Scientific Bulletin. Series F. Biotechnologies, Vol. XXV, No. 2, 2021
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

ANTIOXIDANT AND PESTICIDE POTENTIAL OF SAGE HYDROSOLS

Elena MIHAI[1*#], Daniela ILIE[1#], Alexandra GASPAR-PINTILIESCU[1], Adriana Florina POPESCU[2], Mariana POPESCU[2], Cristina LUNTRARU[2], Miruna NEAGU[2], Oana CRACIUNESCU[1]

[1]National Institute of R&D for Biological Sciences, 296 Splaiul Independentei, 060031, Bucharest, Romania
[2]HOFIGAL Export-Import S.A., 2 Intrarea Serelor, 042124, Bucharest, Romania

[*]Corresponding author email: anele_drag@yahoo.com
[#]These authors have equally contributed to this work.

Abstract

The aim of this study was to evaluate the properties of sage (Salvia officinalis L.*) hydrosols in terms of the antioxidant activity and inhibition of acetylcholinesterase, in order to be used in novel formulas of biopesticides for preventing aphid infestation of crops. Sage hydrosols were obtained as by-products of steam and reflux distillation, respectively, after essential oil extraction from dried aerial parts of the plant. The hydrosols were analyzed for terpenes and polyphenols composition by GC/MS and HPLC. The antioxidant activity of sage hydrosols was evaluated using Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical inhibition assay and cupric reducing antioxidant capacity assay (CUPRAC). Their pesticide potential was assessed using an experimental model in vitro and colorimetric measurement of acetylcholinesterase inhibition kinetics. The results showed that sage hydrosols contained significant amounts of phenolic acids and flavonoids, exhibiting a positive correlation with the antioxidant activity values. At the same time, sage hydrosols were responsible for acetylcholinesterase inhibition in a dose-dependent manner. In conclusion, sage hydrosols are natural effective products recommended for pest management solutions.*

Key words*: acetylcholinesterase, aphids, sage hydrosol, polyphenols, terpenes.*

INTRODUCTION

Hydrosols, also known as hydrolates or floral waters, are by-products of the essential oils extraction process through aromatic plants distillation. In the recent years, they were valorized as natural ingredients in aromatherapy, skin care products and preparation of cakes and drinks (Paolini et al., 2008; Aazza et al., 2011). Hydrosols consist of water-soluble plant compounds (polyphenols) and the aqueous fraction of essential oils, which gives them a pleasant odor. Therefore, the rose and orange hydrosols are the most used in food industry, in the Mediterranean area.

Several studies were focused on the chemical composition analysis of hydrosols resulted from aromatic plants present in different geographical areas, such as Morrocan thyme (*Thymus vulgaris*) (Aazza et al., 2011), Polish lavender (*Lavandula angustifolia*) (Prusinowska et al., 2015), Algerian *Calendula arvensis* (Belabbes et al., 2017) or Mexican oregano (*Poliomintha longiflora*) (Cid-Perez et al., 2019).

Moreover, the evaluation of their antioxidant, antibacterial and antifungal properties indicated their potential to act as natural pesticides, as such, or in combination with surfactants (Tornuk et al., 2011; Hay et al., 2015; Georgiev et al., 2019). It was reported that the redox and neuro-toxic/protective processes could be controlled by bioactive phytochemicals, such as terpenes, phenols, alkaloids, present in hydrosols or essential oils (Degirmenci & Erkurt, 2020). Some compounds, such as rotenone, an isoflavone extracted from *Derris elliptica* and azadirahtin, a secondary metabolite from neem oil, were identified and could act as plant biofungicides and bioinsecticides due to their toxicity and repellent activity (Spochacz, 2018; Hernandez-Carlos, 2019).

All these studies showed that, unlike synthetic pesticides, the hydrosol - and essential oil - based biopesticides had rapid biodegradation,

low toxicity, low risk of developing pest resistance and low cost (Rizvi, 2019).

In this context, the present study aimed to evaluate the chemical composition of Romanian sage hydrosols obtained by steam and reflux distillation and their antioxidant and pesticide activity, in order to be used in eco-agrosystems pest control.

MATERIALS AND METHODS

Extraction of sage hydrosols

The hydrosols were obtained from flowering aerial parts of sage (*Salvia officinalis* L.) by hydrodistillation using a Clevenger-type extraction apparatus (J.P. Selecta). The plant material was air-dried, powdered and an aliquot (25 g) was moistened in ultrapure water. Two extraction methods were applied, one based on steam distillation and the other on reflux using ultrapure water (750 mL), at 100°C, for 2 h. At the end of the incubation step, the essential oil was separated from the aqueous fraction, representing the sage hydrosol, based on the density difference. The steam distilled sage hydrosol (SSH) and the reflux sage hydrosol (RSH) were filtered and stored in the dark, at 4°C, until analysis.

Gas-chromatography/mass-spectrometry analysis

Sage hydrosols were analyzed by gas-chromatography coupled to mass-spectrometry (GC/MS) using a Focus GS-type equipment coupled to a mass spectrometer DSQ II (Thermo Electron Corporation, USA). The separation was performed on Macrogol 20,000 R capillary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1 mL/min. The mass spectrometer was operated at 70 eV with a scan interval of 0.5 s and scan range between 40-1000 m/z. The identification of the main constituents was performed by comparing the spectra with NIST mass spectral database.

HPLC analysis

Polyphenolic compounds from sage hydrosols were investigated by HPLC analysis. A reverse phase column C18 Zorbax Eclipse XDB (150 x 4.6 i.d. mm) was mounted on an Agilent 1200 HPLC system consisting of a quaternary pump, thermostated autosampler and diode array detector (Agilent, Germany). A sample (10 µl) was injected and then eluted using mobile phase A consisting of 2 mM sodium acetate, pH 3 and mobile phase B, acetonitrile, using the following gradient: 2-20% B, 0-30 min; 20-30% B, 30-40 min; 30% B, 40-50 min; 30-2% B, 50-60 min (Craciunescu et al., 2012). The identification of compounds was performed by comparison to the retention times (RT) of phenolic acids (gallic acid, caffeic acid, chlorogenic acid, ferulic acid) and flavonoids (rutin, luteolin, quercetin 3-O-glucoside (isoquercetin), kaempferol 3-O-glucoside (astragalin), luteolin-7-O-glucoside (cynaroside), quercetin, apigenin, kaempferol) standards (Sigma), at 280 and 370 nm. Quantification of the identified compounds was performed by peak area integration using standard curves built on the range of standard concentrations between 10-500 µg/mL.

Determination of total phenolic and flavonoid content

Total phenolic content (TPC) was determined by Folin-Ciocalteu method, as previously described (Moldovan et al., 2011). Briefly, the sample was mixed with Folin-Ciocalteu reagent (1:5, v/v) and incubated in the dark, for 5 min. Then, 2 mL of 12% sodium carbonate was added and the mixture was incubated at the room temperature, for 30 min. The solutions optical density (OD) was measured at 765 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The standard curve was built using different concentrations of caffeic acid in the range of 0-500 µg/mL. The results were expressed as caffeic acid equivalents (CAE) per 100 g of sample in dry weight (d.w.).

Total flavonoids content (TFC) was determined by aluminum chloride method, as previously described (Gaspar et al., 2014). Briefly, the sample was mixed with methanol (1:3, v/v), 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water. After incubation at the room temperature, for 30 min, the OD of the mixtures was read at 415 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The standard curve was built using different concentrations of quercetin in the range of 0-150 µg/mL. The results were expressed as quercetin equivalents (QE) per 100 g of sample in d.w.

Determination of free radicals scavenging activity

The Trolox equivalent antioxidant capacity (TEAC) assay was based on sample's ability to scavenge cationic free radicals of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and it was performed according to a previous protocol with minor modifications (Hay et al., 2015). Briefly, the stock solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1, v/v) and incubation at the room temperature, in the dark, for 16 h. Then, the solution was diluted to obtain an OD of 0.70 ± 0.02 at a wavelength of 734 nm. Different concentrations of sample were mixed with ABTS solution (1:10, v/v) and incubated at the room temperature, in the dark, for 10 min. The OD was read at 734 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The standard curve was built using an analog of vitamin E, Trolox, on the range of concentrations 0-250 µM. The results were expressed as Trolox equivalents (TE) per 100 mg of sample in d.w.

Determination of free radicals inhibition

The assay was based on sample's capacity to inhibit the formation of free radicals of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and it was performed according to Sugahara et al. (2015) protocol with slight modifications. Briefly, 150 µL of sample at different concentrations (10-500 µg/mL) were mixed with 0.9 mL of 0.1 M Tris-HCl buffer, pH 7.4 and 1.35 mL of 0.25 mM DPPH solution. The sample was replaced by an equal volume of buffer to prepare the blank. The mixtures were incubated at the room temperature, in the dark, for 30 min and then, the OD was read at 517 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The results were calculated using the following formula:

$$\%\ DPPH\ inhibition = (1 - OD\ sample)/OD\ blank \times 100$$

A known synthetic antioxidant agent, butylated hydroxytoluene (BHT) and a natural agent, ascorbic acid were similarly processed to serve as controls. The sample concentration (µg/mL) that inhibited 50% free radicals represented the IC50 value.

Determination of the antioxidant capacity

The assay was based on cupric ion reducing antioxidant capacity (CUPRAC) of the sample and it was performed according to an adapted protocol (Georgiev et al., 2019). Briefly, 1 mL of 10 mM $CuCl_2$ solution was mixed with 1 mL 7.5 mM neocuproine and 1 mL of 1 M ammonium acetate buffer, pH 7. The mixture was vortexed and incubated at the room temperature, for 10 min, to develop the complex. Then, 100 µL of sample and 1 mL of distilled water were added and incubation continued at the room temperature, for 1 h. The OD was read at 450 nm using a V-650 UV-VIS spectrophotometer (Jasco, Japan). The sample was replaced by an equal volume of distilled water to prepare the blank. The standard curve was built using Trolox in the range of concentrations 0.1-1.0 mM. The results were expressed as TE per 100 mg of sample in d.w.

Determination of acetylcholinesterase inhibition *in vitro*

The colorimetric assay was based on thiocoline reaction with Ellman's reagent and it was performed using a 96-well microplate adapted protocol (Mathew & Subramanian, 2014). Briefly, in the wells of a microplate, 100 µL of 3 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) reagent was mixed with 20 µL of acetylcholinesterase (AChE) (0.125 U/mL) in 50 mM Tris buffer, pH 8 containing 0.1% BSA and 20 µL sample of different concentrations. The plate was incubated at 25ºC, for 15 min and the OD was read at 412 nm (OD control) using a SpectroStar nano microplate reader (BMG Labtech, Germany). Then, 20 µL of 7.5 mM acetylthiocholine iodide were added as enzymatic substrate and the kinetics of the hydrolysis reaction was recorded as OD at 412 nm, every 3 min, for 30 min (OD sample). A blank was similarly processed after sample replacement with an equal volume of buffer (OD blank). The results were calculated using the following formula:

$$\%\ AChE\ inhibition = \frac{(OD\ sample - OD\ control)}{(OD\ blank - OD\ control)} \times 100$$

The sample concentration (µg/mL) that inhibited 50% AChE activity represented the IC50 value.

Statistical analysis

The experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) (n = 3). Statistical differences were calculated by two-tailed, two-sample equal variance Student *t*-test and they were considered significant at $p < 0.05$.

RESULTS AND DISCUSSIONS

In this study, sage hydrosols were obtained as brown solutions with pleasant odor by steam (SSH) and reflux (RSH) hydrodistillation of the aromatic plant after separation of the essential oil. The yield of SSH extraction was 11.92 ± 1.09% (w/w), while the yield of RSH extraction was 18.11 ± 1.22% (w/w).

Chemical composition of sage hydrosols

Chemical analysis of RSH by GC/MS showed the presence of 15 volatile compounds (Figure 1), while in SSH they were detected only in traces. The identified constituents, their RT and abundance (%) are presented in Table 1.

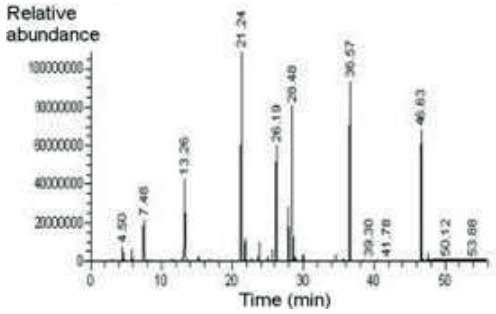

Figure 1. GC/MS chromatogram of sage hydrosol

Table 1. Percentage composition of volatile compounds determined by GC/MS in sage hydrosol

No.	Major compound	RT	% of total peak area
1.	α-Pinene	4.50	1.67
2.	Camphene	5.78	1.58
3.	β-Pinene	7.48	7.44
4.	1,8-cineole	13.26	12.69
5.	Trans-β-ocimene	15.14	0.57
6.	α-Thujone	21.23	17.06
7.	β-Thujone	21.80	2.50
8.	Camphor	23.73	1.57
9.	Bornyl acetate	25.63	0.94
10.	α-Caryophyllene	26.19	14.59
11.	β-Caryophyllene	27.90	5.87
12.	Borneol	28.48	9.53
13.	Cadinene	29.96	0.72
14.	Viridifloral	36.57	11.81
15.	Epimanool	46.63	11.46

RSH consisted mainly of monoterpenes and their oxygenated derivatives. The main components were β-pinene (7.44%), 1,8-cineole (eucalyptol) (12.69%), α-thujone (17.06%), α-caryophyllene (14.59%), borneol (9.53%), viridifloral (11.81%) and epimanool (11.46%). A similar composition was reported for the essential oil extracted from Romanian sage, but components abundance was different, the oil being rich in α-thujone (34.63%), β-thujone (13.10%) and camphor (16.02%) (Popescu et al., 2018). GC-MS analysis of the essential oil extracted from Italian *S. officinalis* seeds showed that the main constituents were 1,8-cineole (6.67%), α-thujone (14.77%) and camphor (13.08%), which indicated a similar composition to that of sage flowering aerial parts (Taarit et al., 2014). The presence of these water-soluble volatile compounds in sage hydrosols indicated their potential to be used as valuable, quality products, instead of by-products and wastes (Baydar et al., 2013).

Polyphenolic composition of sage hydrosols

HPLC chromatograms of SSH and RSH are presented in Figure 2.

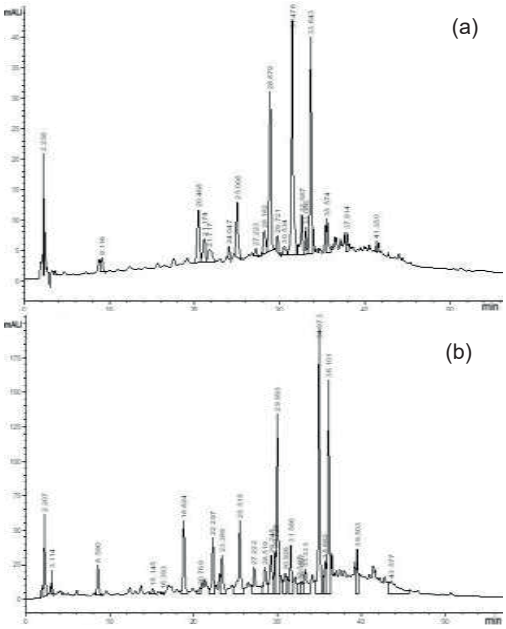

Figure 2. HPLC chromatograms of sage hydrosols obtained by steam (SSH) (a) and reflux (RSH) (b) hydrodistillation

SSH had 8 major peaks at 2.23, 20.46, 25.00, 28.87, 31.47, 32.58, 33.64 and 35.61 min. RSH had 10 major peaks at 2.20, 8.59, 18.82, 22.29, 23.38, 25.51, 29.99, 31.58, 34.97 and 36.10 min. The identified and quantified polyphenolic compounds based on the RT and integrated areas, respectively, are presented in Table 2. The results showed that SSH had higher quantities of phenolic acids, such as caffeic and ferulic acid, and flavonoids, like rutin and the glycosylated derivatives of quercetin, luteolin

and kaempferol, compared to those in RSH (Table 2). Free flavonoids of quercetin, luteolin and kaempferol were not detected, as against their glycosylated forms.

Table 2. HPLC analysis of phenolic acids and flavonoids in sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation

No.	Compound	SSH (mg/g d.w.)	RSH (mg/g d.w.)
1.	Gallic acid	ND	ND
2.	Chlorogenic acid	ND	1.05
3.	Caffeic acid	7.08	1.84
4.	Ferulic acid	10.70	5.63
5.	Rutin	56.62	10.72
6.	Isoquercetin	27.75	4.13
7.	Luteolin-O-glucoside	29.53	5.28
8.	Kaempferol-O-glucoside	17.71	8.63
9.	Quercetin	ND	ND
10.	Luteolin	ND	ND
11.	Apigenin	ND	ND
12.	Kaempferol	ND	ND

The results of TPC and TFC determination are presented in Table 3.

Table 3. Total polyphenolic (TPC) and flavonoid (TFC) content and antioxidant activity of sage hydrosols

Sample	TPC (mg CAE/ 100 g d.w.)	TFC (mg QE/ 100 g d.w.)	TEAC (mM TE/ 100 mg d.w.)	CUPRAC (mM TE/ 100 mg d.w.)
SSH	32.55 ± 1.71	5.93 ± 0.03	245.17 ± 11.68	241.04 ± 13.83
RSH	29.89 ± 2.20	3.08 ± 0.05*	208.75 ± 6.94*	210.02 ± 9.54*

*p<0.05, compared to SSH value

Data showed similar content of polyphenols in both sage hydrosols (~30 mg CAE/100 g d.w.), but flavonoids content was double in SSH obtained by steam distillation (6 mg QE/100 g d.w.), compared to RSH (3 mg QE/100 g d.w.).

Antioxidant activity of sage hydrosols

Three methods based on different scavenging mechanisms were used to assess the antioxidant activity of sage hydrosols, i.e. TEAC assay based on hydrogen atom transfer (HAT) mode, CUPRAC assay based on single electron transfer (SET) mode and DPPH assay with mixed mode of action. The results of the antioxidant activity of sage hydrosols determined by TEAC and CUPRAC assays are presented in Table 3. The capacity to scavenge ABTS radicals was significantly ($p<0.05$) higher for SSH (245 mM TE/100 mg d.w.) than for RSH (208 mM TE/100 mg d.w.). Similar, the capacity to reduce Cu (II) ions was significantly ($p<0.05$) higher for SSH (241 mM TE/100 mg d.w.) compared to RSH (210 mM TE/100 mg d.w.).

We have analyzed the correlation between the antioxidant activity and TPC values. A linear, positive correlation between TEAC and CUPRAC antioxidant activity and the polyphenolic content of SSH ($r^2 = 0.848$) and RSH ($r^2 = 0.929$), respectively, was observed (Figure 3). These data confirmed that the presence of polyphenolic compounds, in particular of flavonoids, could ensure numerous hydroxyl groups and, accordingly, a significant antioxidant activity.

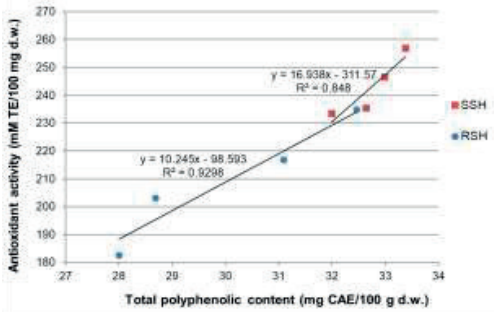

Figure 3. The correlation degree between TEAC and CUPRAC antioxidant activity and the polyphenolic content of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation

The antioxidant activity of sage hydrosols was also determined as their capacity to inhibit DPPH free radicals. The variation of the inhibition percentage as a function of hydrosol concentrations is presented in Figure 4.

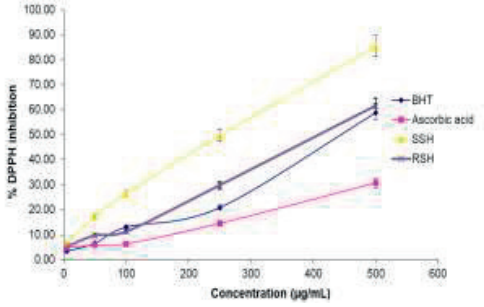

Figure 4. Inhibition of DPPH free radicals in the presence of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation, BHT and ascorbic acid

The inhibition of DPPH radicals increased in a dose-dependent manner in the presence of sage

hydrosols. The slope was linear between 100-500 µg/mL range of concentrations and values of 85.33% and 61.48% were recorded at a concentration of 500 µg/mL SSH and RSH, respectively. At the same concentration value, lower DPPH inhibition was registered for the synthetic antioxidant agent BHT (58.76%) and the natural antioxidant ascorbic acid (30.58%).
In the same time, the IC50 value of SSH (264 µg/mL) was significantly ($p<0.05$) lower than that of BHT (449 µg/mL) and ascorbic acid (883 µg/mL), confirming that the steam distilled hydrosol had higher antioxidant activity (Table 4). In the case of RSH, the IC50 value (429 µg/mL) was not significantly ($p>0.05$) different from that of BHT, but it was lower than that of ascorbic acid, indicating better capacity to inhibit DPPH free radicals.

Table 4. IC50 values of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation, BHT and ascorbic acid determined for DPPH free radicals inhibition and acetylcholinesterase (AChE) activity

Sample	IC50 DPPH (µg/mL)	IC50 AChE (mg/mL)
SSH	264.14 ± 9.23	9.79 ± 0.68
RSH	429.33 ± 12.27*	18.83 ± 2.05*
BHT	449.80 ± 17.44*	ND
Ascorbic acid	883.67 ± 23.26*	ND

ND - not determined; *$p<0.05$, compared to SSH value

Pesticide potential of sage hydrosols

The pesticide potential of sage hydrosols was assessed *in vitro* using an experimental model mimicking their neurotoxic effect on aphides. The inhibition of AChE in the presence of sage hydrosols is presented in Figure 5, while the calculated IC50 values are given in Table 4.

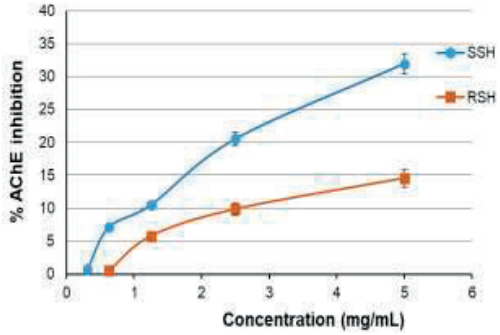

Figure 5. Capacity of sage hydrosols obtained by steam (SSH) and reflux (RSH) hydrodistillation to inhibit the activity of acetylcholinesterase (AChE)

The results showed that both sage hydrosols inhibited AChE activity, but SSH in a higher proportion (32%) than RSH (15%). No AChE inhibition activity was recorded for BHT and ascorbic acid, indicating that an important role was that of the polyphenolic compounds content. The IC50 value of SSH (9.79 mg/mL) was significantly ($p<0.05$) higher than that of RSH (18.83 mg/mL) (Table 4).
In a recent study, the IC50 value of the methanolic extract of *S. officinalis* was 24 mg/mL in the same model of AChE activity *in vitro* (Sharma et al., 2020), confirming the neurotoxic activity of sage and its potential use as biopesticide. At the same time, rutin present as main flavonoid in sage extracts exhibited an IC50 value of 2.5 mg/mL against AChE activity (Neagu et al., 2015).
In regard of volatile compounds, it was previously showed that they did not present AChE inhibition and neurotoxic capacity (Lopez & Pascual, 2009). However, 1,8-cineole also present in sage hydrosols exhibited pests toxicity (Abdelgaleil et al., 2009). It was proposed a mechanism of neurotoxic action from hydrosol compounds based on changes at hormonal level, neurologic effect by gustatory effects and olfactory responses, and imbalance of pro-oxidant/antioxidant reactions, all of these processes leading to disturbances in pests development (Spochacz et al., 2018).

CONCLUSIONS

Two sage hydrosols with different composition were prepared by steam (SSH) and reflux (RSH) distillation methods. GC-MS results indicated that RSH contained 15 types of volatile compounds, unlike SSH presenting traces. In turn, significantly higher polyphenolic content and correlated antioxidant activity were recorded for SSH.
Both hydrosols presented DPPH and AChE inhibition variation in a dose-dependent manner, but SSH demonstrated better radical scavenging and neurotoxic activity than RSH, with IC50 values lower than those of known antioxidant agents, like BHT and ascorbic acid.
The hydrosol obtained by steam distillation of *S. officinalis* aerial parts presented higher flavonoid and lower volatile compounds content, in comparison to that of the reflux

obtained sage hydrosol. A positive correlation was found between the polyphenolic composition and the antioxidant activity of sage hydrosols.
The AChE inhibition activity of sage hydrosols varied in a dose-dependent manner and was higher for the steam-distilled product, rich in flavonoids.
All these results demonstrated that steam distillation of sage could provide a product with practical applications not only in food industry, but also as functional ingredient of novel biopesticide formulas for pest control in eco-agrosystems.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian Ministry of Education and Research, CCCDI-UEFISCDI, project no. PN-III-P2-2.1-PED-2019-3561, within PNCDI III.

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