolites isolated from plants, in addition to the different roles in processes of the plant adaptation, exhibit many in vitro and in vivo biological effects. The mechanism of their biological actions is the stimulation or inhibition of the different metabolic processes (Faggio et al. 2015a, b, 2016; Korkina 2007; Trischitta and Faggio 2006, 2008). Therefore, the investigation of plant secondary molecules and their biological potential have the possibility of a number of scientific and practical applications (Stanković et al. 2015). Biosynthesis of phenylpropanoid compounds is passed from shikimate pathway and regulated by different enzymatic pathways (Ziaei et al. 2012). In the first step of the general phenylpropanoid biosynthetic pathway, the phenylalanine is deaminated by the enzyme phenylalanine ammonium lyase (PAL), and metabolites resulting from PAL activity can be classified as phenolic compounds including phenolic acids, coumarins, flavonoids, lignin, and other compounds (Ziaei et al. 2012; Rigano et al. 2016).

14.2.1 Phenolic Derivatives

The general structural characteristic of all phenolic derivatives is the hydroxyl group attached to the aromatic ring. A large group of natural products with this structural fragment is known as "natural aromatic compounds." They can be systematized in two groups: phenolic compounds and flavonoids. The first group consists of phenols, phenolic acids, and condensation products of phenol – dephides, phenylpropane, tannins, and stilbene – while the other group consists of flavonoids and anthocyanins (Crozier et al. 2006; Quideau et al. 2011). Different amounts of phenolic compounds from plant sources depend on their function in the plant itself, i.e., role in photosynthetic apparatus protection and then protection against herbivores and pathogens, as well as other biotic and abiotic factors to which the plant is exposed during growth and development (Alonso-Amelot et al. 2004). Physiological drought, as one of the common abiotic factors in saline habitats, caused increased intensity of synthesis and accumulation of secondary metabolites (usually phenolic compounds) in order to prevent the negative consequences of drought.

In the previous investigations of quantitative and qualitative characteristics of secondary plant metabolites, several thousand types of phenolic derivatives have been identified. Among them, phenolic compounds are present in all plant organs and are most commonly found in leaves (Stanković et al. 2012). The diversity of the phenolic compounds is the result of their ability to bond different sugar components that occupy different positions in the binding process (Boskou 2006). Phenolic compounds are characterized by a variety of applications in the pharmaceutical industry, medicine, and agricultural and food production. A wide spectrum of the use of phenolic compounds of plant origin is related to the structural properties of the molecule, in which their antioxidant, antimicrobial, and anticancer activity is observed, as well as numerous therapeutic effects (Manach et al. 2004; Tapas et al. 2008; Quideau et al. 2011). Due to the diverse chemical structure, phenolic compounds exhibit an effect at all levels of the organization in biological systems, which is the reason for their intensive application (Fraga 2010). The mechanism of antimicrobial action of phenolic acids is based on an intense increase in the permeability of the membrane, resulting in a loss of selective permeability where important biomolecules are uncontrolled out of the cell (Zaixiang et al. 2011). In addition, the mechanism of antimicrobial activity of phenols is based on interfering or completely blocking some of the functions of the cytoplasmic membrane, as well as inhibition of the enzymes necessary in the process of replication of bacteria. Characteristic of terpenoids is effects of disintegration of the cell membrane; coumarins exhibit antimicrobial activity by inhibition of cellular respiration, while tannins are characterized by inhibition of cell membrane functions and the activity of some important enzymes (Cushine and Lamb 2005). The mechanism of antitumor activity of secondary plant metabolites is different – some of them are carcinogenic inactivation, cell cycle blocking, proliferation inhibition, apoptosis induction, angiogenesis inhibition, control of the enzyme activity involved in the regulation of the cell cycle, and transport through the membrane (Ren et al. 2003; Khantamat et al. 2004; Khoo et al. 2010; Duangmano et al. 2010)

Phenolic acids are one of the many classes of phenolic compounds in plants. Based on their origin, they can be divided into benzoic acid or cinnamic acid derivatives (Robbins 2003). The biological role of phenolic acids as secondary metabolites in herbs is multiple. A very important role is reflected in the part of biochemical communication in the ecosystem, i.e., in the process of adaptation to biotic and abiotic stress (Mandal et al. 2010). Phenolic acids are presented in all types of herbs, especially in medicinal and herbal plants, fruits, and vegetables. They can be found in leaves, flowers, fruits, and seeds, but their quantitative and qualitative relationship varies and is not the same in all plant organs during the vegetative phases because of the influence of the abiotic and biotic factors under which the plant is exposed (Wang et al. 2011). Phenolic acids are with ether or ester bonds related to some protein molecules, carbohydrates, other phenolic acids, or some organic acids and secondary metabolites such as terpenes, lignin, or lignans. In addition, phenolic acids are necessary precursors in the process of biosynthesis of some flavonoids and other complex phenolic acids (Gorshkova et al. 2000; Ascensao and Dubery 2003; Bunzel et al. 2004). In addition to the high number of biological effects in plants, phenolic acids isolated from the plants show strong biological effects in in vitro and in vivo conditions by exhibiting antimicrobial, antioxidant, anticancer, and many other activities (Merkl et al. 2010; Maistro et al. 2011; Kang et al. 2011). Previous investigations of halophyte species in terms of major specialized metabolites revealed the significant amount of different phenolic acids. Among them, caffeic, chlorogenic, gallic, syringic, ellagic, rosmarinic, and ferulic acid were found to be predominant in extracts obtained from *Statice gmelinii*, *Mentha pulegium*, *Salvadora persica*, and *Crithmum maritimum* and species from the genus *Artemisia* (Serkerov and Aleskerova 1984; Korulkina et al. 2004; Meot-Duros and Magne 2009; Ivanescu et al. 2010; Tahira et al. 2011; Pereira et al. 2017a, b; Qasim et al. 2017). The chemical structures of several phenolic acids from halophyte species are presented in Fig. 14.1.

14.2.2 Flavonoids

To date, about 9000 different flavonoids of plant origin are known. Main structural characteristic of these secondary metabolites is the presence of a heterocyclic molecular structure consisting of two benzene rings associated with an aliphatic sequence. Due to numerous modifications of the basic skeleton regarding the change in the oxidation state of the aliphatic sequence, plant possess wide variety of structurally different flavonoids located in plant cell vacuoles (Buer et al. 2010; Mierziak et al. 2014).

Flavonoids are the derivatives of 2-phenyl-benzyl-γ-pyrone whose biosynthetic pathway, as part of phenylpropanoid metabolism, begins with the condensation of one *p*-coumaroyl-CoA molecule with three molecules of malonyl-CoA with the formation of chalcones (4,2,4,6-tetrahydroxychalcone). The next step in the synthesis of flavonoids is the isomerization of chalcones to flavanone, and from this step,

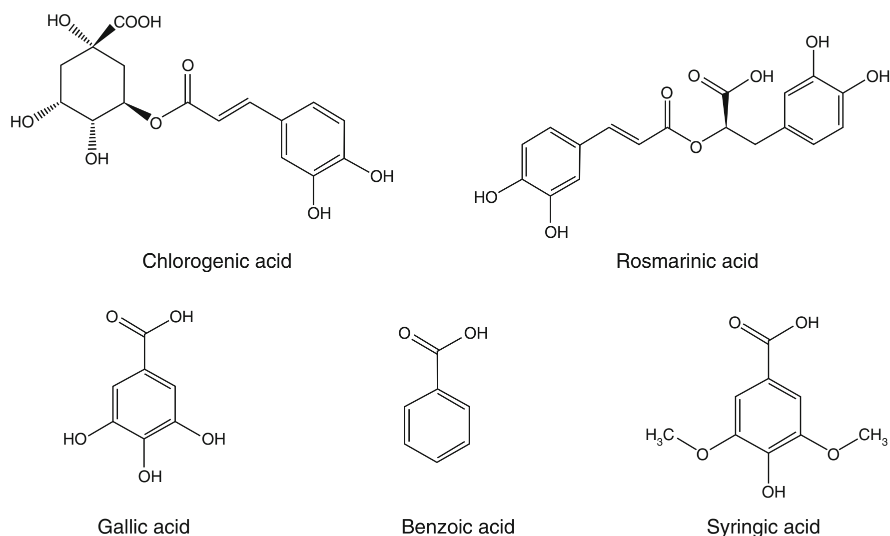


Fig. 14.1 The chemical structures of some phenolic acids from halophyte species

the synthetic pathway branches to several different classes of flavonoids, including aurones, dihydrochalcones, isoflavones, flavones, flavonols, anthocyanins, proanthocyanidins, and others (Buer et al. 2010; Mierziak et al. 2014).

The resulting flavonoids undergo further modifications followed by changes in solubility, reactivity, and stability. Due to the diversity in the chemical structure resulting from the diversity of substituents, flavonoids exhibit several significant functions in plants such as protection against pathogens, pollen germination, ultraviolet radiation protection, antioxidant functions, etc. The antioxidative role of flavonoids is based on the ability to neutralize free radicals, thereby preventing cell damage and allowing plant adaptation to different environmental conditions. This antioxidative role of flavonoids in correlation with the number and position of hydroxyl groups bound to the phenolic ring (Gill and Tuteja 2010; Mierziak et al. 2014). The important biological role of flavonoids in plants is associated with biochemical communication of plants in ecosystems, and the distribution together with the quantitative and qualitative composition of these compounds varies depending on both genetic and ecological factors (Stanković et al. 2012).

The predominance of the polyphenol family in particular the flavonoids was found in different plant species from the saline habitats. The naringin and naringenin 7-O-glucoside are very common in species of Chenopodiaceae family (Al-Jaber et al. 1991). High concentration of flavonoids was obtained from *Atriplex hortensis* (Yang et al. 2008) and *A. halimus* (Benhammou et al. 2009). Moreover, the flavonol class forms the major chemical compounds of species from the *Atriplex* genus (Sanderson et al. 1988). Also, Chenopodiaceae family is characterized by the presence of glycinebetaine as an alternative osmolyte to protect from salt and water stress (Benhammou et al. 2009). High content of flavonoids was also reported

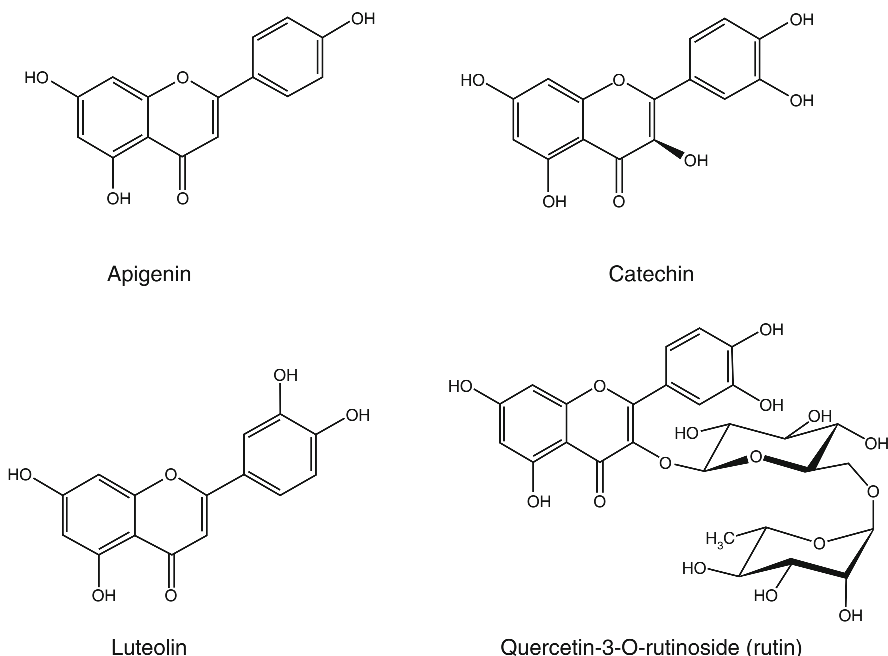


Fig. 14.2 The chemical structures of some flavonoids from halophyte species

in the case of *Limoniastrum guyonianum* (Trabelsi et al. 2012); *Limonium algarvense* (Rodrigues et al. 2015); species from saline habitats of the south of Portugal including *Halopeplis amplexicaulis*, *Cladium mariscus*, *Frankenia pulverulenta*, and *Salsola vermiculata* (Lopes et al. 2016); as well as Tunisian halophytes *Thespesia populneoides*, *Salvadora persica*, *Ipomoea pes-caprae*, *Suaeda fruticosa*, and *Pluchea lanceolata* (Qasim et al. 2017). Different studies also revealed the presence of epicatechin, pyrocatechol, catechin, rutin, luteolin, quercetin, myricetin, apigenin, avicularin, phloretin, and procyanidin B2 as major flavonoids in different halophyte species (Falleh et al. 2011; Ksouri et al. 2009, 2012; Rodrigues et al. 2014; Medini et al. 2015; Jdey et al. 2017; Oueslati et al. 2012a; Pereira et al. 2017a, b; Qasim et al. 2017). The chemical structures of several flavonoids from halophyte species are presented in Fig. 14.2.

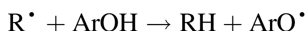
14.3 Antioxidant Activity of Phenolic Compounds

The overproduction of reactive species (ROS, RNS), together with the imbalance between their production and the detoxification system capacity, leads to the generation of oxidative stress. The equilibrium between positive and negative effects of free radicals is essential for living organisms, since the oxidative stress may

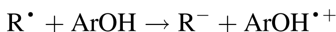
represent underlying causes of several stress-related human diseases including cancer, diabetes, cardiovascular disorders, inflammatory disease, and Alzheimer (Shoham et al. 2008; Benhammou et al. 2009; Hajhashemi et al. 2010). Humans possess endogenous antioxidant defense system against free radicals; however, this system sometimes is not sufficient to prevent the occurrence of cell damage (Rechner et al. 2002). Since it is well known that antioxidants neutralize harmful effects of free radicals, synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are widely used in food industry. Although synthetic antioxidants have widespread use in many countries, there are growing evidences that these molecules may be implicated in toxic and carcinogenic effects (Sasaki et al. 2002; Jennings and Akoh 2009; Sindhi et al. 2013). Hence, it is necessary to replace synthetic compounds with natural antioxidants.

Natural antioxidants are secondary metabolites produced naturally in plant organism. They are mainly phenolic compounds in the function of plant defense mechanisms to counteract reactive oxygen species (ROS) to avoid consequences of oxidative stress. Antioxidant properties of secondary metabolites from the group of phenolic compounds arise from their redox properties and high reactivity as hydrogen or electron donors, from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), ability to scavenge a variety of reactive species (superoxide, hydroxyl, and peroxyl radicals), and ability to chelate transition metal ions (Halliwell et al. 2005; Rohma et al. 2010; Falleh et al. 2012; Jdey et al. 2017). Additionally, polyphenolic constituents demonstrate several biological properties useful in the management of the stress-related diseases (Sousa et al. 2015; Zengin et al. 2015).

Plants represent a significant source of natural antioxidants. In this sense, in recent years, extracts of many plant species become popular in attempts to characterize their antioxidant activity and potential utilization as rich sources of antioxidants (Gourine et al. 2010; Ksouri et al. 2012). Plant phenolic compounds, among the various types of natural antioxidants, represent the leading and most powerful plant secondary metabolites with multiple applications such as additives, functional food, and medicinal and pharmaceutical materials (Povichit et al. 2010). The mechanism of action of plant antioxidant substances is based on their role as a donor of an electron or a hydrogen atom. The antioxidant ability of phenolic compounds depends on the number and position of hydroxyl groups. In addition, bond dissociation energy (BDE) and ionization potential (IP) are also very important characteristics. One of the main mechanisms of the antioxidant activity of phenolic compounds is the role of hydrogen donor (*HAT – hydrogen atom transfer*) and the role of electron donors (*SET – single electron transfer)* to the molecule exposed to the oxidation process (Quideau et al. 2011). The mechanism of these reactions has the following form:



$$R^{\bullet} + ArOH \rightarrow RH + ArO^{\bullet}$$



$$R^{\bullet} + ArOH \rightarrow R^{-} + ArOH^{\bullet +}$$

As natural antioxidants, phenolic compounds significantly contribute to antioxidant activity of the plants. To date, halophytes are recognized as plants with high amount of total phenolic content together with significant antioxidant activity. Significant content of phenolics as well as antioxidant activity has been confirmed in the case of *Atriplex halimus* (Benhammou et al. 2009); *Inula crithmoides* (Jallali et al. 2014; Jdey et al. 2017); *Crithmum maritimum* (Meot-Duros and Magne 2009; Jallali et al. 2014; Pereira et al. 2017a); *Halimione portulacoides* (Vilela et al. 2014); *Limoniastrum guyonianum* and *L. monopetalum* (Ksouri et al. 2008; Trabelsi et al. 2012; Lopes et al. 2016); *Limonium algarvense*; *L. tetragonum* and *L. gmelinii* (Korulkina et al. 2004; Lee et al. 2011; Rodrigues et al. 2015, 2016); *Mesembryanthemum edule* (Falleh et al. 2011); *Frankenia laevis* and *F. pulverulenta* (Lopes et al. 2016; Jdey et al. 2017); *Lythrum salicaria*, *Cladium mariscus*, *Aster tripolium*, and *Typha domingensis* (Lopes et al. 2016); species from the genus *Suaeda* (Oueslati et al. 2012a, b; Stanković et al. 2015; Qasim et al. 2017); *Tamarix gallica* (Ksouri et al. 2009); *Plantago coronopus* (Jdey et al. 2017); *Statice gmelinii*, *Mentha pulegium*, *Camphorosma monspeliaca*, *Salicornia europaea*, *Suaeda maritima*, *Artemisia santonicum*, *Achillea collina*, and *Aster tripolium* (Stanković et al. 2015); and *Thespesia populneoides*, *Salvadora persica*, *Ipomoea pes-caprae*, *Suaeda fruticosa*, and *Pluchea lanceolata* (Qasim et al. 2017). Halophyte species from the genus *Mentha* also demonstrated significant content of phenolic compounds. According to Sarikurkcu et al. (2012) high content of phenolic compound can be obtained from methanolic extracts of *Mentha pulegium* (97.20 mg GA/g of extract), while, according to Hajlaoui et al. (2009), extracts from *Mentha longifolia* also possess high content of phenolics (89.1 mg GA/g).

Ksouri et al. (2007) analyzed content of phenolic compounds together with antioxidant activity of *Cakile maritima* leaves from dry and humid habitats, under limited substrate conditions. It has been found that plants from dry habitat are more resistant, and the resistance is probably associated with higher values of phenolic compound and antioxidant activity comparing the plants from the humid habitat. Meot-Duros and Magne (2009) analyzed the content of total phenolic compounds, the amount of chlorogenic acid, and the antioxidant activity in leaves obtained from populations of this species in different habitats – sand and rocks. It was found that the plants from the sand habitat had a higher content of chlorogenic acid than plants with rocky substrate, but certainly both populations had a high content of phenolic compounds. By comparing physiological parameters of the typical coastal species *Calystegia soldanella* from natural habitats and cultivated conditions, obtained results indicated that the phenol content was significantly higher in plants from natural habitats compared to cultivated plants. This may suggest that phenolic substances play a key role in response to these plants to the limitations of coastal habitats (Spano et al. 2013). Quantitative and qualitative composition and antioxidant activity of metabolites from halophyte species depend on taxonomic affiliation of a

species, biological (organ or developmental stage), environmental, and technical (extraction solvent) factors (Ksouri et al. 2008).

14.4 Example for Experimental Screening of Phenolic Content and Antioxidant Activity of Halophytes

Due to the natural specificity of the composition of secondary metabolites – active substances with biological activity – halophytes are the subject of many aspects of laboratory testing. As in the case of testing of other potential medicinal plants from different ecological groups, from natural or laboratory conditions, laboratory testing of halophytes is differentiated into two important ways – testing of quantitative and qualitative composition of secondary metabolites and testing of biological activity using different model systems. In the experimental process, extracts are obtained using a variety of solvents such as methanol, ethanol, water, acetone, ethyl acetate, etc. as well as tinctures and decocts prepared according to the different procedures.

Before a detailed analysis using chromatographic methods, a group of spectrophotometric methods is used to determine their total amount or preliminary quantification of important groups and subgroups of secondary metabolites. Chemical identification and detailed determination of the amount of secondary metabolites are carried out using various chromatographic methods. Chromatography methods are also used to isolate the target components and their further laboratory processing. Biological activity testing involves the application of several levels of in vitro testing of antioxidant, antimicrobial, anticancer activity, etc., followed by in vivo testing on different systems. Standard substances, such as synthetic compounds with antioxidant activity, or extracts of known herbs with the described industrial use are indispensable in both directions of the assay. In the study of the amount of active substances, their content in extracts, decocts, tinctures or other, is expressed in the form of equivalents of standard substances. In the study of biological activity, standard substances are examined in parallel in the goal of the comparison and evaluation of the results obtained. For the purpose of efficient presentation and interpretation of the obtained results, a large number of special statistical programs are used. In addition to noninfectious statistical evaluation, tests are used to determine the correlation between the amount of active substances and their biological activity, determining the dosage dependence, etc.

14.4.1 Experimental Procedure

The experimental procedure of the presented model for the screening of the antioxidant activity of the halophyte species contains a description of the methods starting from the preparation of the samples to the presentation of the obtained results. The

methods enable preliminary screening of the total amount of phenolic compounds, flavonoids as an important phenolic group, as well as in vitro antioxidant activity using the DPPH reagent.

14.4.1.1 Plant Material

Halophytes *Echinophora spinosa* L., *Crithmum maritimum* L. and *Eryngium maritimum* L. (Apiaceae), *Medicago marina* L. (Fabaceae), *Halimione portulacoides* (L.) Aellen (Amaranthaceae), *Calystegia soldanella* L. (Convolvulaceae), *Cakile maritima* Scop. (Brassicaceae), and *Limonium gmelinii* (Willd.) Kuntze (Plumbaginaceae) were collected from natural habitats in south Adriatic coastal zone (Fig. 14.3). *Crithmum maritimum* L. and *Cakile maritima* Scop. were collected from coastal rocks of Mogren, Budva (Montenegro), while other species were sampled from the location of Ada Bojana, Ulcinj (Montenegro), in May 2014. The voucher specimens were confirmed and deposited at the Herbarium of the Department of Biology and Ecology of Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at ambient temperature. The dried plant material was cut up and stored in tightly sealed dark containers until needed.

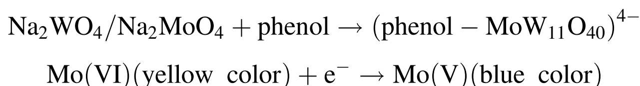
14.4.1.2 Preparation of Plant Extracts

The air-dried plant material (10 g) was coarsely crushed in small pieces of 2–6 mm by using the cylindrical crusher and extracted with ethanol 200 ml. The extract was filtered using a paper filter (Whatman, No. 1) and evaporated under reduced pressure by the rotary evaporator. The obtained extracts were stored in dark glass bottles until use.

14.4.1.3 Determination of Total Phenolic Content in the Plant Extracts

The content of phenolic compounds in the plant extracts was determined by using spectrophotometric method with Folin-Ciocalteu reagent (Singleton et al. 1999). Folin-Ciocalteu reagent contains, in its constitution, phosphomolybdic/phosphotungstic acid complexes. The determination assay is based on the transfer of electrons in alkaline medium from plant phenolic compounds to molybdenum, forming blue complexes that can be monitored spectrophotometrically at 750–765 nm. Therefore, the spectrophotometric measurement is based on quantification of the reducing capacity of phenolic compounds from the plant extract. Proton and a phenoxide anion formed in their discharge reaction reduce the Folin-Ciocalteu molecule to the ion responsible for the blue color of the reaction mixture (Magalhães et al. 2008). The reaction mechanism can be presented according to the following equations:

Fig. 14.3 *Echinophora spinosa* L., *Crithmum maritimum* L., *Eryngium maritimum* L., *Medicago marina* L., *Halimione portulacoides* (L.) Aellen, *Calystegia soldanella* L., *Cakile maritima* Scop., and *Limonium gmelinii* (Willd.) Kuntze

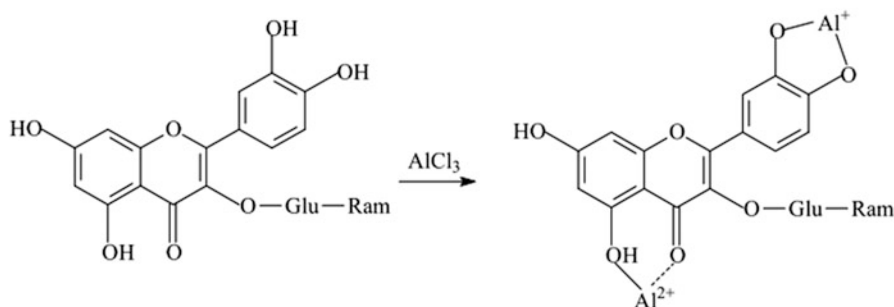


$$Na_2WO_4/Na_2MoO_4 + \text{phenol} \rightarrow (\text{phenol} - MoW_{11}O_{40})^{4-}$$
$$Mo(VI)(\text{yellow color}) + e^{-} \rightarrow Mo(V)(\text{blue color})$$

The methanolic solution of the plant extract in concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of the extract, 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water, and 2 ml of 7.5% $NaHCO_3$. The blank was concomitantly prepared containing 0.5 ml of methanol, 2.5 ml of 10% Folin-Ciocalteu reagent dissolved in water, and 2 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated at 45 °C for 15 min. The absorbance was measured spectrophotometrically at $\lambda_{max} = 765$ nm. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained. The same procedure was repeated for the gallic acid, and the calibration curve was construed. Based on the obtained absorbance, the content of phenolics was calculated (mg/ml) from the calibration curve; then the content of phenolics in the extracts was expressed in terms of gallic acid equivalent, GAE (mg of GA/g of extract).

14.4.1.4 Determination of Total Flavonoid Content in the Plant Extracts

The values for flavonoid content in the examined plant extracts were measured using spectrophotometric method (Quettier et al. 2000). This assay is based on formation of acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavonoids in addition with aluminum chloride. Aluminum chloride also forms acid labile complexes with the ortho-dihydroxyl groups in the A or B ring of flavonoids. The spectrophotometric measurement is based on the quantification of the color change in this reaction, at 400–420 nm. The mechanism of this reaction – the formation of metallocomplex in the case of the rutin molecule – is the following:

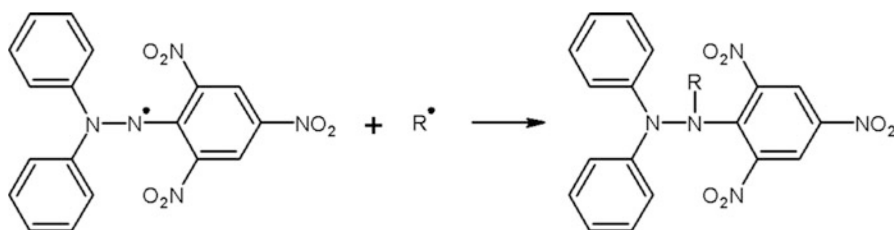


The sample contained 1 ml of methanolic solution of the examined extract in concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ methanolic solution. The samples were incubated for an hour at room temperature. The absorbance was determined

using spectrophotometer at of $\lambda_{max} = 415$ nm. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was measured. The same procedure was repeated for the solution of rutin, and the calibration curve was construed. Based on the measured absorbance, the content of flavonoids was calculated (mg/ml) on the calibration curve; then, the amount of flavonoids in extracts was expressed in terms of rutin equivalent, RUE (mg of RU/g of extract).

14.4.1.5 Evaluation of Antioxidant Activity of the Plant Extracts

The capacity of the plant extract to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was determined by the standard method (Takao et al. 1994), adopted with some corrections (Kumarasamy et al. 2007). Molecules of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) are, based on the chemical structure, stable free radicals. In dry form, the DPPH powder is characterized by a dark-purple to black color with absorption peak at 517 nm. After dissolution, color loses its intensity, depending on the concentration. After reduction, the color of the solution changes to yellow, depending on the concentration of the reduced molecules in the reaction mixture. Antioxidant compounds as donors of the hydrogen atom reduce the stable 1,1-diphenyl-2-picrylhydrazine radical by transforming it into 1,1-diphenyl-2-(2,4,6-trinitrophenyl)-hydrazine. The reduction process can be presented by the following reaction:



During this process, the purple color of the initial solution derived from the stable DPPH radical is changed to yellow due to the presence of a newly formed 1,1-diphenyl-2-(2,4,6-trinitrophenyl)-hydrazine resulting in a decrease in absorbance at 517 nm. This property allows visual and spectrophotometric monitoring of the reaction, i.e., the quantity of radicals at the beginning can be determined by changing the absorbance at 517 nm or by the ECP signal of the DPPH radical. In an experimental study of the antioxidant activity of plant extracts, the intensity of color change from violet to yellow is in correlation with the antioxidative potential of the plant extract (Fig. 14.4), which can be monitored by spectrophotometric measurement. Based on this, the DPPH method is widely used to measure the ability of different substances to act as free radical scavengers or hydrogen donors as well as to evaluate antioxidant activity of plant compounds as plant extracts or isolated metabolites.

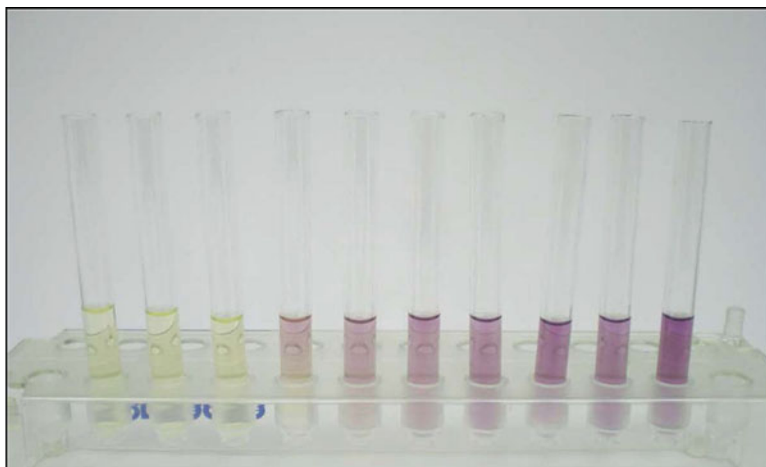


Fig. 14.4 Series of samples with a decreasing gradient of the plant extract concentration after the reaction with the DPPH reagent

For the experimental procedure when applying this method, DPPH (20 mg) was dissolved in methanol (250 ml) to obtain the concentration of 80 μg/ml. The start solution of the examined extracts was prepared in methanol to obtain the concentration of 1 mg/ml. Dilutions were made to achieve concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, and 0.97 μg/ml. Obtained extract samples (1 ml each) were combined with DPPH solution (1 ml).

After 30 min of incubation in darkness at ambient temperature, the absorbance was measured at 517 nm. The control samples contained all the reagents except the plant extract. The percentage inhibition was obtained using Eq. (14.1), while IC_{50} values were estimated from the percentage inhibition versus concentration plot, using a nonlinear regression algorithm. The data were presented as mean values ± standard deviation ($n = 3$):

$$\%\ \text{inhibition} = \left(\frac{A\ \text{of control} - A\ \text{of sample}}{A\ \text{of control}}\right) \times 100 \tag{14.1}$$

14.4.2 Results and Further Implications

14.4.2.1 Total Phenolic Content of the Plant Extracts

The obtained values of total phenolic content for the studied halophytes, quantified by measuring of the amount in the ethanolic extracts using Folin-Ciocalteu's reagent, are presented in Table 14.1. The values obtained for the total content of

Table 14.1 Total phenolic content in the extracts of analyzed halophytes, expressed as gallic acid equivalent – mg GA/g extract

Species	mg GA/g of extract
Echinophora spinosa	104.66 ± 0.82
Crithmum maritimum	106.08 ± 1.37
Eryngium maritimum	44.79 ± 0.58
Medicago marina	34.30 ± 0.87
Halimione portulacoides	30.51 ± 0.41
Calystegia soldanella	74.39 ± 0.72
Cakile maritima	62.06 ± 1.37
Limonium gmelinii	90.81 ± 0.79

Values in the table are shown as the mean value of the obtained results for the three samples ± standard deviation

phenolic compounds in plant extracts of analyzed halophytes were expressed as the equivalent of gallic acid – mg of gallic acid per gram of extract (mg GA/g).

The obtained values for the total amount of phenolic compounds in the plant extracts of analyzed halophytes have a range from 30.51 to 106.08 mg GA/g of extract. The highest value was obtained for the species *Crithmum maritimum* (106.08 mg GA/g) and *Echinophora spinosa* (104.66 mg GA/g), followed by *Limonium gmelinii* (90.81 mg GA/g). The average amount was obtained for plant extracts of *Calystegia soldanella* (74.39 mg GA/g) and *Cakile maritima* (62.06 mg GA/g), as well as the lowest value for *Eryngium maritimum* (44.79 mg GA/g), *Medicago marina* (34.30 mg GA/g), and *Halimione portulacoides* (30.51 mg GA/g).

14.4.2.2 Total Flavonoid Content of the Plant Extracts

The obtained values of flavonoid content in plant extracts of the studied halophytes, determined by described method using ethanolic extracts and $AlCl_3$ as reagent, are showed in Table 14.2. The values obtained for the flavonoid content were presented as the equivalent of rutin – mg of rutin per gram of extract (mg Ru/g). The values obtained for the total content of flavonoids in the plant extracts of the investigated halophytes are in the range from 15.71 to 22.15 mg Ru/g. The highest value was obtained for the species *Limonium gmelinii* (22.15 mg Ru/g) and *Cakile maritima* (22.13 mg Ru/g), followed by *Medicago marina* (21.86 mg Ru/g), *Crithmum maritimum* (21.36 Ru/g), and *Halimione portulacoides* (20.93 mg Ru/g). The average amount was obtained for plant extracts of *Echinophora spinosa* (18.69 mg Ru/g) and *Calystegia soldanella* (18.24 mg Ru/g), as well as the lowest value for *Eryngium maritimum* (15.71 mg Ru/g).

14.4.2.3 Antioxidant Activity of the Plant Extracts

Obtained values for antioxidant activity of examined plant extracts, expressed in terms of IC_{50} ($\mu g\ ml^{-1}$) values, are presented in Table 14.3.

Table 14.2 Total flavonoid content in the extracts of analyzed halophytes, expressed as rutin equivalent – mg Ru/g extract

Species	mg Ru/g of extract
Echinophora spinosa	18.69 ± 0.25
Crithmum maritimum	21.36 ± 0.45
Eryngium maritimum	15.71 ± 0.15
Medicago marina	21.86 ± 0.19
Halimione portulacoides	20.93 ± 0.41
Calystegia soldanella	18.24 ± 0.20
Cakile maritima	22.13 ± 0.32
Limonium gmelinii	22.15 ± 0.07

Values in the table are shown as the mean value of the obtained results for the three samples ± standard deviation

Table 14.3 Antioxidant activity of the extracts of analyzed halophytes, expressed as IC_{50} values (μg/ml)

Species	IC_{50} (μg/ml)
Echinophora spinosa	163.55 ± 0.62
Crithmum maritimum	195.26 ± 1.41
Eryngium maritimum	827.85 ± 0.67
Medicago marina	1317.95 ± 1.98
Halimione portulacoides	1237.14 ± 1.81
Calystegia soldanella	547.52 ± 0.95
Cakile maritima	365.36 ± 0.42
Limonium gmelinii	196.79 ± 0.56

Values in the table are shown as the mean value of the obtained results for the three samples ± standard deviation

For the results of antioxidant activity displayed in this way, the intensity of activity is inversely proportional to the numerical IC_{50} (a lower numerical value indicates better activity of the plant extracts) values. Values for antioxidant activity of examined plant extracts ranged from 163.55 to 1317.95 μg ml^{-1}. The strong antioxidant activity was detected in the *Echinophora spinosa* plant extract (163.55 μg ml^{-1}), followed by *Crithmum maritimum* (195.26 μg ml^{-1}) and *Limonium gmelinii* (196.79 μg ml^{-1}). The average antioxidant activity was measured for plant extracts of *Cakile maritima* (365.36 μg ml^{-1}) and *Calystegia soldanella* (547.52 μg ml^{-1}) and *Eryngium maritimum* (827.85 μg ml^{-1}) as well as the lowest value for *Halimione portulacoides* (1237.14 μg ml^{-1}) and *Medicago marina* (1317.95 μg ml^{-1}).

The obtained results point to a wide variability of phenolic compounds among the analyzed species, as well as different variations among species of the same genus. As shown in Table 14.1, the total phenolic content for *E. spinosa* species is 104.66 mg GA/g of extract, which is approximately the value measured for the *C. maritimum*. Bearing in mind that these two species belong to the same family (Apiaceae), it can be said that there is a similarity in the origin and evolution of plants, and this is the reason for their similar content of phenolic compounds. However, the species *Eryngium maritimum*, which also belongs to this family, showed a deviation when it comes to the content of phenolic compounds. The value obtained for this species is

44.79 mg GA/g extract, which is significantly less than the value of previous two species. Based on this, it can be said that species from the same family do not always have the same or similar values of phenolic compounds. Differences in the content of total phenolic compounds in the investigated halophytes from the same family may refer to specific evolutionary paths of this taxon, and the reason may be the specific response of species to increased salinity, which relates to tolerance of these plants on salinity stress (Quideau et al. 2011).

The results obtained in Table 14.1 confirm the existence of halophytes characterized by a relatively high content of secondary metabolites such as phenol compounds (Oh et al. 2009). Based on the values shown in Table 14.1, it has been established that ethanol is a good solvent for the extraction of secondary metabolites during the examination of selected halophytes and that the measured high content of phenolic compounds in certain species originates from their high solubility in ethanol.

As shown in Table 14.2, the highest content of flavonoids among the examined halophytes has *Limonium gmelinii* (22.15 mg Ru/g), which is very similar to the value obtained for the *Cakile maritima* (22.13 mg Ru/g). It can be noticed that the mean values among the examined halophytes are of the species *Echinophora spinosa* of 18.69 and *Calystegia soldanella* with 18.24 mg Ru/g extract. The convincingly minimal flavonoid concentration (15.71 Ru/g of extract) was measured in the *Eryngium maritimum* which, according to this value, is distinguished from all investigated species.

Based on the obtained results, it can be concluded that the quantities of flavonoids, as well as the values of phenolic compounds, differ in the extracts of overground parts of investigated halophytes. The obtained flavonoid values indicate that these secondary metabolites constitute the most represented group among phenolic compounds, where some of the examined halophytes have a high, while others have a lower content of flavonoids. Thus, the differences in amount of phenolic compounds are reflected in the difference in concentration of flavonoids, as their most important groups. The differences in concentration of flavonoids in the examined halophytes are related, both with different phylogenies and morphologies and with physiological and molecular factors that are related to the response to stressful environmental conditions.

By comparing the values obtained in the first three species in Table 14.2 (*Echinophora spinosa*, *Crithmum maritimum*, and *Eryngium maritimum*) belonging to the same family (Apiaceae), it was concluded that the amount of flavonoids in them significantly varies and that all of three species have different contents of these secondary metabolites. Based on this, it can be said that species from the same family do not necessarily have the same or similar values of flavonoids, since different factors affect the plants and therefore the content of phenolic compounds and flavonoids in them. Factors that affect the difference in the quantities of phenolic compounds cause a difference in flavonoid concentrations in the investigated halophytes.

When assessing the content of phenolic compounds and the antioxidant activity of certain halophytes, it was determined that their variability depends to a great

extent on biological (different types, organs, and developmental phases), ecological, and technical (extraction) factors.

Considering that the three species (*Echinophora spinosa*, *Crithmum maritimum*, and *Eryngium maritimum*) among the investigated halophytes belong to the same family (Apiaceae), it is useful to compare the value of the antioxidant effect obtained from the extracts of their overground parts. It can be concluded that the first two species have the highest antioxidant activity among the examined halophytes, while the third significantly deviates with its value and among the examined halophytes belongs to the group with a small antioxidant effect. The first two species can be said to have similar evolutionary pathways (Quideau et al. 2011), and the deviation in the third species can be attributed to biotic and abiotic factors that affect plants and their antioxidant activity (Lattanzio et al. 2006).

Based on a parallel comparison of the values for the total amount of phenolic compounds and the antioxidant value for the abovementioned three species from the same family, it can be concluded that there is a correlation between the obtained values. Namely, the species *E. spinosa* and *C. maritimum* had the highest amount of phenolic compounds, as well as the highest antioxidant activity among the examined halophytes, while *E. maritimum* deviated from the previous and had lower values of the amount of phenolic compounds and antioxidant activity. A comparative analysis of the value of phenolic compounds and antioxidant activity in extract of overground parts of other investigated halophytes also confirms the connection between these two parameters, since species with higher content of phenolic compounds have a higher antioxidant effect and vice versa. This correlation indicates that phenolic molecules in these halophytes are the key active substances that exhibit antioxidant activity.

14.5 Conclusion

Based on the attention that is given in recent years together with more intensive and significant researches directed toward the plants from saline habitats, it may be concluded that halophytes represent interesting ecological group of plants in several aspects. Findings from plenty of studies indicate that halophyte species generally represent important sources of secondary metabolites with significant biological activity, in particular antioxidant activity, and that habitat specificity leads to specific composition of secondary metabolites in these plants. However, in order to obtain significant amount of metabolites from interests originated from halophytes, moderations are needed between plant yield, plant exploitation, and biodiversity conservation. Therefore, the appropriate screening method together with the proper methods of extractions and applications of obtained results should be studied firstly and precisely. Additionally, the further search for biologically active metabolites from halophyte species must take into account the developmental stage and yield of individual plant species together with environmental factors.

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