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Infusions of gamma irradiated *Aloysia citrodora* L. and *Mentha x piperita* L.: Effects on phenolic composition, cytotoxicity, antibacterial and virucidal activities

Eliana Pereira[a,b], Andreia I. Pimenta[c], Ricardo C. Calhelha[a], Amilcar L. Antonio[a], Lillian Barros[a], Celestino Santos-Buelga[b], Sandra Cabo Verde[c], Isabel C.F.R. Ferreira[a,*]

[a] Centro de Investigação de Montanha (CIMO), ESA, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 1172, 5300-253 Bragança, Portugal
[b] GIP-USAL, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain
[c] Centro de Ciências e Tecnologias Nucleares, Instituto Superior Técnico, Universidade de Lisboa, Estrada Nacional 10 (km 139,7), 2695-066 Bobadela LRS, Portugal

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ABSTRACT

The incorporation of plants in industry requires high standards of hygiene, being the raw materials obligatorily decontaminated. Irradiation is increasingly applied as a decontamination technique in several countries. This work aimed to evaluate the effects of gamma radiation on the phenolic composition, cytotoxicity, virucidal and antimicrobial properties of *Aloysia citrodora* L. (lemon verbena) and *Mentha x piperita* L. (peppermint) infusions. Phenolic compounds profile was obtained by HPLC-DAD-ESI/MS and cytotoxicity was evaluated in human tumour and non-tumour cell lines; in virucidal efficacy, MNV-1 and HAdV-5 viral titers were determined by plaque assay in Raw264.7 and A549 cell lines, respectively; and the antibacterial potential was evaluated against Gram-negative and Gram-positive bacteria. Radiation treatment caused a significant increase in total phenolic compounds of both plants, while the virucidal efficacy was depend on the plant, the virus, and absorbed dose. *S. aureus*, with MIC and MBC values of 5 mg/mL, was the only bacteria sensible to the infusions of non-irradiated and 1 kGy irradiated lemon verbena and peppermint samples; gamma radiation appears to cause no alteration in the antibacterial potential of the studied plants. Peppermint infusion (irradiated at 10 kGy) showed the highest cytotoxic potential in all tumour cell lines.

1. Introduction

The use of plants to improve the taste of foods, due to its sensory and preservative properties, has been a common practice throughout generations (Güler et al., 2015). However, its employment also covers the area of traditional medicine (Baydoun et al., 2015). The increasing demand for healthier diets and the use of natural products in prevention and/or treatment of health problems (Dhami and Mishra, 2015) has led to an intensive search for plant bioactive compounds (Gerson-Cwilich et al., 2006; Agyare et al., 2016; Giovannini et al., 2016; Jacobo-Herrera et al., 2016).

Plants are rich in phenolic compounds, terpenoids, alkaloids, quinones, carotenoids, sterols, glucosinolates and other sulphur-containing compounds (Embuscado, 2015; Gupta et al., 2016), which provide health benefits due to their wide range of biological properties (Li et al., 2015; Gupta et al., 2016). Phenolic compounds are potent antioxidants due to their capacity to scavenge reactive oxygen species and chelate metal ions (Port's et al., 2013). The most common compounds are flavonoids and phenolic acids, exhibiting bioactive properties in terms of the cardiovascular, nervous and gastrointestinal systems, displaying anticarcinogenic, antihypertensive, antimutagenic, anti-nociceptive, anti-inflammatory, immunomodulatory and hepatoprotective actions, as also angiogenesis inhibitory activity (Vaquero et al., 2010; Acosta-Estrada et al., 2014; Bao et al., 2015).

However, plants easily suffer contaminations being absolutely essential the application of techniques to eliminate microorganisms such as fungi and bacteria, insects and others (Lung et al., 2015). The use of safe methodologies both for consumers and environment is mandatory (Machhour et al., 2011). Thus, irradiation is increasingly attracting attention, because it consists on a safe and non-thermal decontamination and preservation technology, suit-

* Corresponding author.
E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira).

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able for various food matrices, including medicinal herbs and spices (EU, 1999; EFSA, 2011).

The application of gamma radiation to different plant species has proven its capacity to preserve chemical and nutritional properties (Pereira et al., 2014; Pinela et al., 2016). The present study was extended to evaluate gamma radiation (1 and 10 kGy) effects on the phenolic composition, cytotoxicity, virucidal and antimicrobial activity of the most consumed forms of *Menta x piperita* L. (peppermint) and *Aloysia citrodora* L. (lemon verbena): infusions.

2. Materials and methods

2.1. Dried samples and gamma irradiation

Aloysia citrodora Paláu (lemon verbena) and *Mentha piperita* L. (peppermint) were provided by Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal – a local producer. Dried samples were divided into three groups: control (non-irradiated, 0 kGy), and samples irradiated with different gamma radiation doses (1 and 10 kGy).

The irradiation was performed using a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with an activity of 165 TBq (4.45 kCi), in September 2015, and Fricke dosimeter was used to estimate dose rate for the applied irradiation geometry. During irradiation process, Amber Perspex routine dosimeters (batch X, from Harwell Company, U.K.) was used for estimate the dose, following a procedure previously described by Pereira et al. (2015a). The estimated dose rate was 1.5 kGy/h, and the average absorbed doses by the plants samples were 0.9 ± 0.1 kGy and 10.2 ± 0.6 kGy. The dose uniformity ratio (D_{max}/D_{min}) was 1.1 In the text and tables, for simplicity, we considered the values 0, 1 and 10 kGy, for the doses of non-irradiated and irradiated groups, respectively.

2.2. Standards and reagents

2.2.1. For gamma radiation

Fricke dosimeter (a chemical solution sensitive to ionizing radiation) to estimate the dose and dose rate of irradiation was used. The preparation of this chemical solution was performed in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Company, Oxfordfordshire, UK). For prepare the acid aqueous Fricke dosimeter solution was used ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid; all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, Billerica, NA, USA).

2.2.2. For chemical analysis

Acetonitrile 99.9% was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic compound standards: apigenin-6-*C*-glucoside, caffeic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside and rosmarinic acid were purchased from Extrasynthese (Genay, France).

2.2.3. For cytotoxicity assays

Fetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2.4. For virucidity determination assays

DMEM media, FBS, L-glutamine, penicillin and streptomycin, sodium pyruvate, non-essential amino acids, HEPES buffer were from Gibco, Life Technologies (Carlsbad, CA, USA). Agarose was from SeaKem ME; Lonza (Rockland, ME, USA) and neutral red solution was from Sigma (St. Louis, MO, USA). Deionized filtered water (DI) was produced by the Milli-Q system Millipore (Millipore, model A10, Millipore Iberica, Spain).

2.3. Infusion's preparation

The infusions were obtained from the dried plant material. Each sample (2 g) was added to 200 ml of boiling distilled water and left to stand at room temperature for 5 min, and subsequently filtered under reduced pressure through Whatman No. 4 paper (Pereira et al., 2015a).

2.4. Evaluation of cytotoxic potential

2.4.1. General

The cytotoxicity evaluation was accomplished according to a procedure previously described by Pereira et al. (2016a). The results were expressed in GI_{50} values, i.e., sample concentration that inhibited 50% of the net cell growth. For positive control, ellipticine was used.

2.4.2. In tumour cell lines

Four human tumour cell lines, (MCF-7), non-small cell lung cancer (NCI-H460), cervical carcinoma (HeLa) and hepatocellular carcinoma (HepG2) were used to determine the cytotoxicity (Pereira et al., 2016a). The cell growth inhibition was measured using sulforhodamine B assay (SRB), were the amount of pigmented cells is directly proportional to the total protein mass and therefore to the number of bounded cells. The bounded sulforhodamine B was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

2.4.3. In non-tumour cell line

This methodology was performed following a procedure previously performed by Pereira et al. (2016a). A cell culture PLP2 was obtained from porcine liver in the laboratory. SRB assay was followed to evaluate the growth inhibition.

2.5. Virucidal activity evaluation

2.5.1. Viruses and cell cultures

Human Adenovirus type 5 (HAdV-5, ATCC® VR-1516™) was propagated in confluent monolayers of human lung carcinoma cells A549 (ATCC® CCL-185™). Murine Norovirus type 1 (MNV-1) strain P3 (kindly provided by Dr. Christiane E. Wobus at the University of Michigan Medical School, USA) was propagated in confluent monolayers of mouse macrophages Raw 264.7 (ATCC® TIB-71™). Cells were maintained at 37 °C and 5% CO_2, in Dulbecco's modified Eagle medium supplemented with 1 mM L-glutamine, 10% Fetal Bovine Serum (FBS; Heat inactivated), 1 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 1 mM HEPES buffer.

For preparing HAdV-5 and MNV-1 stocks, confluent A549 cells and Raw 264.7, respectively, were infected. After 1 h of incubation at 37 °C with mild agitation, the cellular monolayer was washed twice with phosphate buffered saline solution (PBS) and supplemented DMEM was added. The viruses were harvested after 7 days and 3 days post-infection for HAdV-5 and MNV-1, respectively, by three freeze-thaw cycles at low centrifugation at 3000 rpm

Table 1
Cytotoxicity of lemon verbena and peppermint infusions prepared from non-irradiated and irradiated samples.

Doses	MCF-7 (breast carcinoma)	NCI-H460 (non-small cell lung cancer)	HeLa (cervical carcinoma)	HepG2 (hepatocellular carcinoma)	Hepatotoxicity PLP2 (non-tumour cells)
Lemon verbena					
0 kGy	318 ± 31^{a}	234 ± 19^{a}	245 ± 3^{a}	87 ± 1^{a}	>400
1 kGy	302 ± 12^{a}	235 ± 4^{a}	233 ± 22^{a}	79 ± 8^{ab}	>400
10 kGy	305 ± 1^{a}	234 ± 9^{a}	249 ± 14^{a}	74 ± 4^{b}	>400
Peppermint					
0 kGy	267 ± 17^{a}	140 ± 13^{b}	242 ± 14^{b}	43 ± 3^{c}	>400
1 kGy	296 ± 28^{a}	99 ± 8^{c}	254 ± 4^{ab}	55 ± 4^{b}	>400
10 kGy	300 ± 20^{a}	205 ± 18^{a}	263 ± 10^{a}	66 ± 7^{a}	>400

Positive control (Ellipticine) – MCF-7: 0.91 ± 0.04; NCI-H460: 1.03 ± 0.09; HeLa: 1.91 ± 0.06; HepG2: 1.14 ± 0.21; PLP2: 3.22 ± 0.67. GI_{50} values (μg/mL) correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

Table 2
Antiviral efficacy of lemon verbena and peppermint infusions.

	Log_{10} reduction (mean ± SE)	
	Human Adenovirus 5	
Lemon verbena	3.5 mg/mL	7 mg/mL
0 kGy	0.21 ± 0.10	0.23 ± 0.12
1 kGy	$0.04 \pm 0.01^{a,b}$	$0.24 \pm 0.10^{b,c}$
10 kGy	0.07 ± 0.00^{A}	$-0.14 \pm 0.05^{a,c}$
Peppermint	3.5 mg/mL	7 mg/mL
0 kGy	0.02 ± 0.02^{a}	0.29 ± 0.06
1 kGy	0.11 ± 0.08	0.14 ± 0.02
10 kGy	0.07 ± 0.02	0.04 ± 0.01^{a}
Control	0.13 ± 0.01	
	Murine Norovirus 1	
Lemon verbena	3.5 mg/mL	7 mg/mL
0 kGy	0.16 ± 0.06	0.11 ± 0.05
1 kGy	0.05 ± 0.02	0.20 ± 0.09
10 kGy	$-0.08 \pm 0.03^{a,b}$	$0.20 \pm 0.06^{a,b}$
Peppermint	3.5 mg/mL	7 mg/mL
0 kGy	$0.22 \pm 0.01^{a,b,c}$	0.07 ± 0.04^{b}
1 kGy	0.04 ± 0.01	0.04 ± 0.00^{a}
10 kGy	$-0.26 \pm 0.09^{a,c}$	0.05 ± 0.01
Control	0.10 ± 0.01	

Results represent the log10 reductions in cell culture infectivity of murine norovirus and human adenovirus (initial titer, 10^5–10^6 PFU/mL) after 24 h of exposures to lemon verbena and peppermint infusions (irradiated and non-irradiated) at two concentrations. The experiment was conducted in duplicate.

[a] Reduction was statistically significant ($p \leq 0.05$) in comparison to the control (with no infusion) at the same time exposure.

[b] Reductions were significantly different ($p \leq 0.05$) between 3.5 mg/mL and 7 mg/mL of infusion.

[c] Reductions were significantly different ($p \leq 0.05$) between doses of irradiation.

Table 3
Antibacterial efficacy of lemon verbena and peppermint infusions.

	E. feacalis	*E. coli*	*B. cereus*	*S.* Typhimurium	*S. aureus*
MIC (mg/mL)[a]					
Lemon verbena					
0 kGy	≥10	≥10	≥10	≥10	5
1 kGy	≥10	≥10	≥10	≥10	5
10 kGy	≥10	≥10	≥10	≥10	5
Peppermint					
0 kGy	≥10	≥10	≥10	≥10	5
1 kGy	≥10	≥10	≥10	≥10	5
10 kGy	≥10	≥10	≥10	≥10	≥10
PEN/STREP	0.002	0.008	≤0.001	0.08	≤0.001
MBC (mg/mL)[a]					
Lemon verbena					
0 kGy	≥10	≥10	≥10	≥10	5
1 kGy	≥10	≥10	≥10	≥10	5
10 kGy	≥10	≥10	≥10	≥10	5
Peppermint					
0 kGy	≥10	≥10	≥10	≥10	5
1 kGy	≥10	≥10	≥10	≥10	5
10 kGy	≥10	≥10	≥10	≥10	≥10
PEN/STREP	0.002	0.008	0.007	0.239	≤0.001

[a] Concentrations of PEN/STREP are expressed in 10^3 U/mL of penicillin and mg/mL of streptomycin.

(Beckman J2-21M, rotor J20-1) for 30 min at 18 °C. The resulting supernatant was aliquoted and stored at −80 °C.

2.5.2. Virucidal efficacy experiments

Lemon verbena and peppermint infusions were evaluated in separate experiments at concentrations of 3.5 and 7.0 mg/mL in sterile ultrapure water. The experiments were performed at room temperature in triplicate for each virus and infusion, based on the procedure previously described by Gilling et al. (2014). MNV-1 and HAdV-5 were added separately to each one of the tubes (to a final concentrations of 10^7–10^8 PFU/mL) and the tubes were placed in an orbital incubator with mild agitation. Control tubes (no anti-viral added) containing MNV-1 or HAdV-5 in PBS were also included in each experiment. At 0 h and 24 h an aliquot of 100 μl of the samples were removed from each tube and diluted in 900 μl of supplemented DMEM to neutralize the extracts. All samples were placed at −80 °C until subsequent assays were performed. The final MNV-1 and HAdV-5 viral titers were determined by plaque assay in Raw264.7 and A549 cell lines, respectively. Briefly, cells were seeded into 60-mm plates at a density of 7.5×10^5 cells per plate. After reached 70–80% of confluence the cellular monolayer was infected with 300 μl of 10-fold serial dilutions of treated viral samples. Duplicates were made for each sample. Then, plates were incubated for 1 h at 37 °C and 5% CO_2, with mild agitation every 15 min. After removal of the inoculum, cells were overlaid with 3 ml of overlay medium (2× DMEM) with 0.5% agarose. For HAdV-5 infection, after incubation for 72 h, a second 1.5 ml overlay of 2× DMEM with 0.5% agarose was added. Plaques were subsequently counted 8–24 h after a third agarose overlay (1.5 ml) with 1% of a neutral red solution (3.3 g/liter). For MNV-1 infection, after incubation for 72 h a second 3 ml overlay of 2× DMEM with 0.5% agarose and 1% of a neutral red solution was added and the plaques were counted after 8–24 h of additional incubation. Virus titer was expressed in PFU per milliliter of substrate (PFU/mL).

2.5.3. Anti-viral neutralization experiments

Neutralization assays following the procedure reported by Gilling et al. (2014) were performed with both 3.5 mg/mL and 7.0 mg/mL concentrations of Lemon verbena and Peppermint infusions. One ml of the infusion was placed into 9 ml o supplemented DMEM. The solution was mixed thoroughly and then approximately 10^7 PFU/ml of MNV or HAdV was added. After vortex, the suspension was allowed to sit for 5 min. Ten-fold serial dilutions were plaque assayed on Raw264.7 or A549 cells as described previously. If the anti-virals were completely neutralized in DMEM, it

was expected that there would be no reduction of the initial viral titer.

2.6. Antibacterial activity evaluation

Antibacterial activity was evaluated against Gram-negative bacteria: *Escherichia coli* (ATCC 8739), *Salmonella enterica* Typhimurium (ATCC 14028), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (SSIC 1/1), and *Enterococcus feacalis* (ATCC 29212), following the procedure previously described by Soković et al. (2010). Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The infusions were dissolved in sterile ultrapure water at a final concentration of 100 mg/mL and added in tryptic soy broth (TSB) with bacterial inoculum (1.0×10^4 CFU per well) to achieve the wanted concentrations (1–10.0 mg/mL). The microplates were incubated for 24 h at 37 °C. The minimum bactericidal concentrations (MBSs) were determined by sub-cultivation of 2 μl sample into 96-well plates containing 100 μl of fresh TSB per well and further incubation for 24 h at 37 °C. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. Streptomycin and penicillin were used as standard in the antibacterial assays and sterile ultrapure water was used as a negative control.

2.7. Analysis of phenolic compounds

The infusions were analysed using a HPLC-DAD (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, US), couple to a mass spectrometer detector (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyser, and by following a methodology previously described by Barros et al. (2013). The identification of the phenolic compounds was performed by comparison, when available, with commercial standards, otherwise the compounds were tentatively identified by comparing the obtained information with available data reported in the literature. Quantitative analysis was performed using calibration curves obtained for each available phenolic standard and for the identified phenolic compounds for which a commercial standard was not available, the quantification was performed using another compound from the same phenolic group. The results were expressed as mg per mL of infusion.

2.8. Statistical analysis

The results were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. The correlation between phenolic compounds and cytotoxicity of infusions for both plants was performed using Pearson's correlation factor, and the normality of data was checked with Shapiro-Wilk's test. Three samples for each one of the species were used and all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD) or standard error (SE). This treatment was made using SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Cytotoxic properties of infusions from non-irradiated and irradiated samples

Table 1 shows the results obtained for the cytotoxic evaluation on non-tumour (PLP2) and four human tumour cell lines (MCF-7, NCI-H460, HeLa and HepG2) of infusions prepared from non-irradiated and irradiated lemon verbena and peppermint samples. It can be seen that all samples exhibited antiproliferative activity on the four explored tumour cell lines, with GI_{50} values ranging between 74 and 318 μg/mL and 43–300 μg/mL for lemon verbena and peppermint, respectively. Hepatocellular carcinoma cell line (HepG2) was the most sensitive cell line against the cytotoxic effect of both plants, regardless of the dose applied, giving rise to the lowest GI_{50} values, i.e., 74 μg/mL (lemon verbena infusion irradiated at 10 kGy) and 43 μg/mL (infusion of peppermint control sample). No significantly different effects on almost all the assayed cell lines were observed for the distinct lemon verbena samples, either irradiated or not, with the exception of the most sensitive cell line (HepG2). Otherwise, regarding peppermint samples, particular relevant effects were found for the sample irradiated at 1 kGy on the line NCI-H460 (GI_{50} = 99 μg/mL) and of all the studied samples on HepG2, especially in the case of the non-irradiated one (GI_{50} = 43 μg/mL). MCF-7 was the only cell line for which no significant difference was observed for the cytotoxic activity among the different samples. In the previous study performed with methanolic extracts of peppermint (Pereira et al., 2016a), similar cytotoxic activity was also observed on all the explored cell lines.

3.2. Virucidal activity of infusions from non-irradiated and irradiated samples

Table 2 shows the antiviral efficacy of lemon verbena and peppermint infusions prepared from non-irradiated and irradiated samples. The virucidal activity was tested against two enteric viruses – HAdV-5 and MNV-1 (as a human norovirus surrogate), after 24 h of exposure to two concentrations of plant infusions. The data are expressed in $\log_{10}$ variation (t_0–t_{24}) of the viral titer (PFU/mL). The obtained results indicated variations inferior to 1 log10 for both viruses when exposed to the infusions, regardless of the used infusion concentration and gamma radiation treatment dose of the plant. The titer variation for both controls- HAdV and MNV, was very similar and represent the natural loss of infectivity of the viral particles during the 24 h of incubation. Concerning the exposure to the samples, only some of the titer variations are significantly different ($p \leq 0.05$) in comparison to the control. For adenoviruses, the 3.5 mg/mL infusions of lemon verbena, irradiated at 1 kGy and 10 kGy, and peppermint non-irradiated; and 7 mg/mL infusion of lemon verbena and peppermint irradiated at 10 kGy caused a viral titer variation significantly inferior to the control. The presence of 7 mg/mL of lemon verbena infusion of the sample irradiated at 10 kGy caused a negative variation on adenovirus viral titer, indicating that the viral titer after 24hs of exposure was superior to the initial one. This particular result could indicate an increase of the infectivity state after viral exposure to the sample. In general, it seems that the plant samples evaluated do not have anti-viral effects on human adenoviruses.

Regarding MNV, the exposure to infusions of lemon verbena irradiated at 10 kGy (3.5 and 7 mg/mL) and peppermint non-irradiated (3.5 mg/mL) and irradiated at 1 kGy (7 mg/mL) and 10 kGy (3.5 mg/mL) causes significant variations in the viral titer in comparison to the control. Opposite to what occur with HAdV, the exposure to the 7 mg/mL infusion of 10 kGy irradiated lemon verbena and to non-irradiated peppermint infusion (3.5 mg/mL) seems to have an inhibitory effect over MNV, causing log10 reductions in titer significantly higher than in PBS control.

The radiation treatment of the plant prior to infusion procedure seems to cause different effects on the virucidal efficacy, depending on the plant, the virus, and absorbed dose. The irradiation effect over human adenoviruses seems to be only detected for the higher applied concentration of infusion of lemon verbena irradiated at 1 kGy (virucidal effect) and 10 kGy (enhancement effect). In murine noroviruses, the virucidal efficacy point out to be

Table 4a
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in lemon verbena infusions prepared from non-irradiated and irradiated samples.

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative identification	Quantification (μg/mL)		
						0 kGy	1 kGy	10 kGy
1	4.1	280	461	315(8), 135(28)	Verbasoside	2.6 ± 0.5a	2.9 ± 0.4a	2.4 ± 0.4a
2	15.0	344	637	351(100), 285(89)	Luteolin-7-*O*-diglucuronide	68.7 ± 0.3b	64.2 ± 0.2c	70.0 ± 0.5a
3	16.7	314	163	119(100)	*p*-Coumaric acid	2.1 ± 0.1b	2.2 ± 0.1b	2.5 ± 0.1a
4	18.0	338	621	351(100), 269(20)	Apigenin-7-*O*-diglucuronide	9.5 ± 0.2a	9.9 ± 0.1a	10.1 ± 0.6a
5	18.2	330	623	461(18), 315(5)	Verbascoside	114 ± 2b	83 ± 2c	134 ± 1a
6	20.2	350	651	351(100), 299(5)	Chrysoeriol-7-*O*-diglucuronide	10.4 ± 0.1a	9.3 ± 0.4b	9.5 ± 0.3b
7	20.4	330	623	461(18), 315(5)	Isoverbascoside	1.2 ± 0.1b	1.5 ± 0.3ab	1.7 ± 0.1a
8	21.2	330	623	461(15), 315(10)	Forsythoside	27 ± 1a	21.9 ± 0.6b	19 ± 2c
9	21.8	350	491	315(100), 300(23)	Isorhamnetin-7-*O*-glucuronide	4.4 ± 0.1a	4.36 ± 0.06a	4.44 ± 0.07a
10	23.1	330	637	491(5), 461(60), 315(13)	Eukovoside	1.00 ± 0.01a	0.9 ± 0.1ab	0.8 ± 0.1b
11	29.2	330	651	505(7), 475(22)	Martinoside	0.52 ± 0.06a	0.40 ± 0.01b	0.56 ± 0.03a
					Total caffeoyl derivatives	146.0 ± 0.1b	111 ± 1c	159 ± 2a
					Total phenolic acids	2.1 ± 0.1b	2.2 ± 0.1b	2.4 ± 0.1a
					Total flavonoids	92.9 ± 0.1a	87.7 ± 0.4b	94 ± 1a
					Total phenolic compounds	241.04 ± 0.01b	201 ± 1c	255 ± 4a

In each row different letters mean significant differences ($p < 0.05$).

influenced by gamma radiation in the 3.5 mg/mL infusions of peppermint non-irradiated (positive effect) and irradiated at 10 kGy (negative effect). Taken together, the data suggested that the irradiation treatment of lemon verbena and peppermint samples seems to preserve the native properties of the plant against enteric viral pathogens.

There are few studies about the effect of herbal infusions against viral particles. Some studies indicated the potential of peppermint as an anti-viral agent either in methanol (Yucharoen et al., 2011) and tea-like extracts (Geuenich et al., 2008). Nevertheless, the virucidal activity of herbal samples was only reported for enveloped virus, like herpes simplex (HSV) and HIV-1. Geuenich et al. (2008) have reported the efficacy of peppermint infusions to limit the infection mechanism of HIV-1 through the inhibition of virus-host cell interaction, but the same effect was not detected against the non-enveloped HAdV-5. It seems likely that the enveloped viruses are more sensible to herbal infusions than non-enveloped ones, perhaps due to intrinsic natural resistance of non-enveloped enteric virus that are more stable when exposed to environmental conditions or even during the infection.

3.3. Antibacterial activity of infusions from non-irradiated and irradiated samples

Table 3 shows the results for the antibacterial assessment. The effect of herbal infusions was determined for five different bacterial species: Gram-positive (*Enterococcus feacalis*, *Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative (*Escherichia coli* and *Salmonella enterica* Typhimurium). Considering the obtained data, it seems that lemon verbena and peppermint infusions up to 10 mg/mL have no inhibitory effect on the growth of *E. feacalis*, *E. coli*, *B.cereus* and *S.* Typhimurium. Moreover, the gamma radiation dose appears to cause no alteration on the antibacterial potential of the studied plants. These results are in concordance with Saeed et al. (2006) that evaluated the activity of peppermint infusions and concluded that they have no effect over several Gram-negative bacteria, including *E. coli.*

S. aureus, with MIC and MBC values of 5 mg/mL, was the only species that seems to be sensible when exposed to the infusions of non-irradiated and 1 kGy irradiated lemon verbena and peppermint samples. However, when the samples were irradiated at 10 kGy the inhibitory effect over *S. aureus* decreases (MIC ≥ 10 mg/mL; MBC ≥ 10 mg/mL). When this dose is applied it is likely to occur the radiolytic degradation of some compounds of lemon verbena and peppermint that could in consequence alter their antibacterial potential, at least for *S. aureus.*

Altogether, it is possible to verify that excluding *S. aureus*, the infusions of lemon verbena and peppermint reveal a low antimicrobial potential for the studied bacterial species. Nevertheless, considering the public health risk associated with *S. aureus* the use of herbal infusions as a tool of bacterial inhibition could be a feasible mitigation strategy.

3.4. Comparative analysis of phenolic compounds in non-irradiated and irradiated samples

The identification and quantification of the phenolic compounds in non-irradiated (0 kGy) and irradiated samples (1 and 10 kGy) of lemon verbena and peppermint are shown in Tables 4a and 4b , respectively. Eleven and fourteen phenolic compounds were tentatively identified in lemon verbena and peppermint, respectively.

Lemon verbena infusions presented in its composition four flavonoids (peaks 2, 4, 6 and 9), six caffeoyl phenylethanoid derivatives (including verbasoside) (peaks 1, 5, 7, 8, 10 and 11) and a phenolic acid (peak 3). All these compounds have been identified in a previous study (Pereira et al., 2016b), in order to evaluate the effect of gamma radiation in the preservation of phenolic compounds, and on the decontamination of ochratoxin A and aflatoxin B_1.

Up to eleven phenolic compounds were detected in lemon verbena infusions, and as it can be seen in Tables 4a, four flavonoids (peaks 2, 4, 6 and 9), five caffeoyl derivatives (peaks 5, 7, 8, 10 and 11), a phenylethanoid glycoside (peak 1), and a hydroxycinnamic acid (peak 3) were tentatively identified. The compounds were identified based on their mass and UV–vis spectra and retention characteristics. The majority of the detected compounds (verbasoside, luteolin-7-*O*-diglucuronide, apigenin-7-*O*-diglucuronide, verbascoside, chrysoeriol-7-*O*-diglucuronide,

Table 4b
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in peppermint infusions prepared from non-irradiated and irradiated samples.

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	Tentative identification	Quantification (μg/mL)		
						0 kGy	1 kGy	10 kGy
1′	5.3	328	353	191(100), 179(27), 173(5), 161(15), 135(30)	3-*O*-Caffeoylquinic acid	1.5 ± 0.1b	1.03 ± 0.03c	2.34 ± 0.01a
2′	7.2	328	353	191(100), 179(90), 173(50), 161(20), 135(57)	5-*O*-Caffeoylquinic acid	1.7 ± 0.1b	1.54 ± 0.02b	4.4 ± 0.3a
3′	11.3	326	179	135(100)	Caffeic acid	0.4 ± 0.2b	0.32 ± 0.01b	3.0 ± 0.3a
4′	14.7	348	637	285(100)	Luteolin-*O*-dihexoside	16.45 ± 0.04c	18.7 ± 0.3b	19.9 ± 0.7a
5′	16.1	288,330sh	537	493(45), 313(18), 295(36), 269(55), 197(36), 179(64), 135(100)	Caffeic acid trimer	6.0 ± 0.3c	9.0 ± 0.2a	7.2 ± 0.3b
6′	16.3	284,332sh	595	287(100)	Eriodictyol-7-*O*-rutinoside	127 ± 2c	170 ± 6b	205 ± 3a
7′	17.5	286,336sh	449	287(100)	Eriodictyol-*O*-hexoside	2.7 ± 0.2b	2.30 ± 0.03c	5.2 ± 0.3a
8′	19.4	350	593	285(100)	Luteolin-7-*O*-rutinoside	34.4 ± 0.3b	34.5 ± 0.1b	41 ± 1a
9′	20.2	348	461	285(100)	Luteolin-7-*O*-glucuronide	15.2 ± 0.5b	17.8 ± 0.3b	20 ± 1a
10′	20.5	282,330sh	579	271(100)	Naringenin-*O*-rutinoside	4.5 ± 0.2b	3.76 ± 0.04c	6.2 ± 0.1a
11′	21.9	278,338sh	717	537(34), 519(50), 493(39), 339(29), 321(37), 313(6), 295(100), 197(3), 179(11), 161(5), 135(11)	Salvianolic acid B/E/L	20.6 ± 0.5c	21.7 ± 0.1b	27.6 ± 0.2a
12′	23.2	286,338sh	609	301(100)	Hesperetin-*O*-rutinoside	5.8 ± 0.2b	5.7 ± 0.1b	11.0 ± 0.3a
13′	24.1	330	359	197(13), 179(20), 161(100), 135(21)	Rosmarinic acid	34 ± 1b	35.1 ± 0.4b	51 ± 1a
14′	24.5	288,340sh	493	313(5), 295(100), 279(3), 197(14), 179(8), 135(5)	Salvianolic acid A	15.7 ± 0.5b	17.9 ± 0.4b	23 ± 2a
					Total phenolic acids	80 ± 3c	86.6 ± 0.5b	119 ± 1a
					Total flavonoids	206 ± 4c	253 ± 6b	308 ± 6a
					Total phenolic compounds	286 ± 7c	340 ± 7b	427 ± 6a

In each row different letters mean significant differences ($p < 0.05$).

Table 5a
Correlations with phenolic compounds and cytotoxicity of infusions prepared from lemon verbena submitted to gamma radiation.

Peak	Compounds	MCF-7		NCI-H460		HeLa		HepG2	
		Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*
1	Verbasoside	0.452	0.222	0.749	0.020	0.063	0.871	0.255	0.509
2	Luteolin-7-*O*-diglucuronide	0.219	0.571	−0.103	0.793	0.653	0.056	−0.072	0.853
3	*p*-Coumaric acid	−0.014	0.972	0.300	0.432	0.524	0.148	−0.706	0.034
4	Apigenin-7-*O*-diglucuronide	−0.213	0.582	0.450	0.224	0.162	0.677	−0.861	0.003
5	Verbascoside	0.145	0.710	−0.119	0.761	0.578	0.103	−0.226	0.559
6	Chrysoeriol-7-*O*-diglucuronide	0.627	0.071	0.156	0.689	0.537	0.136	0.763	0.017
7	Isoverbascoside	−0.625	0.068	−0.271	0.480	−0.237	0.539	−0.917	<0.001
8	Forsythoside	0.557	0.119	0.222	0.565	0.072	0.855	0.812	<0.001
9	Isorhamnetin-7-*O*-glucuronide	−0.625	0.072	−0.422	0.258	0.288	0.453	−0.548	0.008
10	Eukovoside	0.200	0.605	0.178	0.648	−0.273	0.477	0.443	0.233
11	Martinoside	0.424	0.255	0.207	0.592	0.569	0.110	−0.167	0.668
	TCP	0.238	0.537	−0.077	0.844	0.606	0.084	−0.110	0.778
	TPA	−0.014	0.972	0.300	0.432	0.524	0.148	−0.706	0.034
	TF	0.270	0.482	−0.021	0.956	0.705	0.034	−0.039	0.920
	TPC	0.242	0.531	−0.068	0.862	0.620	0.075	−0.107	0.785

TPC-total caffeoyl phenylethanoid derivatives (including verbasodide); TPA – total hydroxycinnamic acids; TF – total flavonoids; TPC – total phenolic compounds.

isoverbascoside, forsythoside, eukovoside and martinoside) have been already reported in *A. citrodora* (Bilia et al., 2008; Quirantes-Piné et al., 2009, 2010), which has been used to support compounds identities. The identity of compounds 3 (*p*-coumaric acid) and 9 (isorhamnetin-3-*O*-glucuronide) was confirmed by comparison with authentic standards. As far as we know, these two compounds have not been previously reported in *A. citrodora*. The most abundant compound detected was verbascoside (peak 5), with contents

Table 5b
Correlations with phenolic compounds and cytotoxicity of infusions prepared from peppermint submitted to gamma radiation.

Peak	Compounds	MCF-7		NCI-H460		HeLa		HepG2	
		Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*
1′	3-*O*-Caffeoylquinic acid	0.204	0.599	0.985	<0.001	0.421	0.259	0.552	0.123
2′	5-*O*-Caffeoylquinic acid	0.452	0.222	0.936	<0.001	0.617	0.077	0.797	0.010
3′	Caffeic acid	0.429	0.250	0.906	0.001	0.674	0.047	0.781	0.013
4′	Luteolin-*O*-dihexoside	0.759	0.018	0.457	0.216	0.766	0.016	0.960	<0.001
5′	Caffeic acid trimer	0.568	0.111	−0.450	0.225	0.357	0.346	0.437	0.240
6′	Eriodictyol-7-*O*-rutinoside	0.697	0.037	0.558	0.118	0.818	0.007	0.953	<0.001
7′	Eriodictyol-*O*-hexoside	0.370	0.327	0.953	<0.001	0.605	0.061	0.735	0.024
8′	Luteolin-7-*O*-rutinoside	0.505	0.166	0.912	0.001	0.644	0.061	0.836	0.005
9′	Luteolin-7-*O*-glucuronide	0.702	0.035	0.537	0.136	0.814	0.008	0.923	<0.001
10′	Naringenin-*O*-rutinoside	0.265	0.491	0.977	<0.001	0.500	0.170	0.625	0.072
11′	Salvianolic acid B	0.541	0.133	0.842	0.004	0.731	0.025	0.871	0.002
12′	Hesperetin-*O*-rutinoside	0.495	0.176	0.924	<0.001	0.599	0.088	0.817	0.007
13′	Rosmarinic acid	0.486	0.185	0.878	0.002	0.706	0.034	0.833	0.005
14′	Salvianolic acid A	0.690	0.040	0.781	0.013	0.679	0.044	0.954	<0.001
	Total phenolic acids	0.566	0.112	0.840	0.005	0.721	0.029	0.888	0.001
	Total flavonoids	0.676	0.046	0.645	0.061	0.804	0.009	0.954	<0.001
	Total phenolic compounds	0.650	0.058	0.709	0.033	0.788	0.012	0.944	<0.001

ranging between 83 and 134 μg/mL of infusion. Verbascoside is a phenylethanoid glycoside with advantageous potentials for human health, specifically antioxidant, anti-inflammatory and antimicrobial properties in addition to wound-healing and neuroprotective effects (Alipieva et al., 2014). The antioxidant potential of this compound is related to the presence of two catechol groups in its structure (D'Imperio et al., 2014). Thus, its increase (caused by irradiation at 10 kGy) will provide the plant with higher bioactivity. This increase of compounds can be explained because some bonds can be broken resulting in smaller molecules. Nevertheless, the use of high irradiation doses might also lead to higher compound's extractability. This can explain the higher values of phenolic compounds concentration observed for the dose of 10 kGy when compared with those found in non-irradiated and 1 kGy irradiated samples (Stewart, 2001; Molins, 2001). The obtained results showed that a statistically significant degradation of chrysoeriol-7-*O*-diglucuronide (peak 6), forsythoside (peak 8), and eukovoside (peak 10) was produced at the dose of 10 kGy. In contrast, the levels of luteolin-7-*O*-diglucuronide (peak 2), *p*-coumaric acid (peak 3), verbascoside (peak 5) and isoverbascoside (peak 7) increased after irradiation at 10 kGy.

In peppermint infusions seven phenolic acids (peaks 1′, 2′, 3′, 5′, 11′, 13′ and 14′) and seven flavonoids (peaks 4′, 6′, 7′, 8′, 9′, 10′ and 12′) were detected. All these compounds have been already identified in methanolic extracts of *Menta x piperita* L. (Pereira et al., 2016a). In accordance to the previous study, eriodictyol-*O*-rutinoside (peak 6′) was the majority compound present in the infusions. Eriodictyol has been reported to possess a relevant bioactive potential, expressing scavenging activity of intracellular free radicals (Imen et al., 2015). The obtained results revealed a statistically significant ($p < 0.05$) increase in the concentration of all detected phenolic compounds in the sample irradiated at 10 kGy, with the exception of caffeic acid trimer (peak 5′) whose levels were higher in the sample submitted to 1 kGy. The increase in the levels of phenolic compounds following gamma irradiation at 10 kGy was also observed in other samples studied herein, such a *Ginkgo biloba* (Pereira et al., 2015b). The changes in the phytochemicals contents that can occur using irradiation would depend on different factors (kind of radiation, applied dose, exposure time or type sample) and, as previously commented, they might be due to either an increase in enzymatic activity that would favour the release of matrix-linked compounds or an increased accessibility and subsequent extractability from the tissues (Alothman et al., 2009).

A correlation analysis was also performed between results obtained in the cytotoxicity assays (MCF-7, NCI-H460, HeLa and HepG2) and the phenolic composition for lemon verbena and peppermint samples (Tables 5a and 5b), using a Pearson's correlation analysis, in which the normality was verified through a Shapiro-Wilk test. In general, hardly statistically significant ($p < 0.05$) correlations were found for the samples of lemon verbena, but just for NCI-H460 with verbasoside (peak 1, Pearson's $r = 0.749$), and HepG2 with chrysoeriol-7-*O*-diglucuronide (peak 6, Pearson's $r = 0.763$) and forsythoside (peak 8, Pearson's $r = 0.812$). The main compound (verbascoside) did not show correlation with the effects on any cellular line studied, and similarly happened with eukovoside and martinoside. Nonetheless, total flavonoid contents correlated well with the results obtained on the HeLa cell line (Pearson's $r = 0.705$).

In the case of peppermint infusions statistically significant correlations ($p < 0.05$) were found within all cell lines, especially HepG2, for which the most significant correlations were obtained for most of the compounds, and in particular with peaks 4′ (Pearson's $r = 0.960$), 6′ (Pearson's $r = 0.953$), 9′ (Pearson's $r = 0.923$), and 14′ (Pearson's $r = 0.954$). Total phenolic acids (Pearson's $r = 0.888$), total flavonoids (Pearson's $r = 0.954$) and total phenolic compounds (Pearson's $r = 0.944$) also presented high correlation factors with the citotoxic effect on HepG2 cell line. The results on MCF-7 cell line presented statistically significant correlation with the content of luteolin-*O*-dihexoside (Pearson's $r = 0.759$), and the NCI-H460 cell line correlated with various compounds, although the best correlation was with 3-*O*-caffeoylquinic acid (Pearson's $r = 0.985$). Effects on HeLa cell line were highly correlated with eriodictyol-7-*O*-rutinoside (Pearson's $r = 0.818$).

Salvianolic acid A (peak 14′) correlated significantly with the effects observed in all cell lines (MCF-7: 0.690; NCI-H460: 0.781; HeLa: 0.679 and HepG2: 0.954), whereas caffeic acid trimer (peak 5′) was the only compound for which no correlation could be established with any of the cell lines studied. The main compound, eriodictyol-7-*O*-rutinoside (peak 6′), showed significant correlation factors with all cell lines, with the exception of NCI-H460.

4. Conclusion

According with international phytosanitary regulations, irradiation is a validated, authorized and feasible alternative to chemical fumigation for dry plants processing. In this study, it was intended to follow the process from the irradiation until the consumer, to

evaluate the impact of this industrial process on the main components of herbal infusions.

Regarding the effects on cytotoxic potential, in general, lemon verbena did not reveal significant statistically differences considering the different doses applied (0, 1 and 10 kGy). Nonetheless, peppermint at the dose of 10 kGy stood out with the highest cytotoxic potential in all tested cell lines. Regarding the evaluation of the effects of gamma radiation on phenolic composition, peppermint showed statistically significant increase with the doses applied, being observed an increase in total phenolic compounds in both studied species. The phenolic composition presented higher correlations factors for the cytotoxic activity using peppermint infusion, then with lemon-verbena. Concerning virucidal efficacy, gamma radiation treatment seems to cause different effects depending on various factors such as plant species, viruses, and the absorbed dose. For antibacterial activity, it was evident that *S. aureus*, with MIC and MBC values of 5 mg/mL, was the only bacteria that was sensible to the infusions of non-irradiated and 1 kGy irradiated lemon verbena and peppermint samples. On the other hand, the antibacterial activity of infusions from non-irradiated and irradiated samples showed that the used irradiation doses (1 and 10 kGy) did not cause significant changes in the antimicrobial activity of the studied plants.

The results of this study can contribute to a wider knowledge of the effects of gamma irradiation on several relevant bioactive potentials of aromatic and medicinal plants; and showed that gamma radiation is recommended for application in these matrices.

Conflict of interest

The authors declare no conflict of interest.

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