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Pharmacological perspectives from Brazilian *Salvia officinalis* (Lamiaceae): antioxidant, and antitumor in mammalian cells

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ABSTRACT

Salvia officinalis (Lamiaceae) has been used in south of Brazil as a diary homemade, in food condiment and tea-beverage used for the treatment of several disorders. The objective of this study was to characterize chemical compounds in the hydroalcoholic (ExtHS) and aqueous (ExtAS) extract from *Salvia officinalis* (L.) by gas chromatography-mass spectrometry (GC-MS) and by high-resolution electrospray ionization mass spectrometry (ESI-QTOF MS/MS), evaluate *in vitro* ability to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}), catalase (CAT-like) and superoxide dismutase (SOD-like) activity, moreover cytotoxic by MTT assay, alterations on cell morphology by giemsa and apoptotic-induced mechanism for annexin V/propidium iodide. Chemical identification sage extracts revealed the presence of acids and phenolic compounds. *In vitro* antioxidant analysis for both extracts indicated promising activities. The cytotoxic assays using tumor (Hep-2, HeLa, A-549, HT-29 and A-375) and in non-tumor (HEK-293 and MRC-5), showed selectivity for tumor cell lines. Immunocytochemistry presenting a majority of tumor cells at late stages of the apoptotic process and necrosis. Given the results presented here, Brazilian *Salvia officinalis* (L.) used as condiment and tea, may protect the body against some disease, in particularly those where oxidative stress is involved, like neurodegenerative disorders, inflammation and cancer.

Key words: antioxidant, apoptosis, cancer, chemical compounds, *Salvia officinalis* (L.).

INTRODUCTION

The importance of plants traditionally used by the population can lead us to search for new active compounds with application in chemoprevention or anticancer therapy. In Brazil, the use of plants to

treat numerous diseases is part of the local culture (Ceolin et al. 2014).

Originally found in the East Mediterranean region, *Salvia officinalis* (Linneu, 1753), is a perennial shrubby herb belonging to the Lamiaceae, that is being extensively researched, plants of this family commonly used as a diary condiment in food, hydroalcoholic tincture and

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tea is the most consumed beverages used for the treatment of several disorders in traditional and folk medicine (Berdowska et al. 2013). Currently, many reports are using *S. officinalis* (L.) to analyze its pharmacological activity and studies have shown its potential as anti-inflammatory (Rodrigues et al. 2012), antimicrobial (Garcia et al. 2012), hypoglycemic (Shafiee-Nick et al. 2012), anti-diabetic (Christensen et al. 2010), antioxidant (Generalic et al. 2012), to prevent neurodegenerative disease (Takano et al. 2011) and antitumor activity (Al-Barazanji et al. 2013, Akaberi et al. 2015).

Drug development derived from natural products became a highly promising strategy for identification of new antitumor agents. Tumor cells exposed to sage extracts may reduce cell viability by mechanisms not elucidated, but it is believed that this plant exerts its antitumor effect by changing the redox equilibrium, inducing apoptosis pathways and cell cycle arrest (Zare Shahneh et al. 2013, Vasko et al. 2014).

Given sage extract promising biological activities, the aim of this study is to investigate the major compounds presented in the hydroalcoholic and aqueous sage extract, its antioxidant activity and cytotoxic effect against well-known tumor cell lines: human laryngeal carcinoma (Hep-2), human cervix adenocarcinoma (HeLa), human lung carcinoma (A-549), human colorectal adenocarcinoma (HT-29) and human melanoma (A-375). Cytotoxic effects were also tested in normal cell lines: non-malignant human embryonic kidney (Hek-293) and human lung fibroblast (MRC-5). Moreover, this study also evaluated the morphological alterations and induction of apoptosis and necrosis after exposure to hydroalcoholic and aqueous sage extract.

MATERIALS AND METHODS

Salvia officinalis (L.) SAMPLE

Salvia officinalis (L.) was collected in September in Farroupilha city, district of Rio Grande do Sul,

Brazil (Lat. -29°13'30"S by Lon. -51°20'52"W) and deposited at the *UCS Herbarium* (HUCS - 37944), University of Caxias do Sul (UCS). The leaves were dried in a circulating air oven (Fabbe) at 40-45±1 °C for seven days. The dried material was ground in a knife mill (Wiley) and stored in amber bottles for further tests.

Salvia officinalis (L.) EXTRACTS

The hydroalcoholic extract of *Salvia officinalis* (L.) (ExtHS) was obtained from a dried ground sample (1 g) with 80% (v/v) EtOH-H₂O mixture (10 mL) through maceration process at room temperature for seven days in an amber bottle, under controlled shaking conditions. The extract was filtered and the solution evaporated to dryness, yielding a dark-green fine powder. The powder was resuspended in 70% (v/v) EtOH-H₂O at different concentrations (5-625 µg mL⁻¹) and aliquots were ultrafiltered through a 0.22 µm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland).

The aqueous extract of *S. officinalis* (L.) (ExtAS) was obtained from a dried ground sample (1 g) with infusion, using distilled water at 100 °C for 10 minutes (10 mL). The extract was filtered with reduced pressure and lyophilized, producing a dark-brown fine powder. The powder was resuspended in distilled water, at different concentrations (5-5000 µg mL⁻¹), and further ultrafiltered through a 0.22 µm polyethersulfone membrane (TPP, Techno Plastic Products, Switzerland).

CHEMICAL CHARACTERIZATION

The major chemical constituents of dried *S. officinalis* hydroalcoholic and aqueous extracts were evaluated by high-resolution electrospray ionization mass spectrometry (ESI-QTOF MS/MS) on a Bruker Daltonics micrOTOF-Q II instrument in negative mode. The samples were solubilized in HPLC-grade methanol containing formic acid, 99.9/0.1 (v/v), and injected into the ESI source by

means of a syringe pump at a flow rate of $5.0 \mu\text{L min}^{-1}$. Diagnostic ions were identified by the comparison of their ESI(-)-MS/MS dissociation patterns with previous studies. GC-MS analyses were performed with an Agilent 6890 chromatograph equipped with an electron impact detector at 70 eV and a DB-5 fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 μm) using helium as carrier gas with a constant flow rate of 1.0 mL min^{-1} (split = 1:20). Identification of the constituents was carried out comparing the obtained mass spectra with NIST 2005 library spectra.

TOTAL PHENOLIC CONTENT ANALYSIS

ExtHS and ExtAS total phenolic content ($10 \mu\text{g} \mu\text{L}^{-1}$) was measured using the Folin-Ciocalteu colorimetric method as described by Singleton and Rossi (1965). The absorbance was measured at 765 nm (Shimadzu UV-1700 spectrophotometer, Kyoto, Japan) after 30 min of reaction. Gallic acid was used as standard to produce the linear regression curve ($r^2=0.998$). Total phenolic content was expressed in μg of gallic acid equivalents (GAE) μL^{-1} of extract.

RADICAL SCAVENGING ACTIVITY, SUPEROXIDE DISMUTASE-LIKE AND CATALASE-LIKE ACTIVITIES

The radical 2,2-diphenyl-1-picrylhydrazyl (DPPH $^{\cdot}$) assay was performed using a modified Yamaguchi method, 1998 (Yamaguchi et al. 1998). Briefly, the hydroalcoholic and aqueous stock solutions of *S. officinalis* were diluted to different concentrations (0.12 to $2.5 \mu\text{g} \mu\text{L}^{-1}$) using hydroalcoholic solution (70% v/v) for ExtHS and distilled water for ExtAS. The results are expressed as IC_{50} (the amount of the extract needed to scavenge 50% of DPPH $^{\cdot}$).

The capacity of the extracts to reduce radical cation 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS $^{+\cdot}$) was determined following a previously published method (Re et al. 1999). ABTS $^{+\cdot}$ radical scavenging ability was calculated as percentage of inhibition (% I) = $(A_{734} \text{ of blank} - A_{734} \text{ of sample}) / A_{734} \text{ of blank} \times 100\%$. The blank

solution was previously prepared with ABTS $^{+\cdot}$ and ethanol only for ExtHS and ABTS $^{+\cdot}$ and distilled water only for ExtAS. The results are expressed in IC_{50} (amount of extract needed to reduce 50% of the ABTS $^{+\cdot}$ radical cation), obtained by linear regression of the % I values. Gallic acid was used as positive control.

To evaluate enzyme-like activities, ExtHS and ExtAS were prepared in a concentration of $10 \mu\text{g mL}^{-1}$. The superoxide dismutase (Sod)-like assay was measured as the inhibition of the self-catalytic adrenochrome formation rate at 480 nm in a reaction medium containing 1 mmol L^{-1} adrenaline (pH 2.0) and 50 mmol L^{-1} glycine (pH 10.2) (Bannister and Calabrese 1987). This reaction was performed at 30°C for 3 min. The results are expressed as superoxide dismutase units (USOD). Catalase-like assay was measured according to the method described by Aebi (1984). The assay determines the rate of H_2O_2 decomposition at 240 nm. The reaction was conducted at 30°C for 1 min. The results are expressed as catalase units (UCAT). Gallic acid was used as positive control ($0.05 \mu\text{g} \mu\text{L}^{-1}$).

CYTOTOXIC ACTIVITY

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann 1983, Alley et al. 1988). Briefly, tumor (Hep-2, HeLa, HT-29, A-549, A-375) and non-tumor (HEK-293 and MRC-5) cells were seeded at a density of $8 \times 10^4 \text{ cells mL}^{-1}$ in $100 \mu\text{L}$ of supplemented culture media. After 24 h, cells were treated with different concentrations ($5\text{--}625 \mu\text{g mL}^{-1}$) of ExtHS and different concentrations ($5\text{--}5000 \mu\text{g mL}^{-1}$) of ExtAS. Negative controls were treated with the same amounts of hydroalcoholic (residual amount ethanol = 3% on cells) and aqueous solution without extract, incubated at 37°C in 5% CO_2 for 24 h. The medium was removed

and 1 mg mL⁻¹ MTT dye in serum-free medium was added to the wells. Plates were incubated at 37 °C for 2 h in humidified 5% CO₂ atmosphere. Subsequently, the MTT solution was dissolved in 100 µL DMSO. Absorbance was measured using a microplate reader at 570 nm (Spectra Max190, Molecular Devices, USA). The readings were compared with the control, which represented 100% viability. All the cells used in the study were acquired commercially from Banco de Células do Rio de Janeiro.

MORPHOLOGICAL EXAMINATION AND INDUCTION OF APOPTOSIS IN DIFFERENT CELL LINES

Cell morphology of Hep-2, HeLa, HT-29, A-549, A-375, HEK-293 and MRC-5 lines was monitored using an inverted microscope under a conventional Giemsa staining protocol (Nersesyan et al. 2006). Changes in the cellular morphology were observed and documented after 24 h ExtHS and ExtAS treatment exposure at its IC₅₀. The negative control group was treated with EtOH-H₂O 70% (v/v), respecting the residual amount ethanol of 3% for ExtHS and destilate water for ExtAS for the same period of time.

For apoptosis analysis, all cell lines were seeded on coverslips into 24-well plates at a density of 8x10⁴ cells mL⁻¹ in 500 µL of supplemented culture media (DMEM). After 24 hours, cells were treated with different concentrations of ExtHS and ExtAS at its IC₅₀, followed by extract incubation at 37 °C in 5% CO₂ for 24 hours. Next, treatment with sage extract was removed, washed with phosphate buffered saline, followed by primary antibody incubation using 2.5 µg mL⁻¹ of Annexin V (Abcam® - ab14196) at 37 °C, in 5% CO₂ atmosphere, for 1 hour. Samples were washed with ligation buffer solution and incubate with secondary antibody, 2.5 µg mL⁻¹ Dylight® 488 (Abcam® - ab115637) at 37 °C, in 5% CO₂ atmosphere for 30 minutes. Next, cells were stained with 0.5 µg mL⁻¹ propidium iodide (PI) (Sigma-Aldrich®- P4170)

for 30 minutes, washed with ligation buffer and fixed with formaldehyde 4% at room temperature, for 15 minutes. The percentage of cells undergoing early apoptosis (annexin V positive), late apoptosis (annexin V positive and PI positive) and/or necrosis (PI positive) was estimated by counting four fields for each condition using a fluorescence microscope (BX43, Olympus). To confirm cell apoptosis, we used the technique of staining with dye acridine orange (100 µg mL⁻¹) and ethidium bromide (100 µg mL⁻¹), data not shown.

STATISTICAL ANALYSIS

All biological measurements were conducted on the basis of three independent experiments. Statistical significance was evaluated using *t*-test and oneway analysis of variance (ANOVA) with post hoc multiple comparisons test (Tukey) to assess statistical differences in case of normal distribution. Significance was accepted at *p* lower than 0.05 using the Statistical Package for Social Sciences (SPSS, version 19.0) for Windows.

RESULTS

CHEMICAL CHARACTERIZATION

The GC-MS analysis showed major essential oil components present in the *S. officinalis* hydroalcoholic extract (**Table I**). Identified molecules include monoterpene eucalyptol, α-thujone, β-thujone, camphor; sesquiterpenes β-caryophyllene, α-caryophyllenene, viridiflorol and diterpenemannol. These compounds were commonly found in the essential oil of *S. officinalis*, however, with different concentrations depending on plant geographic origin and harvest season.

The highest intense peaks shown in the ESI-QTOF analysis (negative ionization mode) from the hydroalcoholic extract (**Table II, entries 1-4**) were the deprotonated molecules [M-H]⁻ at *m/z* 345.2086 and *m/z* 455.3773, which correspond to a nominal mass of 346 u and 456 u, respectively.

TABLE I
Major constituents expressed as peak area percentage of hydroalcoholic extract of *S. officinalis* identified by GC-MS analysis.

entry	RT	Identified compound ^a	% peak area
1	8.61	eucalyptol (1,8-cineole)	9.44
2	11.32	α -thujone	23.15
3	11.73	β -thujone	5.94
4	12.82	Camphor	13.26
5	24.18	β -caryophyllene	5.34
6	25.55	α -caryophyllene (α -humulene)	4.08
7	30.93	Viridiflorol	13.12
8	46.60	Manool	10.21

^a The compounds were identified on GC-MS (gas chromatograph coupled with mass spectrometry) and listed according to their elution. RT = retention time.

According to literature data, compounds isolated from *Salvia* genus, such as rosmanol and its isomers, methyl carnosate, and 12-methoxycarnosinic acid possess nominal mass of 346 u. Rosmanol and its isomers were discarded due to high difference between the accurate mass and the exact mass of these compounds. A tandem MS/MS analysis of the peak at m/z 345.2086 shows a major fragment at m/z 301, which represents a loss of neutral CO_2 molecule (MW = 44).

This fragmentation behavior identified the 12-methoxycarnosinic acid it is compared the fragment pathway between 12-methoxycarnosinic acid and methyl carnosate. The deprotonated

molecules $[\text{M-H}]^-$ with m/z 455.3573 (**entry 4**) was attributed to the ursolic acid $[\text{M-H}]^-$; 455.3531 that is present in different *Salvia* species. This assignment was confirmed by comparison with HRMS analysis of commercially available ursolic acid (Sigma-Aldrich - USA). The rosmarinic acid that is frequently found in sage extracts and considered a potent antioxidant molecule was also present in both hydroalcoholic and aqueous extracts (**Table II, entries 3 and 10**). This compound was identified due to the accurate m/z of 359.0783 (**entry 3**) and 359.0785 (**entry 10**). The MS² analyses revealed the major fragments at m/z 197, 179 and 161.

TABLE II
Main compounds identified by ESI-QTOF MS/MS analysis of the hydroalcoholic and aqueous extract of *Salvia officinalis* (L.).

Entry	Observed mass [M-H] ⁻	Major fragments (intensity %)	Calculated mass	Identified compound	Experimental error (ppm)
hydroalcoholic					
1	179.0356	non fragmented	179.0339	Caffeic acid	9.4
2	345.2086	301 (100)	345.2060	12-methoxycarnosinic acid	7.5
3	359.0783	197 (66), 179 (7), 161 (100)	359.0761	Rosmarinic acid	6.3
4	455.3573	non fragmented	455.3530	Ursolic acid	9.0
aqueous					
5	133.0129, [M-H] ⁻	non fragmented	133.0142	Malic acid	-9.0
6	117.0190, [M-H] ⁻	non fragmented	117.0193	Succinic acid	-2.5
7	149.0100, [M-H] ⁻	non fragmented	149.0092	Tartaric acid	5.3
8	179.0357		179.0339	Caffeic acid	10
9	191.0200	non fragmented	191.0197	Citric acid	1.5
10	359.0785	197 (42), 179 (10), 161 (100)	359.0761	Rosmarinic acid	6.8
11	461.0701	285 (100)	461.0725	Luteolin-7-O-glucoronide	-5.2

TOTAL POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITY

The total phenolic content were analyzed through Folin-Ciocalteu assay and showed polyphenols in the ExtHS that presents 1.88 ± 0.01 mgGAE mL⁻¹ and ExtAS shows 1.61 ± 0.95 mgGAE mL⁻¹. The ExtHS and ExtAS were able to donate electrons to the stable radicals DPPH[•] and ABTS^{•+} and showed promising SOD-like and CAT-like activities, being the CAT-like activity of ExtHS and ExtAS higher to that presented by gallic acid, a standard antioxidant (Table III).

CYTOTOXIC ACTIVITY

Cytotoxic activity after 24 hours treatment with ExtHS showed 50% inhibitory concentration that varied according to each tumor cell line investigated,

ranging from 45 to 235 μ g mL⁻¹. The Hep-2 revealed the lowest IC₅₀ (45 ± 1 μ g mL⁻¹) whereas A-549 presented the highest level for IC₅₀ (235 ± 1 μ g mL⁻¹). Non-tumor lines IC₅₀ values were above 335.00 μ g mL⁻¹. The ExtAS treatment showed less promising 50% inhibitory concentration, which varied also according to the tumor cell line, from 1500 to 5000 μ g mL⁻¹. Tumor-cells A-549 revealed the lowest fifty percent inhibitory concentration (1500 ± 1 μ g mL⁻¹), whereas A-375 presented the highest level for IC₅₀ (above 5000 μ g mL⁻¹). Non-tumor cells Hek-293 and MRC-5 IC₅₀ values were again above the ones found in the tumor lines, 5000 μ g mL⁻¹. Both non-tumor cells treated with ExtHS an ExtAS showed higher IC₅₀ compared to the tumor lines, indicating a selective cytotoxic activity (Table IV).

TABLE III

In vitro antioxidant activity, DPPH[•], ABTS^{•+}, superoxide dismutase-like and catalase-like activities in *Salvia officinalis* (L.) hydroalcoholic and aqueous extracts.

Sample	DPPH [•] (radical scavenging %)	ABTS ^{•+} (radical scavenging %)	Superoxide dismutase-like (USOD)	Catalase-like (UCAT)
Hydroalcoholic	0.77 ± 0.03^a	1.57 ± 0.07^a	$2.71 \pm 0.20^*$	$3.56 \pm 0.27^*$
Aqueous	0.73 ± 0.03^a	1.53 ± 0.03^a	$2.87 \pm 0.08^*$	$2.38 \pm 0.22^*$
Gallic acid	4.26 ± 0.025^b	6.77 ± 0.010^b	2.01 ± 0.02	1.22 ± 0.13

Results presented as mean \pm SD. ^aAmount (mg mL⁻¹) of the *S. officinalis* extracts required to scavenge 50% of the DPPH[•] and ABTS^{•+} radical. ^bAmount (μ g mL⁻¹) of the gallic acid required to scavenge 50% of the DPPH[•] and ABTS^{•+} radical. USOD = One unit of Sod is defined as the amount of extract (uL) that inhibits the rate of adrenochrome formation by 50%. UCAT= One unit of Cat is defined as the amount of extract (uL) that decomposes 1 mmol of H₂O₂ in 1 min at pH 7.4. *Values obtained for the extracts were statistically different the Gallic acid using t-test for independent samples ($p \leq 0.05$).

TABLE IV

IC₅₀ values – 50% inhibitory concentration obtained in the MTT assay for tumor and non-tumor cell after *S. officinalis* exposure 24h treatment.

Cell line	IC ₅₀ Hydroalcoholic extract (μ g mL ⁻¹)	IC ₅₀ Aqueous extract (μ g mL ⁻¹)
Tumor		
Hep-2	45.00 ± 1.00	4550.00 ± 2.90
HeLa	75.00 ± 4.10	3500.00 ± 4.10
HT-29	130.00 ± 2.10	4600.00 ± 3.80
A-375	165.00 ± 5.00	>5000.00
A-549	235.00 ± 1.00	1500.00 ± 2.50
Non Tumor		
HeK-293	335.00 ± 4.30	>5000.00
MRC-5	>625.00	>5000.00

MORPHOLOGICAL EXAMINATION OF CANCER CELLS AND APOPTOSIS ANALYSIS

Morphological cells alterations after ExtHS and ExtAS treatment with IC_{50} compared to the untreated groups were monitored under a light microscope (Olympus BX43). Modifications in morphology after Giemsa staining could be observed after 24 h extract treatment. Changes include cell shrinkage and apoptotic body formation, suggesting the induction of apoptosis as a consequence of extracts exposure. Negative controls here treated exclusively with distilled water and hydroalcoholic solvent 70 % (v/v) and presented no alterations on cell morphology. However, modifications in morphology were more expressive in tumor cells when compared a non-tumor cells.

The apoptotic potential was assessed by indirect immunofluorescence with annexin V/FITC - PI and confirmed with acridine orange/ethidium bromide staining (data not shown). Tumor and non-tumor cells were treated with ExtHS and ExtAS at IC_{50} for 24 h. Signaling for cell death was observed in the

majority of the cell treated with ExtHS compared to the untreated control group, similar patterns were observed for ExtAS but in lesser proportions (**Table IV**). Percent of cells immunostained in late apoptosis (Annexin V positive/PI positive) and/or necrosis (PI positive) are represented in **Figure 1**. Significantly lower percentages of late apoptosis/necrosis events were observed for the two extracts of non-tumor cells Hek-293 and MRC-5 compared to the tumor lines Hep-2, HeLa, HT-29, A-549 and A-375.

DISCUSSION

Some of the compounds herein identified, such as tartaric acid, malic acid and citric acid, exhibit antimicrobial activity and are widely used in the food industry to control the oxidative instability, acting as antioxidant by sequestering free radicals. Similarly, we found these compounds in aqueous extract of *S. officinalis* (L.), indicating that sage actually presents molecules with promising antioxidant activity. These organic acids may be

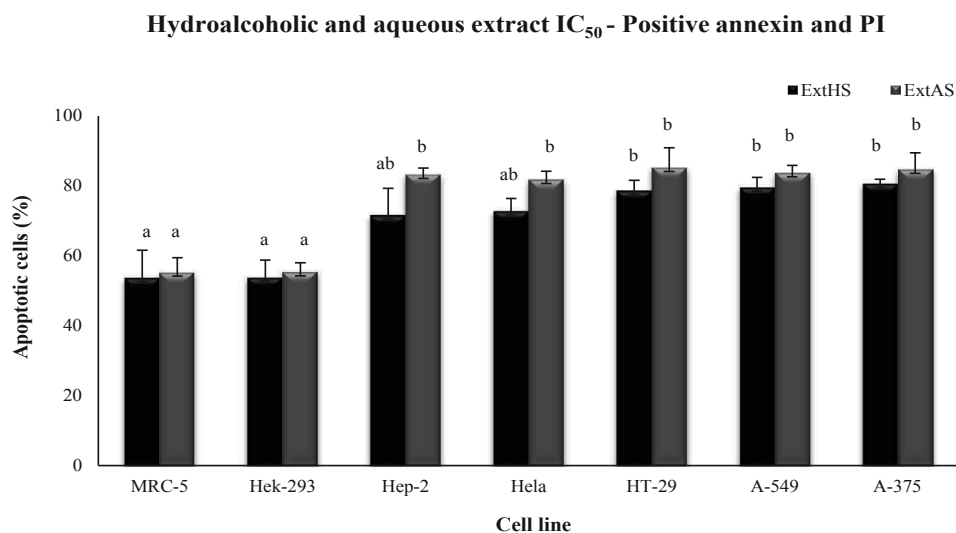


Figure 1 - Percent of cells immunostained in late apoptosis (Annexin V positive/PI positive) and/or necrosis (PI positive). Results were obtained from three independent experiments. Each bar represents the average percentage of late apoptosis/necrosis \pm SD relative to control, which was normalized to 100%. Letters correspond to statistically significant differences using ANOVA-Tukey test ($p \leq 0.05$).

related to the increase in the permeability of the membrane cell, leading to apoptosis (Ryu and Beuchat 1998).

Another interesting molecule found in the aqueous extract (ExtAS) was the succinic acid, an intermediate of the citric acid cycle that may participate on the immune system regulation. It has been attributed that dicarboxylic acids increases mitochondrial calcium levels, forming pores in the mitochondrial membrane and releasing cytochrome c into the cytosol, activating caspases (Rubic et al. 2008). The 12-methoxycarnosinic acid which was found in the hydroalcoholic extract (ExtHS), previously was reported to interact with the nuclear peroxisome proliferator-activated receptor (PPARs) in mouse embryonic fibroblasts, and plays an important role in anti-diabetic responses, once they regulate glucose homeostasis, lipid metabolism and inflammation (Christensen et al. 2010).

Other compounds herein identified participate in apoptosis induction mechanisms. The eucalyptol (50 mM) was able to regulate activity of p38 and caspase-3, inducing apoptosis and suppressing proliferation in the colorectal human cancer (Murata et al. 2013). Camphor has been attributed to present cytotoxic activity in human squamous cell carcinoma (HSC-2) (Cherneva et al. 2012). Ursolic acid was able to induce apoptosis in ovarian cancer cells via activation of caspase and reduction of glycogen and protein synthesis by phosphorylation of the enzyme of glycogen synthase kinase – 3 β (GSK-3 β) (Song et al. 2012). This molecule was able to up-regulate Bax and down-regulate Bcl-2 levels, releasing cytochrome c to the cytosol and decreasing mitochondrial membrane potential, thereby activating extrinsic and intrinsic apoptosis pathway in human breast cancer cell line (Kim et al. 2011, Akaberi et al. 2015).

Furthermore, rosmarinic acid, also found in the hydroalcoholic (ExtHS) and aqueous (ExtAS) extracts, was suggested to inhibit proliferation and induce apoptosis in tumor cells Caco-2 and HSC

T6, by down-regulating Bcl-2 and decreasing the expression of CyclinD1, inhibiting phosphorylation of STAT3, responsible for the progression and invasion of cancer (Zhang et al. 2012). Other compound found in both extracts was the caffeic acid. Previous studies using A-549 cell model reported that this compound may cause apoptosis on S/G2 cell cycle arrest, up-regulating Bax and down-regulating Bcl2 proteins (Ulasli et al. 2013).

Chemical analysis using GC-MS revealed compounds like β -caryophyllene, α -caryophyllene, eucalyptol, α -thujone, β -thujone, viridiflorol, mannol, camphor and luteolin-7-*O*-glucuronide in the ExtHS, that are known to present antioxidant, bactericidal, anti-inflammatory and analgesic activity (Fischedick et al. 2013, Kaliora et al. 2014). β -caryophyllene has been attributed to inhibit growth of cell lines and induce apoptosis in lymphoma and neuroblastoma, through ROS-mediated and cascade of mitogen-activated protein kinases (MAPKs) inhibition (Park et al. 2011). Extracts with α -caryophyllene showed cytotoxic activity in murine macrophage RAW264.7 and colon cancer at IC_{50} 73.3 $\mu\text{g mL}^{-1}$ (El Hadri et al. 2010). Here, ExtHS presented cytotoxic activity in human colorectal adenocarcinoma (HT-29) with IC_{50} 165.00 $\mu\text{g mL}^{-1}$, and was able to induced morphological changes and late apoptosis/necrosis in cells.

The ExtHS and ExtAS obtained from sage leaves were rich in polyphenols compounds with *in vitro* ability to scavenge the free radicals DPPH \bullet and ABTS $^{\bullet+}$, furthermore, cytotoxic activity in tumor cells. Polyphenol compounds are commonly found in sage, however, crude extracts will present different concentrations of polyphenols depending on several conditions like extraction method, plant geographic origin and harvest season (Russo et al. 2013).

It is known that superoxide dismutase and catalase enzymes have an important role in maintaining the physiologic redox equilibrium. The

two extracts used in this study presented important *in vitro* SOD-like and CAT-like activities compared with the standard gallic acid. This study verified the antioxidant properties of hydroalcoholic and water-based extracts, which are widely used in Brazil as plant tincture and tea, and may have an important role in maintaining the redox equilibrium, decreasing oxidative stress. These properties could contribute to play a key role to various diseases, such neurodegenerative disorders, inflammation and cancer.

Cytotoxicity analysis with MTT showed that non-tumor cell lines presented a higher IC_{50} compared to the tumor lines at the same extract exposure time indicating less cytotoxicity. Also to be considered, the ExtAS treatment showed less promising 50% inhibitory concentrations in tumor cell lines. Non-tumor cell lines presented, as observed to the ExtHA, a higher IC_{50} after incubation with ExtAS, indicating increase resistance when exposed to the aqueous extract and selective cytotoxic activity.

Moreover, the cytotoxicity assays of plant extracts presented effect in a dose-dependent manner. Kozics et al. (2013) determined the viability of HepG2 cells with 24-h treatment using various concentrations of hydroalcoholic sage extract. Cells were immediately rescued from culture and MTT analysis was performed showing a IC_{50} of 5.7 mg mL^{-1} , similar to the ExtAS in this study. Whereas, Zare Shahneh et al. (2013) tested on six cancer cell lines; non-Hodgkin's B-cell lymphoma (Raji), human leukemic monocyte lymphoma (U937), human acute myelocytic leukemia (KG-1A), human breast carcinoma (MCF-7 cells), human prostate cancer (PC3) and mouse fibrosarcoma (WEHI-164) and reported a range of IC_{50} 39.95 – 166.89 $\mu g mL^{-1}$, similar for the hydroalcoholic samples tested in this study. In addition, non-tumor cell human umbilical vein endothelial (HUVEC) presented lower cytotoxicity ($IC_{50} > 600 \mu g mL^{-1}$), that is in accordance with our findings.

Here, the antioxidant activity was evaluated using chemical assays, while the cytotoxic potential was performed in mammalian cells. It is already known that the mechanisms of the biological effects of the polyphenols are different in cells than that of chemical assays. So, it is possible than a compound that showed antioxidant activity in DPPH[•] and ABTS^{•+} assays could act as pro-oxidant in tumor cells. The pro-oxidant effect demonstrated in our work (mammalian cells) was dose-dependent, however, we showed only the IC_{50} of the both extracts for the seven cell lines studied.

The colorimetric technique gives a good high-contrast polychromatic stain and shows morphology alterations compatible with apoptosis. Here, negative control cells were shown as rounded-like structures, with abundant and intact cytoplasm. The cells treated with ExtHS and ExtAS at its inhibitory 50% concentration presented several modifications in morphology and reduced number of cells.

Death response mechanisms after extracts exposure was also investigated, indicating that most cells died in late stages of apoptosis or necrosis. Cells in late apoptosis or necrosis demonstrate similar patterns, once both annexin V and PI crosses the cell membrane after permeabilization, thus cannot be distinguished. Overlapping of annexin V and PI was considered to be a late apoptosis and/or necrosis event and signaling for cell death was observed in the majority of the cell treated with ExtHS compared to the untreated control group. Late apoptosis and/or necrosis were observed after ExtAS incubation and presented similar ExtHS induction pathway, but in less proportion, findings that were confirmed using acridine orange and ethidium bromide staining (data not shown).

In summary, the results here presented demonstrate that the cytotoxic potential was more promising in cells after ExtHS incubation, however, changes in extraction methodology may potentiate its activity in tumor cells. In addition, all compounds are of pharmaceutical importance

and the presence or not of synergistic, antagonistic or additive combinations of bioactive molecules should be investigated for better comprehension of the effects of natural products in cell metabolism. These findings evoke additional studies to clarify the relationship of compounds here identified and the molecular mechanisms involved in its biological activity. Brazilian *Salvia officinalis* (L.) extracts presented favorable cytotoxic response, generating morphological changes and induction of apoptosis, and also showing selectivity for tumor cells. Its compounds may lead to new drug discovery with reduction of toxic side effects in cancer patients.

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RESUMO

Salvia officinalis (Lamiaceae) é muito utilizado no sul do Brasil como condimento, tempero e chá para o tratamento de várias doenças. O objetivo deste estudo foi caracterizar compostos químicos no extrato hidroalcoólico (ExtHS) e aquosas (ExtAS) de *Salvia officinalis* (L.) por cromatografia gasosa acoplado a espectrometria de massa (GC-MS) e pela espectrometria de ionização por electrospray de alta resolução acoplado a massas (ESI-QTOF MS/MS), avaliar a capacidade *in vitro* para eliminar o radical livre 2,2-difenil-1-picrilhidrazil (DPPH[•]) e 2,2'-azino-bis (6-3-etilbenzotiazolina-sulfônico) (ABTS^{•+}), atividade da catalase (CAT-like) e superóxido dismutase (SOD-like), além disso, atividade citotóxica por ensaio MTT, alterações na morfologia celular por Giemsa e mecanismo de apoptose induzida por anexina V/iodeto de propídio. A identificação química revelou a presença de ácidos e compostos fenólicos nos extratos de sálvia. Na análise antioxidante *in vitro* ambos os extratos indicaram atividades promissoras. O ensaio citotóxico em células tumorais (HEp-2, HeLa, A-549,

HT-29 e A-375) e em não-tumoral (células HEK-293 e MRC-5), mostraram seletividade para células tumorais. A imunocitoquímica apresentou a maioria das células tumorais nos estágios tardios de processo de apoptose e necrose. Tendo em conta os resultados aqui apresentados, *Salvia officinalis* brasileira (L.) usada como condimento e chá pode proteger o corpo de algumas doenças, em particular aquelas que envolvem estresse oxidativo, como desordens neurodegenerativas, inflamação e câncer.

Palavras-chave: antioxidante, apoptose, câncer, compostos químicos, *Salvia officinalis* (L.).

REFERENCES

- AEBI H. 1984. Catalase in vitro. *Methods Enzymol* 105: 121-126.
- AKABERI M, MEHRI S AND IRANSHAHI M. 2015. Multiple pro-apoptotic targets of abietane diterpenoids from *Salvia* species. *Fitoterapia* 100: 118-132.
- AL-BARAZANJY R, DIZAYE K AND AL-ASADYE A. 2013. Cytotoxic and cytogenetic effects of *Salvia officinalis* on different tumor cell lines. *Middle East J Int Med* 13: 15-25.
- ALLEY MC, SCUDIERO DA, MONKS A, HURSEY ML, CZERWINSKI MJ, FINE DL, ABBOTT BJ, MAYO JG, SHOEMAKER RH AND BOYD MR. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 48: 589-601.
- BANNISTER JV AND CALABRESE L. 1987. Assays for superoxide dismutase. *Methods Biochem Anal* 32: 279-312.
- BERDOWSKA I, ZIELINSKI B, FECKA I, KULBACKA J, SACZKO J AND GAMIAN A. 2013. Cytotoxic impact of phenolics from Lamiaceae species on human breast cancer cells. *Food Chem* 141: 1313-1321.
- CEOLIN T, CEOLIN S, HECK RM, NOGUEZ PT AND ZDANSKI DE SOUZA AD. 2014. Relato de experiência do curso de plantas medicinais para profissionais de saúde. *Rev Baiana Saúde Públ* 37: 501-511.
- CHERNEVA E, PAVLOVIC V, SMELCEROVIC A AND YANCHEVA D. 2012. The effect of camphor and borneol on rat thymocyte viability and oxidative stress. *Molecules* 17: 10258-10266.
- CHRISTENSEN KB, JORGENSEN M, KOTOWSKA D, PETERSEN RK, KRISTIANSEN K AND CHRISTENSEN LPR. 2010. Activation of the nuclear receptor PPAR by metabolites isolated from sage *Salvia officinalis* L. *J Ethnopharmacol* 132: 127-133.
- EL HADRI A, GOMEZ DEL RIO MA, SANZ JS, GONZALEZ COLOMA A, IDAOMAR M, OZONAS BR, GONZALEZ JB AND SANCHEZ REUS MI. 2010. Cytotoxic activity

- of alpha-humulene and trans-caryophyllene from *Salvia officinalis* in animal and human tumor cells. *An R Acad Nac Farm* 76: 343-356.
- FISCHEDICK JT, STANDIFORD M, JOHNSON DA AND JOHNSON JA. 2013. Structure activity relationship of phenolic diterpenes from *Salvia officinalis* as activators of the nuclear factor E2-related factor 2 pathway. *Bioorg Med Chem* 21: 2618-2622.
- GARCIA CSC, ROESCH-ELY M, WASUM RA, DE ANTONI-ZOPPAS BC, WOLLHEIM C, NEVES GA, WEISS-ANGELI V AND DE SOUZA KCB. 2012. Assessment of *Salvia officinalis* (L.) hydroalcoholic extract for possible use in cosmetic formulation as inhibitor of pathogens in the skin. *Rev de Ciênc Farm Básica Apl* 33: 509-514.
- GENERALIC I, SKROZA D, SURJAK J, MOZINA SS, LJUBENKOV I, KATALINIC A, SIMAT V AND KATALINIC V. 2012. Seasonal variations of phenolic compounds and biological properties in sage (*Salvia officinalis* L.). *Chem Biodivers* 9: 441-457.
- KALIORA AC, KOGIANNOU DA, KEFALAS P, PAPASSIDERI IS AND KALOGEROPOULOS N. 2014. Phenolic profiles and antioxidant and anticarcinogenic activities of Greek herbal infusions; balancing delight and chemoprevention? *Food Chem* 142: 233-241.
- KIM KH, SEO HS, CHOI HS, CHOI I, SHIN YC AND KO SG. 2011. Induction of apoptotic cell death by ursolic acid through mitochondrial death pathway and extrinsic death receptor pathway in MDA-MB-231 cells. *Arch Pharm Res* 34: 1363-1372.
- KOZICS K, KLUSOVA V, SRANCIKOVA A, MUCAJI P, SLAMENOVA LH, KUSZNIEREWICZ B AND HORVATHOVA E. 2013. Effects of *Salvia officinalis* and *Thymus vulgaris* on oxidant-induced DNA damage antioxidant status in HepG2 cells. *Food Chem* 141: 2198-2206.
- MOSMANN T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63.
- MURATA S, SHIRAGAMI R, KOSUGI C, TEZUKA T, YAMAZAKI M, HIRANO A, YOSHIMURA Y, SUZUKI M, SHUTO K AND OHKOHCHI N. 2013. Antitumor effect of 1, 8-cineole against colon cancer. *Oncol Rep* 30: 2647-2652.
- NERSESYAN A, KUNDI M, ATEFIE K, SCHULTE-HERMANN R AND KNASMULLER S. 2006. Effect of staining procedures on the results of micronucleus assays with exfoliated oral mucosa cells. *Cancer Epidemiol Biomarkers Prev* 15: 1835-1840.
- PARK KR, NAM D, YUN HM, LEE SG, JANG HJ, SETHI G, CHO SK AND AHN KS. 2011. beta-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. *Cancer Lett* 312: 178-188.
- RE R, PELLEGRINI N, PROTEGGENTE A, PANNALA A, YANG M AND RICE-EVANS C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 26: 1231-1237.
- RODRIGUES MR, KANAZAWA LK, DAS NEVES TL, DA SILVA CF, HORST H, PIZZOLATTI MG, SANTOS AR, BAGGIO CH AND WERNER MF. 2012. Antinociceptive and anti-inflammatory potential of extract and isolated compounds from the leaves of *Salvia officinalis* in mice. *J Ethnopharmacol* 139: 519-526.
- RUBIC T ET AL. 2008. Triggering the succinate receptor GPR91 on dendritic cells enhances immunity. *Nat Immunol* 9: 1261-1269.
- RUSSO A, FORMISANO C, RIGANO D, SENATORE F, DELFINE S, CARDILE V, ROSSELLI S AND BRUNO M. 2013. Chemical composition and anticancer activity of essential oils of Mediterranean sage (*Salvia officinalis* L.) grown in different environmental conditions. *Food Chem Toxicol* 55: 42-47.
- RYU JH AND BEUCHAT LR. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int J Food Microbiol* 45: 185-193.
- SHAFIEE-NICK R, GHORBANI A, VAFABAGHERI F AND RAKHSHANDEH H. 2012. Chronic administration of a combination of six herbs inhibits the progression of hyperglycemia and decreases serum lipids and aspartate amino transferase activity in diabetic rats. *Adv Pharmacol Sci* 2012: ID 789796, 6 p.
- SINGLETON VL AND ROSSI JA. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16: 144-158.
- SONG YH, JEONG SJ, KWON HY, KIM B, KIM SH AND YOO DY. 2012. Ursolic acid from *Oldenlandia diffusa* induces apoptosis via activation of caspases and phosphorylation of glycogen synthase kinase 3 beta in SK-OV-3 ovarian cancer cells. *Biol Pharm Bull* 35: 1022-1028.
- TAKANO N, INOKUCHI Y AND KURACHI M. 2011. Effects of ethanol extracts of herbal medicines on dermatitis in an atopic dermatitis mouse model. *Yakugaku Zasshi* 131: 581-586.
- ULASLI SS, CELIK S, GUNAY E, OZDEMIR M, HAZMAN O, OZYUREK A, KOYUNCU T AND UNLU M. 2013. Anticancer effects of thymoquinone, caffeic acid phenethyl ester and resveratrol on A549 non-small cell lung cancer cells exposed to benzo(a)pyrene. *Asian Pac J Cancer Prev* 14: 6159-6164.
- VASKO L, VASKOVA J, FEJERCAKOVA A, MOJZISOVA G AND PORACOVA J. 2014. Comparison of some antioxidant properties of plant extracts from *Origanum vulgare*, *Salvia officinalis*, *Eleutherococcus senticosus* and *Stevia rebaudiana*. *In Vitro Cell Dev Biol Anim* 50: 614-622.
- YAMAGUCHI T, TAKAMURA H, MATOBA T AND TERAOKA J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-

- picrylhydrazyl. Biosci Biotechnol Biochem 62: 1201-1204.
- ZARE SHAHNEH F, BARADARAN B, ORANGI M AND ZAMANI F. 2013. In vitro Cytotoxic of four plants used in Persian traditional medicine. Adv Pharm Bull 3(2): 453-455.
- ZHANG Y, SMUTS JP, DODBIBA E, RANGARAJAN R, LANG JC AND ARMSTRONG DW. 2012. Degradation study of carnosic acid, carnosol, rosmarinic acid, and rosemary extract (*Rosmarinus officinalis* L.) assessed using HPLC. J Agric Food Chem 60: 9305-9314.