10.31195/ejejfs.599543

Eurasian Journal of Forest Science

2019 7(3): 293-300

http://dergipark.gov.tr/ejejfs

In vitro study on antioxidant, antibacterial and DNA interaction activities of extracts from *Arbutus andrachne* L.

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Abstract

Traditionally, the fruits and leaves of *Arbutus* (*A.*) *andrachne* are well known and used for applications in antimicrobial, antioxidant and anticancer activity. This study was aimed to determine *in vitro* antibacterial, antioxidant and DNA interaction activities of the different extracts of *A. andrachne*.

A. andrachne was collected from Amasya Province during May 2018. Dried leaves powder were mixed with of suitable solvents. Then, the mixtures were extracted with the soxhlet apparatus for 4 hours. The antibacterial effects of extracts were researched on pathogens, namely *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 25953, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, and *Salmonella enteritidis* ATCC 13076 using disc diffusion methods. Gentamicin and cefotaxime were used as the control for bacteria. To try to understand each plant extracts effect on the antioxidant methods, we studied the different methods as named radical scavenging activity (DPPH), metal chelating activity, ferric reducing antioxidant power assays. In the plant extracts, total phenolic and total flavonoid contents were determined by spectrophotometric methods due to investigating the effect of secondary metabolites on antioxidant activity. To explore the beneficial effect of the extracts on hydroxyl radical-mediated DNA damage, plasmid DNA pUC18 was used.

As a result, *A. andrachne* extracts had an antibacterial effect when they compared with control group antibiotics. According to disc diffusion methods, the highest antibacterial effect was identified in ethanol, chloroform and hexane extract, respectively. Ethanol extract of *A. andrachne* showed the best antioxidant activity. Moreover, extracts of *A. andrachne* had repair effects on plasmid DNA in the presence of H_2O_2 condition.

The investigated *A. andrachne* extract showed significant bioactivities. These extracts have been a promising candidate for the preparation of new natural products. However, future studies should be carried out to verify such actions in different matrices.

Keywords: *Arbutus andrachne* L. antioxidant, antimicrobial, DNA interaction

Introduction

In recent years, the root, trunk, leaf or fruit of various plants are utilized as a natural alternative in modern medicine. Secondary metabolites (terpenes, terpenoids, flavonoids) in the content of natural plants help to protect human health against various diseases. The antioxidant content of secondary flavonoids provides inhibition of oxidative damage associated with many diseases such as cancer, coronary heart disease, and paralysis (Özgen et al. 2009). Antioxidants are known to have a protective role against oxidative damage of reactive oxygen species on biomolecules such as DNA, lipid, and protein (Ayvaz et al. 2018; Gül et al. 2017).

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Also, the unconscious and unnecessary use of antibiotics causes a rapid increase in antibiotic-resistant microorganisms day by day. Therefore, diseases caused by resistant microorganisms pose serious public health problems. The use of medicinal herbs to reduce the harmful effects of antimicrobial agents increases with each passing day (Beattie et al. 2005). Already, the World Health Organization (WHO) has acknowledged many years ago that traditional medicine is important in health applications (WHO 2002-2005). Therefore, compounds derived from medicinal and aromatic plants with biological activity are widely investigated to treat various animal and human diseases (Kunduhoglu et al. 2011; Ünal et al. 2008).

A. andrachne L. plant belongs to the Ericaceae family and is known to have antimicrobial, antioxidant, anticancer and anti-inflammatory activity (Sıcak and Eliuz, 2019; Saklani and Kutty, 2008). The local name in Turkey is known as "Sandal tree" and "Davulga" or "Kocayemiş". The small fruit of *A. andrachne* L., which blossomed in May and localized in many different regions of Anatolia, was reported to occur in November (Baytop et al. 1999). The small fruit of *A. andrachne* L., which blossomed in May and localized in many different regions of Anatolia, was reported to occur in November. Its fruits are used in a wide food product such as jam, fruit jelly, marmalade and alcoholic beverages (liquor and wine). Fruits containing tannin, anthocyanin, and carotenoids are generally sweet, although unpleasant taste. The main therapeutic effects of *A. andrachne* L. are related to urinary tract infections, as well as, anticancer, antibacterial and antioxidant activities.

The main purpose of this study is to reveal the antibacterial and antioxidant effects of the *A. andrachne* L. plant obtained from the province of Amasya. For this purpose, the antibacterial effects of *A. andrachne* L. plant extracts were investigated by the disk diffusion method. Secondly, the antioxidant activities of *A. andrachne* L. specimens were determined using radical scavenging activity (DPPH), metal chelating activity, ferric reducing antioxidant power assays methods. In addition, the protective role of *A. andrachne* L. extracts on DNA damage originating from hydroxyl radicals was investigated using plasmid DNA. In the literature search, DNA interaction experiments with *A. andrachne* L. were not found. In this context, the current work differs from previous studies. These analyses were also made to determine which of the three extracts (ethanol, dichloromethane, and n-hexane) is more effective.

Materials and Methods

Sample extraction

A. andrachne L. was collected from a natural population which is located in Devret Hill (Amasya) in May 2018 (Baytop, 1999). The leaves of *A. andrachne* L. were then dried at room temperature. The plants (25 g) were extracted with the soxhlet tool (Isolab, Turkey) for 4 hours in ethanol, dichloromethane, and n-hexane (prepared by using solvents of different polarity). The extracts were filtered by Whatman No. 1 paper. The solvents were removed under a rotary vacuum until dry at 25-35 °C for 3–4 h. (Heidolph Collegiate, LV28798826, New Jersey, USA). Then the residue dissolved in Tetrahydrofuran (THF, Sigma) for antimicrobial activity (20 mg/mL). The extracts samples were stored at 4 °C in dark bottle for investigation (Bouyahya et al. 2016).

Test organisms and culture condition for antibacterial analysis

Extracts of *A. andrachne* L. were tested against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 25953, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, and *Salmonella enteritidis* ATCC 13076. Before use, bacterial strains were subcultured overnight at 37°C for 18 to 24 h in Trypric Soy Broth (TSB, Oxoid, Hampshire, UK).

Antibacterial activity

Antibacterial activity of the *A. andrachne* L. extracts were determined by the disc diffusion method (CLSI 2010). *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 25953, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus cereus* ATCC 7064, *Bacillus subtilus* ATCC 6633, and *Salmonella enteritidis* ATCC 13076 were used in bacterial strains. The

concentrations of the microorganisms were adjusted by turbidity measurements (0.5 McFarland) using serum physiologic solution. The concentration of bacterial suspensions was adjusted to 10^8cells/mL. Then, extracts (20 mg/mL) prepared in THF were loaded 6 mm to diameter sterile blank discs (Oxoid). Inhibition zones were determined after incubation at 37 °C for 24 h. As a positive control for bacteria, gentamicin (10 µg) and ceftriaxone (30 µg) were placed in Petri dishes. All tests were performed in triplicate.

Antioxidant activity

Free radical scavenging activity

The free radical scavenging activity was determined with 1,1-diphenyl-2-picrylhydrazyl (DPPH•) using methods of Brand-Williams (Brand-Williams et al. 1995). Different concentrations of plant material (25, 50, 100, 200, 400 µg/mL) were prepared and 0.75 mL each one of these extracts was added in to the 1.5 mL of 0,1 mM DPPH• solution in methanol. This solution was also added to, butylated hydroxytoluene (BHT), and Trolox (25-400 µg/mL) which were used to as positive controls for comparing. The mixture was shaken vigorously, and the decrease in absorbance at 517 nm was measured for 30 min at room temperature. Water (0.75 mL) in place of the sample was used as control. IC50 value was calculated to use the linear regression as the concentration required for 50% reduction of the DPPH radical. The percent inhibition activity was calculated using the following equation: free radical scavenging effect % = $[(A_0 - A_1)/A_0]\cdot 100$. ($A_0$ = the control absorbance and A_1 = the sample solution absorbance).

Metal chelating activity

The chelating activity of extract on ferrous ions (Fe^{2+}) was measured according to the method of Decker and Welch (Decker and Welch, 1990). Aliquots of 1 mL of different concentrations (100–500 µg/mL) of extracts were mixed with 3.7 mL of deionized water. The mixture was incubated with $FeCl_2$ (2 mM, 0.1 mL) for 30 min. After incubation, the reaction was initiated by addition of ferrozine (5 mM and 0.2 mL) for 10 min at room temperature, and then the absorbance was measured at 562 nm. A lower absorbance indicates a higher chelating power. The chelating activity of the extract on Fe^{2+} was compared with that of EDTA at the same concentrations. Metal chelating activity was calculated using the following equation Metal chelating activity (%) = $[(A_0 - A1)/A0]\cdot 100$.

Estimation of total phenolic content

According to the methods of Slinkard and Singleton (Slinkard and Singleton, 1977), using Folin–Ciocalteu reagent was determined depending on phenolic standard gallic acid. 1 mL of the plant extract was introduced into test tube followed by 1 mL Folin–Ciocalteu's reagent. The solution was kept in the dark for 5 min and then 3 mL of sodium carbonate (2%) was added. The tubes were covered with parafilm and kept again in the dark for 1 h and were measured absorption at 765 nm with a spectrophotometer and compared to a gallic acid calibration curve. The results were expressed as mg gallic acid/g dried sample. Each assay was carried out in triplicate.

Estimation of total flavonoid content

Total flavonoid content was determined with quercetin standard solution using Park methods (Park et al. 2008). The plant extract in 0.3 mL of was introduced into test tubes followed by 3.4 mL 30% methanol, 0.15 mL of 0.5 M $NaNO_2$ and 0,3 M $AlCl_3$ reagent. After 5 min 1 mL of 1 M NaOH was added and absorption was measured at 506 nm with a spectrophotometer and compared to a quercetin calibration curve. Each assay was carried out in triplicate. The total flavonoids were described as mg of quercetin equivalents per g of the dried fraction.

DNA interaction assay

To explore the beneficial effect of the *A. andrachne* L. extracts on hydroxyl radical-mediated DNA damage plasmid pUC18 DNA (Thermo Scientific) was used. Firstly, the *A. andrachne* L. extracts were dissolved in dimethyl sulfoxide (DMSO, concentration range from 12.5 to 100 mg/mL). A reaction mixture (20 µL final volume) containing 0.25 µg/µL plasmid DNA pUC18, 1.0 mM EDTA (Ethylenediaminetetraacetic acid)-FeSO4,1 µL of 3% H_2O_2, 0.1 g/mL *A. andrachne* L. extracts in Tris-EDTA (TE) buffer was prepared. H_2O_2 and 0.1% tetrahydrofuran treated plasmid DNAs were used as control groups. Secondly, the prepared mixture for each *A. andrachne* L. extracts were incubated at 37°C for 24 hours. 2 µL loading dye (bromophenol blue [0.025%] and

sucrose [4%] in dH_2O) was added into the mixture (10 µL total volume) and loaded on to the 1% agarose gel. Electrophoresis process was for 90 min at 80 V in TBE buffer running buffer (pH 8). Then, the agarose gel was stained with 0.5 µg/L ethidium bromide after electrophoresis. The Gel was imaged under UV light (Ayvaz et al. 2018).

Results and Discussion

In the present study, the extracts from *A. andrachne* L. samples collected from Amasya were assayed for antibacterial potency against four Gram-positive and four Gram-negative bacteria of clinical importance. Antioxidant activity was evaluated by measuring with different methods. At the same time, this study exhibited also DNA damage inhibitory activities of *A. andrachne* L.

According to analyzed antibacterial activity, the extracts of the plants indicated varying degrees of activity against bacterial strains that outlined in Table 1. Ethanolic extracts from *A. andrachne* L. is the most effective on *S. aureus* (ATCC 25953), *S. aureus* (ATCC 25953) and *B. cereus*. The antimicrobial activity of dichloromethane extracts against the *P. aeruginosa* (ATCC 9027), *S. enteritidis* and *E. coli* was found to non-effective. Hexane extracts of *A. andrachne* L is the most effective on *B. cereus* and *B. subtilis*.

Table 1. Zone diameters of inhibition (mm) showing the antimicrobial activities of each extract from *A. andrachne*

Microorgansims / **Solvent and antibiotics**	**B.s**	**B.c**	**P.a 9027**	**P.a 27853**	**S.a 25953**	**S.a 25923**	**S.e**	**E.c**
Ethanol	14	10	10	10	19	15	11	11
Dichloromethane	12	14	8	10	14	17	9	9
n-Hexane	11	11	8	10	9	8	8	9
CRO 30 µg	28	13	12	33	21	31	10	34
CN 10 µg	19	21	23	23	20	23	26	23

Microorganisms: B.s.: *Bacillus subtilis*, B.c.: *Bacillus cereus*, P.a.: *Pseudomonas aeruginosa*, S.a.: *Staphylococcus aureus*, S.e.; *Salmonella enteritidis*, E.c.: *Eschericia coli*. CRO: Ceftriaxone, CN: Gentamicin

The literature investigations revealed diverse traditional uses of *Arbutus* species based on antiseptic, anti-hypertensive, anti-diabetic, anti-inflammatory, anticancer and laxative properties. However, only a few of them are determined scientifically for their biological activities (Tenuta et al. 2019). The described characteristics are related to the contents of several biologically active compounds of *Arbutus* sp. (Morgado et al. 2018). One of them reported from Turkey, in the survey, the highest antibacterial activity of *A. andrachne* L. was reported against *S. aureus* (Sıcak and Eliuz, 2019). In another survey carried out in Turkey, *A. andrachne* L. extracts inhibited the growth of five bacteria and the inhibition zones ranged between 8- 17 mm. The highest antibacterial activity shows was observed on *S. aureus* (17 mm) (Ergun et al. 2014). Similarly, in the present study, ethanolic extracts of *A. andrachne* L. was the most effective on *S. aureus* (19 mm) (ATCC 25953). Tenuta et al. (2019) from France reported, they reported that the most promising activity of *Arbutus* sp leaves was found mainly against Gram-positive bacteria. In addition, they showed an intense antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, moderate activity against *Salmonella typhimurium*, *Escherichia coli*.

Although there is a few information in the literature about *A. andrachne* L. extracts biological activity, extracts prepared from *A. unedo* L., another type of *Arbutus* genus, leaves were showed antimicrobial activity against many microorganisms *B. cereus*, *B. subtilis*, *S. aureus*, *S. epidermis*, *E. coli* and *P. aeruginosa*. The biological activity of *A. unedo* L. tree has been the subject of several studies (Delgado-Pelayo et al. 2016; Erkekoglou et al. 2017; Fonseca et al. 2015). One of them reported from Portugual, in the survey, the extracts revealed antibacterial activity against several Gram-positive bacteria, including *Bacillus cereus*, *Enterococcus faecalis* and clinical methicillin-resistant *Staphylococcus aureus* (Morgado et al. 2018; Asmae et al. 2012; Ertabaklar et al. 2009; Ferreira et al.,

2012). In another survey carried out again in Portugual, it was determined that the antimicrobial potential of the extract were more efficient to Gram-positive bacteria (Malherio et al. 2012). Similar results were also reported for different *Arbutus* sp. in the literature. In Gram-positive bacteria, cell wall allows the essential oil and hydrophobic constituents to be in direct contact with the phospholipid bilayer of the cell membrane. Researchers reported that where they bring about their effect, causing either an increase in ion permeability and leakage of vital intracellular constituents, or impairment of the bacterial enzyme systems (Wendakoon and Sakaguchi, 1995; Malherio et al. 2012).

Natural or synthetic antioxidant compound capability which is obtained the quenching the reactive oxygen/nitrogen or radicals is determined via two different chemical processes: electron (ET) and hydrogen atom transfer (Apak et al. 2016). The antioxidant assay performed on the ethanol, dichloromethane, and n-hexane extract of *A. andrachne* L. collected in the Amasya region evidenced the presence of total antioxidant capacity via electron transfer methods. To perform DPPH, metal chelating, total flavonoid, total phenolic assay on these extracts were chosen from similar family plants works literature (Pallauf et al. 2008; Oliveira et al. 2009; Fortalazes et al. 2010). The level of DPPH activity was the highest at ethanolic extracts, so this result showed that ethanol was a good solvent for the extraction of the antioxidant moiety. The hexane solvent of which generally is used for terpenoids extraction, exhibited the lowest activity. The IC_{50} value of ethanolic extracts was lower than dichloromethane and hexane; 146.6, 200.9, 333.3 respectively. In literature was found similar results concerning DPPH activity (Figure 1) (Figure 1) (Bilto et al. 2015).

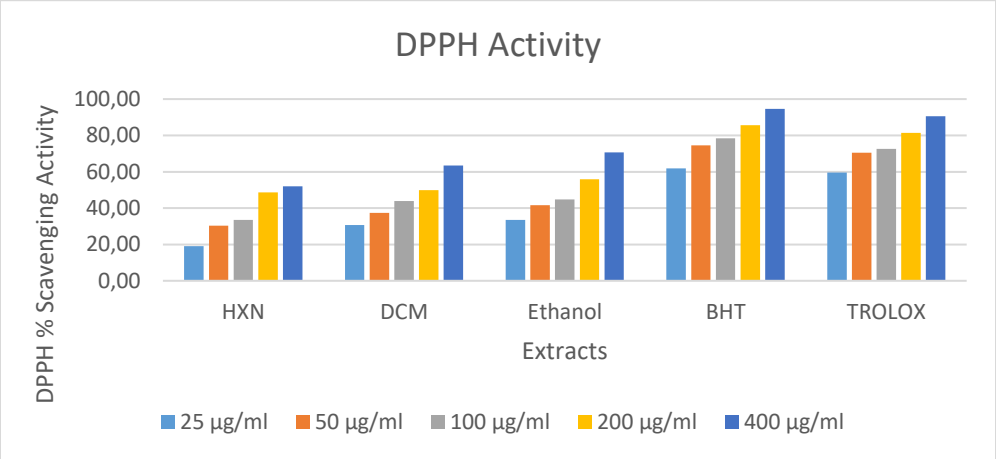

Figure 1. DPPH radical scavenging of extracts

When the metal chelating activity was compared EDTA standard, all extracts exhibited moderated metal chelating activity. Again, ethanol was the highest activity than others (Figure 2).

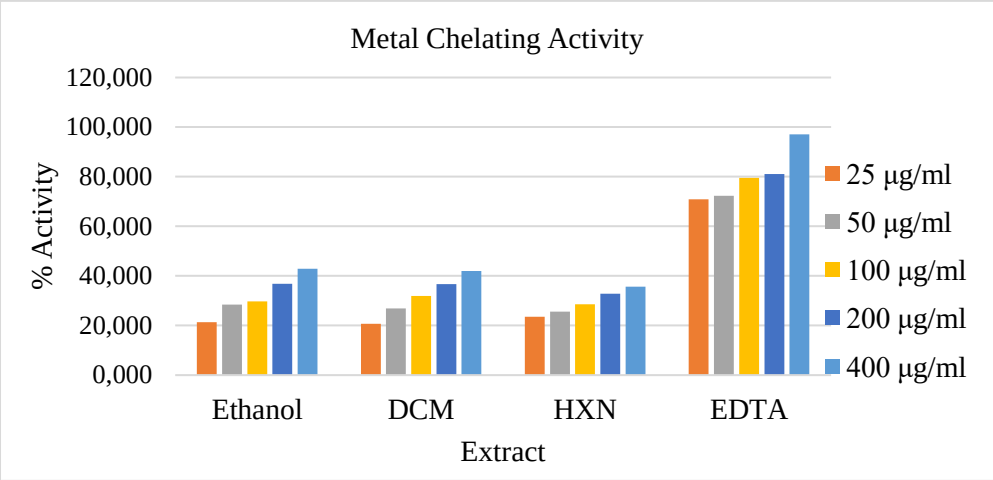

Figure 2. The extracts metal chelating studies.

Total flavonoid and total phenolics content were related to antioxidant capacity. Antioxidant result and these content values were shown similarities for each extract. The total phenolic ranged from 11,6 to 151,9 mg/g (gallic equivalent) and total flavonoid ranged from 65,2 to 71.6. *A. andrachne* L. fruit total penolic content was found 3.3 mg/g in 2010 (Table 2) (Serçe et al. 2010).

Table 2. Total phenolic and flavonoid content of extracts

Chemical Composition	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mgQTE/g)
n-Hexane	11,6061	65,20905
Dicholoromethane	34,2140	66,13783
Ethanol	151,8707	71,64125

Moreover, in this study, inhibitory activities of *A. andrachne* L. on hydroxyl radical-induced deoxyribonucleic acid (DNA) damage of *A. andrachne* L. extracts was investigated. According to the results of agarose gel electrophoresis, extracts were dissolved in DMSO and 0.25 µg/µL plasmid DNA pUC18 was treated with 12.5, 25, 50 and 100 mg/mL extracts respectively (Figure 3).

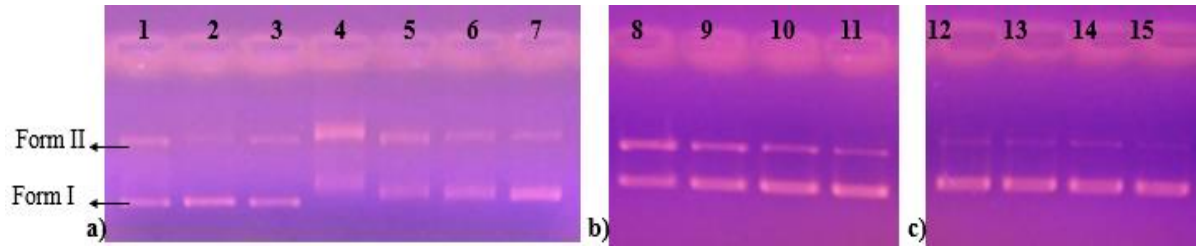

Figure 3. Gel image of extracts of *A. andrachne* L.

a) Lane 1: H_2O_2 and pUC18 plasmid DNA; Lane 2: pUC18 plasmid DNA control (blank); Lane 3: DMSO control; Lane 4-7: H_2O_2, pUC18 plasmid DNA and different concentration of ethanolic extracts (12,5-100 mg/mL)
b) Lane 8-11: H2O2, pUC18 plasmid DNA and different concentration of dichloromethan extracts (12,5-100 mg/mL)
c) Lane 12-15: H_2O_2, pUC18 plasmid DNA and different concentration of n-Hexanolic extracts (12,5-100 mg/mL)

Lane 2 and lane 3 was run with untreated pUC18 plasmid DNA as a control, while lanes 4-15 pointed out plasmid DNA interacted with increasing concentrations of the extracts in H_2O_2 condition. Increasing doses of *A. andrachne* L. extracts had a protective effect on hydroxyl radical-mediated plasmid DNA damage, but a low concentration of all *A. andrache* extract had no protective effect on plasmid DNA in H_2O_2 conditions. It appears that extracts of ethanol, dichloromethane, and n-hexane, exhibit relatively similar effects against plasmid DNA. The extracts did

not cause to change in Form I DNA (supercoil), but transferred from Form II DNA (open circular) (Ayvaz et al., 2018). The extracts have resulted in the cleavage of the plasmid DNA hence converting the plasmid form I to either form II and/or form III. As the concentrations of ethanol dichloromethane and n-hexane extracts increased, the mobility and band density of form I DNA increased slightly.

Conclusion

Several in vitro studies have conducted with different extracts of parts of *Arbutus* sp. Concerning the biological activity of usually *A. unedo* extracts, the majority of studies reported data based on antibacterial and antioxidant activity. We determined to total phenolic, total flavonoid and antioxidant activity of each extract as prepared from the n-hexane, dichloromethane, and ethanol. The ethanol extract was important not only suitable solvent of flavonoid and phenolic content but also antioxidant activity.

This study showed that *A. andrachne* L. species belonging to the Ericaceae family have *in vitro* significant biological activities. In conclusion, *A. andrachne* L. might be considered as a potential source which could be developed as precursors for antimicrobial and antioxidants drugs. After additional studies should be performed to analyze cytotoxic and pharmacological properties.

Conflict of interest

The authors declare no conflict of interests.

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Submitted: 31-07-2019 Accepted: 04-09-2019