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Identification of phenolic compounds and biologically related activities from *Ocotea odorifera* aqueous extract leaves

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ABSTRACT

Ocotea odorifera (Vell.) Rohwer is popularly used as food and flavoring. The aim of this study was to determine the chemical composition of the aqueous extract from *O. odorifera* leaves and evaluate the correlation of their phytochemical composition and biological activities. The antioxidant effect was determined by DPPH radical scavenging, β-carotene-linoleic acid and lipid peroxidation assays; the antibacterial activity was evaluated by the hole plate and MIC techniques and the antimutagenic activity was evaluated by the Ames test. Identification of phytochemicals was performed by LC–ESI/MS and the correlation between the phytochemical composition of the extract and the evaluated activities. The results allowed the identification of 13 phenolic compounds in the extract that exhibited high antioxidant activity and moderate antibacterial and antimutagenic action. Statistical analyses showed correlation of the total phenolic content with biologically related activities. The phytochemical analyses, together with the biological results, support the popular use of *O. odorifera*.

1. Introduction

Ocotea odorifera (Vell.) Rohwer, belonging to the Lauraceae family, is a native species in Brazil that is found naturally in areas of the Atlantic Forest. It is usually known as sassafrás, canela-sassafrás and canela-do-mato and is used as a food flavoring (Oliveira, Yamada, Fagg, & Brandão, 2012; Oltramari et al., 2004) and in traditional medicine for rheumatism treatment and as an antimalarial (Bolsaris, 2007; Tribess et al., 2015). In 2013, this species was entered into the Red Book of the Flora of Brazil as having a risk of extinction and was classified as "endangered" (Martinelli & Moraes, 2013).

Methanolic extracts from *O. odorifera* leaves showed no cytotoxic activity against Vero cells and a lack of genotoxicity by an *in vivo* micronucleus test, while ellagitannins isolated from *O. odorifera* showed potent activity against *Candida parapsilosis* (Yamaguchi et al., 2011). The essential oil of *O. odorifera* is used as source of sassafras oil, which has long been exported from Brazil to Europe, Japan and the USA (Oltramari et al., 2004) due to the antifungal and larvicidal activities that were reported for the essential oil of this species (Castro & Lima, 2011; Pinto et al., 2010). Phytochemical analysis of the *O. odorifera* essential oil showed that the oil contains a high concentration of safrole in addition to phenylpropanoid derivatives, steroids, coumarins and sesquiterpenes (Lordello, Cavalheiro, Yoshida, & Gottlieb, 2000; Oltramari et al., 2004). Safrole was a common flavoring in the beverage, cosmetic, food and medical industries, but this practice was discontinued after safrole was determined to induce carcinogenicity (Miller & Miller, 1983). It is also known that the essential oil of different O*cotea* species exhibits strong antioxidant and antibacterial action (Bruni et al., 2004). However, no extensive characterization of the phytochemical composition of the aqueous extract of *Ocotea odorifera* leaves has been performed thus far, and its biological properties with regard to food use, such antioxidant, antibacterial and antimutagenic properties, have yet to be reported. Effort has therefore been focused on using available experimental techniques to identify antioxidant, antibacterial and antimutagenic compounds from natural products (Gursoy, Sarikurkcu, Cengiz, & Solak, 2009; Trabelsi et al., 2013). Thus, the aim of this study was to determine the chemical composition of the aqueous extract of *O. odorifera* leaves and to evaluate the correlation of its

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phytochemical composition with antioxidant, antibacterial and antimutagenic activities.

2. Materials and methods

2.1. Plant material

Samples of *O. odorifera* plants were collected in the Atlantic Forest biome in December of 2010 on Conservation Unit Parque Estadual da Serra do Brigadeiro in the state of Minas Gerais, Brazil. Voucher specimens were deposited at the Herbário VIC, Universidade Federal de Viçosa, with the code VIC 42.326. The "collection authorization license" at CU was issued by the "Instituto Estadual de Floresta de Minas Gerais" (N°CU 073/07, 050/07 COL). The species identification was carried out by comparison of herbarium specimens.

2.2. Extract preparation

The leaves of *O. odorifera* were dried in a ventilated, dark room at ambient temperature and were subsequently pulverized in a knife mill. The leaf extract of *O. odorifera* was prepared by infusion using distilled water at 90 °C (ratio plant:solvent, 1:20 w/v) for 15 min with shaking. The extract was then vacuum filtered and lyophilized, yielding 5.8% (relative to the weight of the plant material) dry extract of *O. odorifera* (EOO).

2.3. Chemical analyses

2.3.1. Thin layer chromatography analyses

The presence of secondary metabolites, flavonoids, tannins, coumarins, anthraquinones, terpenes/steroids, saponins and alkaloids was detected in EOO by thin layer chromatography (TLC silica gel F_{254}). Mobile phases and visualization spray reagents were used as indicated in Wagner and Bladt (1996) and compared with reference compounds.

2.3.2. Ultra-high performance liquid chromatography–mass spectrometry in series analyses

Analyses by ultra-high performance liquid chromatography coupled to ultraviolet spectroscopy (LC–DAD) and to mass spectrometry (LC–ESI-MS/MS) were performed according to Gattuso et al. (2006) with some modifications in the Laboratório de Fitoquímica at the Faculdade de Farmácia, UFMG. Phenolic compounds were identified based on the typical UV absorption for each phenolic class analyzed, in addition to their typical fragmentation patterns obtained by MS/MS in comparison to the literature data.

The analyses were performed in an ACQUITY® TQD ion trap mass spectrometer (Waters) equipped with a quadrupole instrument fitted with an electrospray source in the positive and negative ESI modes and atmospheric pressure chemical ionization (APCI). Interface that was operated with the following conditions: ion spray voltage: −4 kV; orifice voltage: −60 V; capillary voltage, 3500 V; capillary temperature, 320 °C; source voltage, 5 kV; vaporizer temperature, 320 °C; corona needle current, 5 mA; sheath gas, nitrogen, 27 psi; and the instrument was operated in the full scan mode (100–2000 Da). The LC–ESI-MS/MS analyses were additionally performed with helium as the collision gas and the collision energy was set at 30 eV. Chromatographic separation was done on a reversed-phase column ACQUITY UPLC BEH (1.7 μm, 50 × 2 mm i.d.) (Waters). The mobile phase consisted of aqueous 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The elution protocol was a 0–11 min linear gradient from 5 to 95% B. The flow rate was 0.3 mL min^{-1}, and the sample injection volume was 4.0 μL. UV–Visible spectra were registered from 190 to 450 nm.

2.3.3. Determination of phenolic compounds by UV–Visible spectrophotometry

The total polyphenolic content of the EOO was determined using the Folin-Ciocalteu reagent with tannic acid as the standard, and the absorbance at 760 nm was measured after 60 min of reaction at room temperature in the dark (Verza, Kreinecker, Reis, Henriques, & Ortega, 2007). Folin-Ciocalteu reagent (0.5 mL), 15% aqueous Na_2CO_3 (1.0 mL) and distilled water (8.3 mL) were added to a test tube, followed by the addition of 200 μL of EOO solution (3.0 mg/mL resuspended in water). The results were expressed as μg of tannic acid equivalent (TAE)/mg of EOO.

The total tannin content was calculated according to Verza et al. (2007). Thus, 20 mL of EOO (3 mg/mL resuspended in water) was mixed with 200 mg of hide powder and vortexed at 200 rpm for 60 min. Subsequently, the sample was filtered, and 200 μL of the EOO filtrate was collected. The following steps were identical to those in the experiment for the quantification of total polyphenols. The total tannin content was obtained by the difference between the total polyphenol content and the value obtained in the Folin-Ciocalteu reagent test in the absence of tannins. Similarly, the total tannin content was also expressed as tannic acid equivalents (TAE) using a standard curve generated with tannic acid as standard.

Flavonoids were quantified by the $AlCl_3$ method with rutin as the standard compound, and the absorbance at 420 nm was measured in a UV–Visible spectrometer after 15 min of reaction at room temperature in the dark (Lamaison & Carnet, 1990). In total, 0.5 mL of acetic acid, 10 mL of methanol and 2.5 mL of $AlCl_3{\cdot}6H_2O$ 8% in methanol were added to 5 mL of EOO (50 mg/mL resuspended in methanol), and the sample was brought to a final volume of 25 mL with methanol. The results were expressed as μg of rutin equivalents (RE)/mg of EOO.

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant test was performed by adding 2.0 mL of DPPH (0.1 mM) to 2.0 mL of solutions of EOO (0.5, 25, 50, 75 and 100 μg/mL) diluted in methanol, and the absorbance was measured with a UV–Visible spectrophotometer at 517 nm after 30 min of reaction at room temperature in the dark (Abdel-Hameed, 2009). A synthetic product, butylated hydroxytoluene (BHT), and standard *Ginkgo biloba* leaf extract (EGb 761) were used as antioxidant standards that were tested at the same EOO concentrations. The IC_{50} was determined by performing an exponential regression of the plotted points for EOO, which revealed the concentration required to reduce the initial concentration of DPPH radicals by 50%. The percentage of remaining DPPH was calculated using the following equation:

$$\%\text{DPPH radical capture} = [1 - (A_S)/(A_0)] \times 100$$

where A_S is the absorbance of the sample or standard and A_0 is the absorbance of the control (DPPH only).

2.4.2. β-Carotene-linoleic acid assay

The antioxidant activity was assayed using the β-carotene-linoleate model system according to Yu et al. (2006). In this system, β-carotene (2.2 mg in 10 mL of chloroform), linoleic acid (250 μL) and Tween-20 (2 g) were transferred into a round-bottomed flask, and the chloroform was evaporated in a rotary evaporator under vacuum. Subsequently, 500 mL of distilled water, previously saturated with oxygen for 30 min, was added to the mixture, and then the solution was shaken vigorously to form an emulsion. To 5 mL aliquots of this emulsion was then added 1 mL of EOO (25 μg/

mL, resuspended in methanol), and the tubes were incubated at 50 °C to accelerate oxidation reactions and start the bleaching of β-carotene. The absorbance was read in a UV–Visible spectrophotometer at 470 nm at intervals of 20 min from 0 h to 160 min of incubation time. BHT and EGb 761 standards were also used for comparison with the results obtained for EOO. The results are expressed as the percentage of inhibition of oxidation of the samples (As), which was calculated in relation to the decrease in absorbance of the negative control (Ac).

$$\% \text{ Inhibition} = ((Ac - As)/Ac) \times 100$$

2.4.3. Lipid peroxidation

The antioxidant activity was also evaluated using the lipid peroxidation test, according to the work of Conforti, Statti, Tundis, Menichini, and Houghton (2002). For this procedure, 2.5 mL of distilled water, 500 μL of soy lecithin solution (7700 μg/mL), and 1 mL of ascorbic acid (1750 μg/mL) were added to a test tube, followed by 125 μL of iron(III) chloride (330 μg/mL) and 100 μL of EOO (12 μg/mL). After the addition of these reagents, the tubes were incubated in a shaker for 60 min at 40 °C. After this time, 1 mL of thiobarbituric acid (TBA 3700 μg/mL) was added to all tubes, and the tubes placed in a water bath at 80 °C for 15 min. The tubes were then centrifuged for 10 min at 10,000 rpm. The absorbance at 532 nm of the supernatant from the centrifugation was read in the UV–Visible spectrophotometer. BHT and EGb 761 standards were also used for a comparison with the results obtained for EOO. The results are expressed as the percentage of inhibition of the oxidation of the sample (As), which was calculated in relation to the decrease in the absorbance of the negative control (Ac).

$$\% \text{ Inhibition} = ((Ac - As)/Ac) \times 100$$

2.5. Antibacterial action

2.5.1. Hole plate method

EOO were tested against *Staphylococcus aureus* 3993, *Staphylococcus aureus* 4125 and *Escherichia coli* 24 strains isolated from animals with bovine mastitis. The isolates were grown in 5 mL of brain heart infusion broth for 16 h at 37 °C. The antibacterial activity was evaluated by the diffusion technique in agar using the hole plate method (NCCLS & National Committee for Clinical Laboratory Standards,, 2003). A 100-μL suspension of the bacteria (10^6 UFC/mL of *S. aureus* 3993, *S. aureus* 4125 and *E. coli* 24 strains) was spread on Müeller-Hinton agar. Holes of approximately 5 mm in diameter and 3 mm in height were made in the agar, and 30 μL of EOO (50 mg/mL) prepared in dimethylsulfoxide (DMSO) was added to the holes, followed by 24 h of incubation at 37 °C. After incubation, the inhibition zones were measured in millimeters. Ampicillin (5 mg/mL) was used as the positive control, and DMSO was used as the negative control. The experiments were repeated twice in triplicate. The determination of the antibacterial activity of EOO was performed using the diameter of the bacterial growth inhibition zone and diameter of the growth inhibition zone of the positive control.

$$\% \text{ Antibacterial activity} = \frac{\text{Hole with extract at the evaluated concentration}}{Holewithpositivecontrol} \times 100$$

2.5.2. Minimum inhibitory concentration

The broth microdilution method was used for determination of the minimum inhibitory concentration (MIC) of EOO that still showed activity (NCCLS, 2003). The 96-well plates were prepared by dispensing 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, 4.0, 8.0, 12.0, 16.0, 20.0, 24.0, 28.0, 32.0, 36.0 and 40.0 μL aliquots of EOO, followed by 100 μL of inoculum containing 10^6 UFC/mL of *S. aureus* 3993, *S. aureus* 4125 or *E. coli* 24, with the remainder of the volume in each well being supplied with Müeller-Hinton broth. The microplates were incubated at 37 °C for 24 h. After incubation for 24 h, 4 μL of the salt INT (iodonitrotetrazolium) (2 mg/mL) was added to each well. The bacterial viability was observed by the formation of pink color after the addition of the INT. Ampicillin (5 mg/mL) was used as the positive control and DMSO was used as the negative control.

2.6. Antimutagenic and antigenotoxic activities

2.6.1. Antimutagenicity

The Ames test was chosen to quantify the extract's antimutagenicity. The EOO was preincubated for 20–30 min with the TA97, TA98, TA100 and TA102 strains of *Salmonella typhimurium*/microsome, with and without metabolic activation (S9) and different mutagens (Maron & Ames, 1983). Five different concentrations of EOO (562.5, 1125, 2250, 3375 and 4500 μg/mL) in distilled water were assessed. The concentration employed was based on a cytotoxicity test carried out for EOO with the TA100 strain without metabolic activation. Thus, the upper limit of the dose interval that was tested was either the highest nontoxic dose or the lowest toxic dose that was determined in this preliminary assay. To each concentration of the tested mixture was added 500 μL of phosphate buffer 0.2 M pH 7.4 (without metabolic activation) or 500 μL S9 mix (with metabolic activation), 100 μL of bacteria culture (4×10^8 cells/mL) and 2 μL of ampicillin (50 mg/mL), besides different mutagens (mutagens used in the test without metabolic activation were 50 μL of 4-nitro-O-phenylenediamine (100 μg/mL) for TA97 and TA98, 10 μL of sodium azide (50 μg/mL) for TA100 and 2 μL of mitomycin C (500 μg/mL) for TA102, and 25 μL of 2-aminoanthracene (200 μg/mL) was used for all strains with metabolic activation) incubated at 37 °C for 30 min. The lyophilized rat liver S9 fraction induced by Aroclor 1254 was purchased from Moltox (Molecular Toxicology, Annapolis, USA). The S9 mix (50 mL) was prepared as follows: 19.75 mL of distilled water, 25 mL of 0.2 M phosphate buffer pH 7.4, 2 mL of 0.1 M NADP, 0.25 mL of 1 M d-glucose-6-phosphate, 1 mL of 1.65 M KCl + 0.4 M $MgCl_2 \cdot 6H_2O$ salt solution and 2 mL of the lyophilized S9 fraction that had been reconstituted with distilled water. After the incubation period, 2 mL of top agar and the mixture were plated on glucose minimal medium. The plates were incubated at 37 °C for 48 hours, and the *His+* revertants were counted. The tests were conducted in triplicate. Water distilled was used as a negative control.

The antimutagenic action was determined using the equation from Oh, Kim, Choi, Chung, and Ham (2008):

$$\% \text{ Antimutagenic action} = \frac{(\text{nº revertants control } (\pm) - \text{nº revertants concentration tested})}{(\text{nº revertants control } (+) - \text{nº revertants control } (-))} \times 100$$

2.6.2. Antigenotoxic activity

To assess the DNA-damaging potential of EOO, a plasmid treatment was performed following the method described by De Mattos et al. (2000). EOO samples (562.5, 2250.0 and 4500.0 μg) were incubated with 20 μL of pUC18 (75.7 μg/μL) plasmid DNA and 100 μL of genotoxic agent $SnCl_2$ (750.0 μg/mL) for 1 h at 37 °C. The samples were brought up to a volume of 220 μL with distilled water, and the DNA was precipitated. Plasmid DNA was resuspended in 20 μL of Milli-Q water and run on a 0.8% agarose gel (80 volts) with ethidium bromide (0.1 μg/mL) staining to allow

the visualization of the DNA. The DNA samples treated with distilled water were used as the negative control. The plasmid DNA test was assessed by verifying the presence or absence of the relaxed-circular conformation (form III), the helical conformation (form II) and the strained superhelical conformation (form I). A comparison between the DNA bands of the samples and the positive- and negative-control DNA bands was used to diagnose any possible DNA-damaging action of EOO.

2.7. Data analysis

The data on the quantification of total phenolics, tannins and flavonoids, as well as those of the antioxidant, antibacterial and antimutagenic activities were analyzed by the Shapiro-Wilk and Bartlett tests ($\alpha = 0.05$) to confirm the presence of a normal distribution and homoscedasticity, respectively. Antioxidant activity data were subjected to a one-way ANOVA test, followed by a Tukey test ($\alpha = 0.05$) to separate the means. Antibacterial activity data, which did not meet the assumptions of ANOVA, were submitted to a Kruskal-Wallis test followed by a Dunn's test ($\alpha = 0.05$). The antimutagenic activity data were subjected to a three-way ANOVA ($\alpha = 0.05$) to test the interaction among the effects of strains, the metabolic activation and the concentrations of EOO. If an effect was detected, the means were compared by the Tukey test ($\alpha = 0.05$). Principal component analysis (PCA) using XLSTAT 2014.5.03 software with Pearson correlation model was applied to clarify the relationship between phenolic compounds and the biological activities of the EOO. All statistical analyses were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA).

3. Results and discussion

3.1. Chemical analyses

The phytochemical screening of secondary metabolic groups by TLC detected the presence of saponins, flavonoids and tannins. A UPLC–DAD chromatogram of the EOO monitored at 210 nm is shown in Fig. 1. This fingerprinting corroborated the results obtained from the phytochemical prospection, and peaks 1–13 correspond to identified phenolic constituents, including condensed tannins (proanthocyanidins) and flavonoid glycosides. Analysis of first-order MS spectra recorded for each peak, together with MS-MS experiments in positive and negative ESI modes, literature UV comparisons and retention times (RT), allowed the identification of the extract phytochemicals (Table S1 and Fig. S1). A major peak was detected at 1.61 (compound 1), 2.00 (compound 4) and 2.16 min (compound 5). The use of UPLC coupled to tandem mass spectrometry (MS/MS) can provide abundant information for the structural elucidation of a wide range of compounds (Regueiro et al., 2014).

For fragmentation, the aglycone ion was not observed in the presence of flavone C-glycosides due to the strength of the C—C connection between the sugar and aglycone moieties. This behavior was observed in first-order MS of compounds 8, 9 and 13, suggesting that these compounds are flavone C-glycosides. The MS-MS spectra obtained by focusing on each $[M-H]^-$ ion of compounds 8, 9 and 13 exhibited the same pattern of fragmentation ($[(M-H) - 18\ Da]^-$, $[(M-H) - 90\ Da]^-$, $[(M-H) - 120\ Da]^-$, $[A+113\ Da]^-$, and $[A+83\ Da]^-$), which is typical of di-C-glycosylflavones. Positions 6 and 8 appeared to be substituted in each case since the maximum of band II was located at 270 nm or higher. The presence of the $[(M-H) - 60]^-$ fragment in these MS-MS spectra, which is usually generated by the fragmentation of pentose derivatives, along with the presence of the $[(M-H) - 120\ Da]^-$ (base peak) and $[(M-H) - 90\ Da]^-$ peaks, suggested that the sugar substituents were pentoses and hexoses (Gattuso et al., 2006). The *m/z* of the $[A+113\ Da]^-$ and $[A+83\ Da]^-$ ions correspond to the aglycone-bearing sugar fragments, and they are particularly important for aglycone identification. For positive identification and characterization of flavan-3-ol monomers, dimers, trimers and tetramers, the following points were considered: a UV spectrum with a peak near 280 nm (λ_{max}) and molecular ion peaks in positive- and negative-ion ESI modes of MS. The fragmentation pathway heterocyclic ring fission (HRF) and *retro*-Diels–Alder (RDA) fragmentation give information about the hydroxylation of the B-rings and the bonds between two monomeric unit; quinonemethide (QM) fragmentation defines the two monomeric units and especially the base unit (Jaiswal, Jayasinghe, & Kuhnert, 2012).

Compound 1: The UV spectrum of compound 1 was compatible with a flavan-3-ol type (*epi*)catechin. One peak was detected at *m/z* 1153 in positive ESI mode and assigned to a proanthocyanidin tetramer [(*epi*)catechin-(4,8/2,7)-(*epi*)catechinan-(4,8)-(*epi*)catechin-(4,8)-(*epi*)catechin]. This compound produced the MS-MS base

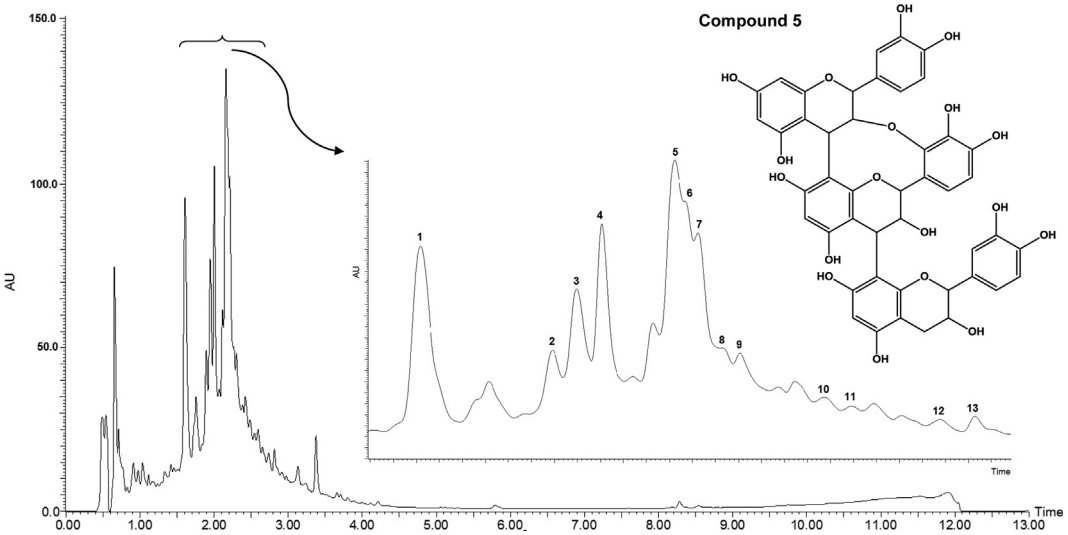

Fig. 1. RP-UPLC–DAD profile of aqueous extract from *Ocotea odorifera* leaves (EOO). Conditions: CHS130 100 RP-18 column (1.7 μm, 50 × 3 mm i.d.). Elution was carried out with a linear gradient of water 0.1% formic acid (A) and acetonitrile 0.1% formic acid (B) (from 5% to 95% of B in 11 min) and the UPLC fingerprints were registered on a ACQUITY Waters apparatus with a UV-DAD detector in 210 nm.

peak in positive ESI mode at *m*/*z* 865 that derived from the QM cleavage; a secondary peak at *m*/*z* 1001 [(M−H) – 152 Da] originates from an RDA fragment. Another fragment was generated at *m*/*z* 983 by the loss of an RDA fragment (152 Da), followed by the loss of a water molecule (18 Da).

Compounds 2, 10 and 12: Three isomers were detected at *m*/*z* 577 in the negative ESI mode and were tentatively assigned as (*epi*)catechin-(*epi*)catechin dimers with B-type linkages. Compounds 2 and 10 produced the MS-MS base peak at *m*/*z* 407 [(M−H) – 170 Da] by losing an RDA fragment (152 Da), followed by the loss of a water molecule (18 Da); compound 2 showed secondary peaks at *m*/*z* 289 ([(*epi*)catechin-H+]), which originates from a QM fragment) and at 425 [(M-H)+-152 Da], which originates from an RDA fragment. Compound 12 produced the MS-MS base peak at *m*/*z* 289 [(M−H) – 288 Da], which originates from a QM fragment, and secondary peaks at *m*/*z* 407, caused by the loss of an RDA fragment (152 Da) and the subsequent loss of a water molecule (18 Da), and at *m*/*z* 299, caused by the loss of an RDA fragment (152 Da) followed by the loss of an HRF fragment (126 Da).

Compound 3: The UV spectrum of compound 3 is compatible with an apigenin aglycone. The negative ESI mode MS-MS experiment focused on the *m*/*z* 577 ion showed the presence of a 308-Da disaccharide on an apigenin skeleton, which is composed of a rhamnose unit ([(M−H) – 146 Da]$^-$) connected to a glucose unit ([(M−H) – 162 Da]$^-$). Since the disaccharide can be considered to be linked to position 7, compound 3 has been identified as apigenin 7-O-rutinoside.

Compounds 4 and 13: Two isomers were detected at *m*/*z* 575 while running in negative ESI mode and were assigned to an A-type (*epi*)catechin dimer. Compound 4 produced the MS-MS base peak at *m*/*z* 151 (RDA) and secondary peaks at *m*/*z* 289 and 285 (QMs) and 449 (HFR). Compound 13 produced the MS-MS base peak at *m*/*z* 289 (QM) and secondary peaks at *m*/*z* 151 (RDA), 285 (QM), and 449 (HFR).

Compound 5: One peak was detected at *m*/*z* 863 while running in negative ESI mode and it was assigned to a proanthocyanidin trimer, [(*epi*)catechin-(4,8/2,7)-(*epi*)catechinan-(4,8)-(*epi*)catechin]. Compound 5 produced the MS-MS base peak in negative ESI mode at *m*/*z* 573 with a neutral loss of an (*epi*)catechin unit; the secondary peak at *m*/*z* 711 [(M-H) -152 Da] originates from an RDA fragment. Other important fragments were observed at *m*/*z* 451 and 411, which are present from the fragmentation of the (*epi*)catechin trimer ((*epi*)catechin→A→(*epi*)catechin→(*epi*)catechin) connection.

Compounds 6 and 7: The first-order MS spectra of compounds 6 and 7 showed the same protonated species [M+H]$^+$ *m*/*z* 291. In both MS-MS spectra, the [(M−H) – 152 Da]$^-$ ions were formed by the loss of an RDA fragment. The base peak in both compounds at [M+H]$^+$ *m*/*z* 147 refer to the loss of an H_2O molecule [(M+H) – 18 Da]$^+$, followed by an HFR fragment (−126 Da). Thus, compounds 6 and 7 are identified as monomeric (*epi*)catechin.

Compounds 8 and 9: Compound 8 and 9 are isomers with glucose in the 6 and 8 positions, respectively. The positive-mode MS-MS spectrum focused on *m*/*z* 565.32 and 565.45 ([M+]$^+$) ions was not particularly useful for structural identification; only peaks derived from the fragmentation of the sugar units were observed. The presence of the [A+113 Da]$^-$ and [A+83 Da]$^-$ peaks in the MS-MS spectrum focused on the *m*/*z* 563.23 and 563.29 ([M−H]$^-$) ions, indicating that the aglycone has a molecular weight of 270. The UV spectrum showed typical absorptions for a disubstituted trihydroxyflavone. The loss of 60, 90 and 120 mass units from the deprotonated species [M−H]$^-$ (as discussed previously), in addition to the shift observed for band II in the UV spectrum, confirmed that the sugar substituents at positions 6 and 8 are pentoses and hexoses. The retention time, UV, and positive- and negative-mode ESI MS-MS spectra led to the identification of compounds 8 and 9 as apigenin 6-C-pentoside-8-C-hexoside and/or apigenin 6-C-hexoside-8-C-pentoside.

Compound 11: The UV spectra showed absorption bands centered at 272 and 334 nm, which can be attributed to bands II and I, respectively, of a flavone structure. The first-order MS spectrum of compound 11 showed the deprotonated species [M−H]$^-$ at *m*/*z* 593.35. In the MS-MS spectra, the [(M−H) – 90 Da]$^-$ and [(M−H) – 120]$^-$ ions suggested the presence of hexoses as substituents at the 6 and 8 positions. Compound 11 was identified as apigenin 6,8-di-C-glucoside (vicenin-2).

3.2. Quantification of phenolic compounds

Quantification of the phenolic compounds in EOO determined the content of total polyphenolics (46.81 ± 3.43 μg TAE/mg of EOO), tannins (29.73 ± 2.23 μg TAE/mg of EOO) and flavonoids (9.61 ± 0.41 μg RE/mg of EOO). Many of these phenolic compounds that have antioxidant activity are used in food products and a number of medical treatments (Li, Hao, Wang, Huang, & Li, 2009).

3.3. Antioxidant activity

The values found for EOO in the DPPH radical scavenging test, the co-oxidation system of β-carotene/linoleic acid and the lipid peroxidation test show the antioxidant activity measured by each of the different methods (Table 1). The correlation analysis among the total polyphenolic, tannin, and flavonoid concentrations and the antioxidant activity of EOO showed that only the lipid peroxidation test directly relates to the content of total polyphenols and total tannins (the angle between the axis of total polyphenol and total tannin parameters and the axis of the lipid peroxidation parameters is nearly 0°). For the system of β-carotene/linoleic acid, there is a weak correlation with the tannin content. For the DPPH radical-scavenging test, there was no correlation with any of the levels of polyphenols evaluated (angles between the axes are nearly 90°). The flavonoid content had a negative correlation with the lipid peroxidation test, since the axis vectors for the two parameters are in opposite directions (the angle between the axes is 180°) (Fig. 2A). Due to the complexity of the antioxidant processes, it is important to use different methodologies to assess this activity.

The mechanism of antioxidant action can include suppressing ROS formation, either by inhibition of enzymes or by chelating trace elements involved in free-radical production, scavenging

Table 1
Antioxidant activity of leaf aqueous extract from *Ocotea odorifera* (EOO).

Treatments/ statistic[1]	Antioxidant activity		
	IC_{50} of DPPH[5] (μg/mL)	β-Carotene/linoleic acid (%)	Lipid peroxidation (%)
NC[2]	–	0.0 ± 0.0 c	0.0 ± 0.0 c
BHT[3]	2.6 ± 0.2 b	79.3 ± 3.8 a	96.2 ± 1.3 a
EGb 761[4]	2.2 ± 0.0 c	0.9 ± 1.8 c	63.5 ± 6.7 b
EOO	4.2 ± 0.2 a	32.5 ± 3.6 b	52.8 ± 6.9 b
F	125.7	541.6	119.2
df	2,6	3,8	3,8
P	<0.001	<0.001	<0.001

[1] One-way ANOVA (α = 0.05). Means (±SD, n = 3) followed by different letters were significantly different within columns (Tukey test's, α = 0.05). – dashes indicate no data.
[2] NC – negative control = without extract.
[3] BHT – butylated hydroxytoluene.
[4] EGb 761 – standardized *Ginkgo biloba* extract.
[5] Concentration of extract necessary to decrease the initial concentration of DPPH radical by 50%.

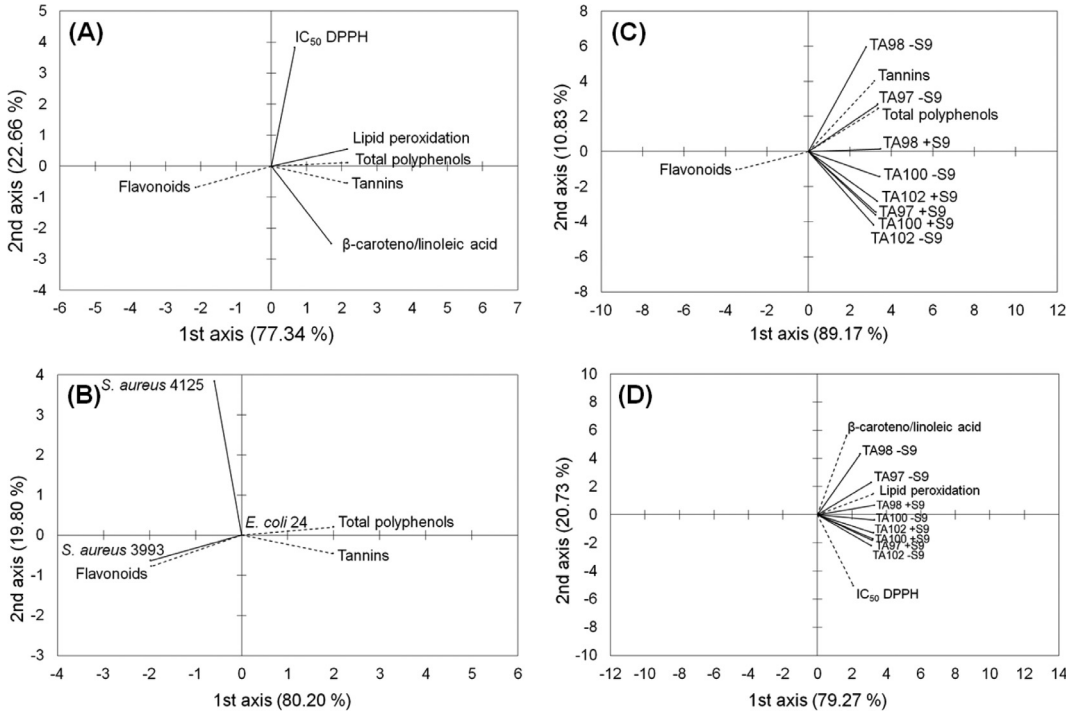

Fig. 2. Ordination diagrams of the principal component analysis (PCA) based on the phenolics compounds and biological activities of aqueous leaf extract from *Ocotea odorifera* (EOO). (A) Antioxidant activity by methods DPPH radical scavenging, β-carotene/linoleic acid and lipid peroxidation and phenolics compounds. (B) Antibacterial activity and phenolics compounds. (C) Mutagenic inhibition in *Salmonella typhimurium*/microsome strains, with (+S9) and without (−S9) metabolic activation and phenolics compounds. (D) Antioxidant activity and mutagenic inhibition in *Salmonella typhimurium*/microsome strains, with (+S9) and without (−S9) metabolic activation.

reactive species, and upregulating or protecting antioxidant defenses (van Acker et al., 1996). Unlike the scavenging of free radicals by DPPH, which is based on electron transfer from an antioxidant compound to an oxidant, the co-oxidation of β-carotene/linoleic acid and lipid peroxidation determine the activity of a compound in protecting a substrate from lipid oxidation by neutralizing free radicals formed in the system (Gursoy et al., 2009). Studies claim that the antioxidant activity of phenolic compounds is mainly due to their reducing properties and chemical structure, which play an important role in the neutralizing or sequestering of free radicals and the chelation of transition metals (Kristinova, Mozuraityte, Storro, & Rustad, 2009).

3.4. Antibacterial action

The results of antibacterial activity showed a hole inhibition of 13.5 ± 1.4 and 11.5 ± 1.2 mm for the gram-positive strains *S. aureus* 3993 and *S. aureus* 4125, respectively (Table 2). The presence of the lipopolysaccharide membrane in gram-negative bacteria, such as *E. coli* 24, strongly controls the penetration of antibacterial agents and explains why this strain was not susceptible to the antibacterial action of the EOO (Nikaido, 2003). For antibacterial diffusion tests in agar MIC, there is no consensus on the inhibition level acceptable to natural products, when compared with antibiotics standards. However, studies indicate a positive result of antibacterial action for an inhibition hole of greater than 7 mm (Nascimento, Locatelli, Freitas, & Silva, 2000) and a MIC of less than 500 μg (Aligianis, Kalpoutzakis, Mitaku, & Chinou, 2001). Thus, this value of MIC (40 < MIC < 60 μg) classifies the EOO as having strong antibacterial action.

Studies point to phenolic compounds as important promoters and metabolites of antibacterial activity (Mingo, Silván, & Martinez-Rodriguez, 2016). Several modes of action of phenolic compounds have been suggested to cause antibacterial inhibition. These mechanisms include damaging the bacterial cell membrane (Lacombe, Tadepalli, Hwang, & Wu, 2013), the inhibition of

Table 2
Antibacterial action (hole inhibition ± SD (mm)) and Minimum Inhibitory Concentration (MIC) of leaf aqueous extract from *Ocotea odorifera* (EOO).

Treatments/statistic[1]	Concentration	Antibacterial action[4] (%)		
		S. aureus 3993	*S. aureus* 4125	*E. coli* 24
AMP[2]	150 μg/disk	100 a (43.0 ± 1.0)	100 a (34.7 ± 0.6)	100 (29.7 ± 0.6)
DMSO[3]	30 μL/disk	0.0 ± 0.0 c (0.0 ± 0.0)	0.0 ± 0.0 c (0.0 ± 0.0)	0.0 ± 0.0 (0.0 ± 0.0)
EOO	1500 μg/test	31.4 ± 3.2 b (13.5 ± 1.4)	33.2 ± 3.5 b (11.5 ± 1.2)	0.0 ± 0.0* (0.0 ± 0.0)
H		16.4	16.4	–
df		2	2	–
P		<0.001	<0.001	–
MIC		40 < MIC < 60 μg	40 < MIC < 60 μg	–

[1] Kruskal-Wallis test's (α = 0.05). Means (±SD, n = 6) followed by different letters were significantly different within columns (Dunn's test's, α = 0.05). – dashes indicate no data analysis.
[2] Ampicillin – positive control.
[3] Dimethyl sulfoxide – negative control.
[4] Antibacterial action = [(inhibition zone (mm) of treatment/inhibition zone (mm) of positive control) × 100].
* Significantly different within rows (different strains) for extract treatment (*H* = 12.2; *df* = 2; *P* = 0.002 and Dunn's test's, α = 0.05).

extracellular enzymes, a direct effect on antibacterial metabolism (Scalbert, 1991) and DNA degradation (Brudzynski, Abubaker, & Miotto, 2012). Thus, Fig. 2B shows high statistical correlation between the flavonoid content and its antibacterial activity against the *S. aureus* 3993 strain (very close axes). Furthermore, the arrangement between the axes of the tannins and polyphenols and axis of the inhibition of the growth of the *S. aureus* 3993 strain shows a negative correlation (axes approximately 180°), showing that the antibacterial activity against this strain is only related to flavonoid content. For the *S. aureus* 4125 strain, the antibacterial activity is not related to any measured polyphenol content (axes arranged near 90°).

3.5. Antimutagenic and antigenotoxic activities

We observed that for the TA97 strain, the antimutagenic activity is observed for 1125.0, 2250.0 and 3375.0 μg of EOO in the absence of S9 metabolism, and mutagenic inhibition is observed for these three statistically similar concentrations (Table 3). According to Caillet, Lessard, Lamoureux, and Lacroix (2011), antimutagenic action greater than 70% is considered strong, between 40% and 70% is considered moderate, and below 40% is considered neutral. Note also that the antimutagenic action of the EOO to the TA97 strain in the absence of S9 metabolism was higher than that found in the presence of S9. For the TA98 strain of EOO, there is a moderate antimutagenic response to EOO in the absence of S9 metabolism, and strong activity for 3375.0 and 4500.0 μg of EOO in the presence of S9 metabolism. The TA100 and TA102 strains already showed no antimutagenic response to EOO in the absence of S9 metabolism; however, increased antimutagenic activity after S9 metabolism was observed (moderate activity against strain TA100 and high antimutagenic activity against the TA102 strain) (Table 3). Thus, we emphasize that the highest antimutagenic activity was observed for the TA97 and TA98 strains, compared with strains TA100 and TA102, in the absence of S9 metabolism. No significant variation among the four different strains was observed in the presence of S9 metabolism. Differences in the mechanism of action of the studied strains are directly related to the obtained results. For TA97 and TA98 strains, the mechanism of action occurs by frameshifts (genes *his*D6610 TA97 and *his*D3052 TA98), while for the TA100 and TA102 strains (genes *his*G46 TA100 and *his*G428 TA102), the mechanism occurs by base-pair substitution (Maron & Ames, 1983). The homogenate S9 fraction of the constituent enzymes promotes oxidation reactions catalyzed by the cytochrome P450 enzymes (Basheer & Kerem, 2015). Thus, since there is a known interaction between the constituent CYP450 enzymes (Rodeiro et al., 2008), it is believed that these oxidative enzymes have promoted the oxidation of polyphenolic compounds present in the EOO, causing decreased antimutagenic activity in the different strains of *S. typhimurium* that were evaluated.

The mechanisms of action of the antimutagenic activity of phenolic compounds include interference with cytochrome P450-mediated metabolism of mutagens, interaction with active mutagenic metabolites, DNA protection against mutagens presenting electrophilic properties, scavenging of the electrophilic mutagens,

Table 3
Number of colonies and mutagenic inhibition in *Salmonella typhimurium* strains by leaf aqueous extract from *Ocotea odorifera* (EOO), without (−S9) and with (+S9) metabolic activation.

Strain	Concentration (μg/plate)	−S9					+S9				
		N°. of colonies	Statistic comparations[3]				N°. of colonies	Statistic comparations			
			Inhibition (%)	1st	2nd	3rd		Inhibition (%)	1st	2nd	3rd
TA97	NC[1]	317.3 ± 6.1	–				474.0 ± 10.0	–			
	562.5	438.7 ± 8.3	71.2 ± 2.0	b	a	a	842.7 ± 30.0	43.0 ± 4.6	a	b	a
	1125.0	333.0 ± 26.3	96.3 ± 6.2	a	a	a	796.0 ± 20.0	50.3 ± 3.1	a	b	ab
	2250.0	275.0 ± 18.1	110.1 ± 4.3	a	a	a	804.0 ± 6.9	49.0 ± 1.1	a	b	a
	3375.0	320.0 ± 20.0	99.4 ± 4.8	a	a	a	825.3 ± 24.1	45.7 ± 3.7	a	b	b
	4500.0	539 ± 26.2	39.8 ± 9.5	c	a	b	774.7 ± 14.0	46.0 ± 13.4	a	a	b
	PC[2]	738.3 ± 15.2	0.0 ± 0.0	d			1121.3 ± 61.5	0.0 ± 0.0	b		
TA98	NC	60.7 ± 10.1	–				40.3 ± 0.6	–			
	562.5	126.3 ± 1.5	35.8 ± 1.5	bc	a	b	1094.7 ± 61.2	25.9 ± 4.3	b	a	a
	1125.0	114.7 ± 12.2	47.2 ± 11.9	ab	a	b	1009.3 ± 115.5	31.9 ± 8.1	b	b	b
	2250.0	115.7 ± 10.1	46.3 ± 9.8	ab	a	b	830.7 ± 30.3	44.5 ± 2.1	b	a	a
	3375.0	143.0 ± 14.7	19.5 ± 14.4	cd	b	c	385.3 ± 36.1	75.8 ± 2.5	a	a	a
	4500.0	86.3 ± 20.1	65.9 ± 26.4	a	a	a	392.0 ± 32.7	74.6 ± 3.4	a	a	a
	PC	163.0 ± 8.0	0.0 ± 0.0	d			1464.0 ± 55.0	0.0 ± 0.0	c		
TA100	NC	448.0 ± 10.6	–				204.0 ± 48.0	–			
	562.5	852.3 ± 4.5	12.7 ± 1.0	b	b	c	1098.0 ± 20.3	28.1 ± 1.6	b	a	a
	1125.0	879.0 ± 11.0	7.0 ± 2.4	b	b	c	958.7 ± 37.3	39.3 ± 3.0	ab	a	ab
	2250.0	854.7 ± 10.1	12.2 ± 2.2	b	b	c	890.0 ± 38.0	44.8 ± 3.1	ab	a	a
	3375.0	725.3 ± 16.2	40.1 ± 3.5	a	a	b	994.0 ± 34.0	36.5 ± 2.7	ab	a	b
	4500.0	712.7 ± 10.3	36.3 ± 13.6	a	a	b	790.0 ± 36.2	50.5 ± 6.8	a	a	b
	PC	911.3 ± 7.6	0.0 ± 0.0	b			1447.7 ± 116.4	0.0 ± 0.0	c		
TA102	NC	196.0 ± 8.0	–				210.0 ± 14.0	–			
	562.5	921.7 ± 24.2	16.0 ± 2.8	ab	a	c	376.0 ± 14.4	27.5 ± 6.3	c	a	a
	1125.0	925.0 ± 24.0	15.6 ± 2.8	ab	b	c	318.7 ± 39.2	52.5 ± 17.1	b	a	a
	2250.0	829.3 ± 40.1	26.7 ± 4.6	a	b	c	326.7 ± 18.5	49.1 ± 8.1	b	a	a
	3375.0	858.3 ± 32.0	23.3 ± 3.7	a	b	bc	278.7 ± 28.9	70.0 ± 12.6	ab	a	a
	4500.0	856.3 ± 26.1	22.3 ± 5.1	a	b	b	240.0 ± 42.3	76.4 ± 36.5	a	a	a
	PC	1060.0 ± 4.0	0.0 ± 0.0	b			439.0 ± 3.6	0.0 ± 0.0	d		

– dashes indicate no data.

[1] Negative control = distilled water (100.0 μg/plate).

[2] Positive control = 5.0 μg/plate of 4-Nitro-*o*-phenylenediamine (TA97-S9 and TA98-S9); 5.0 μg/plate of sodium azide (TA100-S9) and 1.0 μg/plate of mitomycin-C (TA102-S9); 5.0 μg/plate of 2-aminoanthracene (TA97+S9; TA98+S9; TA100+S9 and TA102+S9).

[3] Three-way ANOVA (interaction strain x metabolic activation x concentration: $F = 11.0$; $df = 15,96$; $P < 0.001$). Means (± SD, n = 3) followed by different letters were significantly different (Tukey test's, $\alpha = 0.05$). 1st = comparison among concentrations within for each group of strains; 2nd = comparison within rows and 3rd = comparison among strains within to each concentration.

Fig. 3. Antigenotoxic action of leaf aqueous extract from *Ocotea odorifera* (EOO). Note: 1: negative control (75.5 μg DNA + 100.0 μL H_2O), 2: positive control (75.5 μg DNA + 750.0 μg $SnCl_2$), 3: (75.5 μg DNA + 750.0 μg $SnCl_2$ + 562.5 μg EOO), 4: (75.5 μg DNA + 750.0 μg $SnCl_2$ + 2250 0.0 μg EOO), 5: (75.5 μg DNA + 750.0 μg $SnCl_2$ + 4500.0 μg EOO).

and binding or insertion into the outer membrane transporters, which would lead to the blockage of a mutagen that was transferred into the cytosol (Słoczyńska, Powroźnik, Pękala, & Waszkielewicz, 2014). Thus, the results of statistical correlation between polyphenol content and antimutagenic action for the different of *S. typhimurium* strains in the absence and presence of S9 metabolism shows high correlation between the content of total polyphenols and tannins and the antimutagenic action across the TA97 (-S9), TA98 (+S9) and TA98 (-S9) strains (Fig. 2C). As observed for the frameshift mechanism of these strains, it is believed that the phenolic constituents of the EOO are more likely to act by preventing a frameshift. For the other strains, with and without S9 metabolism, no correlation was observed between the phenolic content and the antioxidant activity, confirming the lower mutagenicity inhibition values shown in Table 3. There is already an association between antioxidant activity and antimutagenic action; the results in Fig. 2D show a high correlation among the results obtained for the antioxidant activity in the lipid peroxidation test and the antimutagenic action of EOO against TA97 (−S9) and TA98 strains (−S9 and +S9). There is also a high correlation between the antimutagenic activity against TA98 strains (−S9) and the antioxidant activity in the co-oxidation of β-carotene/linoleic acid. Due to the similarity of the antioxidant mechanism in these two tests, it is believed that they can be used as pretests for the identification of natural products with potential antimutagenic action. For strains with base-pair substitution mechanisms (TA100 and TA102), there was a low correlation between the antimutagenic results with and without S9 metabolism and the lipid peroxidation test. However, the DPPH test for scavenging free radicals showed poor correlation with antimutagenic results in different *S. typhimurium* strains.

Finally, the antigenotoxic activity of EOO was not observed in any of the test concentrations in the presence of the genotoxic agent $SnCl_2$ (Fig. 3). Thus, it is noted that the appearance of a linear arrangement of the plasmid (form III) in lane 3 (lowest concentration EOO) is similar to the electrophoretic profile seen in lane 2 (positive control), in which we notice the absence of the helical conformations and tensioned superhelices, forms II and I, respectively, as were observed in lane 1 (negative control). For the two highest concentrations tested, it was not possible to see the presence of the plasmid DNA in agarose gel. Although the EOO has a high phenolic content, the antioxidant activity of the extract was not able to inhibit the redox reactions promoted by the genotoxic agent $SnCl_2$ that destabilize DNA.

4. Conclusion

The results obtained allowed the identification of 13 phenolic compounds in the aqueous extract of *Ocotea odorifera* leaves; these compounds were identified as tetramers, trimers and dimers of (*epi*)catechin, in addition to other flavonoids and tannins. Moreover, high *in vitro* antioxidant activity was observed in the different tests, with a high correlation between the content of total phenolic compounds and the antioxidant activity in the lipid peroxidation test. The results showed antibacterial action against *Staphylococcus aureus* strains that highly correlated with the flavonoid content. Finally, we obtained high antimutagenic action of the EOO against TA97 and TA98 *Salmonella typhimurium*/microsome strains in the absence of S9 metabolism; there is also a correlation among total phenolic content, antimutagenic activity and antioxidant action. The aqueous extract of *O. odorifera* leaves proved to possess interesting properties, emerging from both its chemical composition and from the evaluation of its *in vitro* biological activities. It is believed, therefore, that the observed therapeutic activities can add value to the use of *O. odorifera* as a food condiment. In fact, food products with *O. odorifera* aqueous extract could be an interesting possibility, with accentuated therapeutic activities in addition to those of the essential oil (without the recognized toxicity of the safrole compound).

Conflict of interest statement

There are no conflicts of interest involved in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.03.087.

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