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CROP SCIENCE

Bioactivity and molecular properties of Phenoxyacetic Acids Derived from Eugenol and Guaiacol compared to the herbicide 2,4-D

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Abstract: Herbicides are agrochemicals applied in the control of weeds. With the frequent and repetitive use of these substances, serious problems have been reported. Compounds of natural origin and their derivatives are attractive options to obtain new compounds with herbicidal properties. By aiming to develop compounds with potentiated herbicidal activity, phenoxyacetic acids were synthesized from eugenol and guaiacol. The synthesized compounds were characterized and the herbicidal potential of phenoxyacetic acids and precursors was evaluated through bioassays regarding the germination and initial development of *Lactuca sativa* and *Sorghum bicolor* seedlings, with the induction of DNA damage. The induction of changes in the mitotic cycle of meristematic cells of roots of *L. sativa* was also analyzed. At the concentration of 3 mmol L^{-1}, phenols and their respective phenoxyacetic acids presented phytotoxic and cytotoxic activities in *L. sativa* and *S. bicolor*. Eugenol and guaiacol also presented genotoxic action in *L. sativa*. The toxic effect of eugenoxyacetic acid was more pronounced in *L. sativa* than in *S. bicolor*, similar to the commercial 2,4-D herbicide. Molecular properties of the phenols and their derivatives phenoxyacetic acids were compared with the ones obtained for the herbicide 2,4-D, where it was found a correlation between their molecular properties and bioactivity.

Key words: Eugenoxyacetic acid, guaiacoxiacetic acid, natural phenols, semisynthetic herbicides, 2,4-D.

INTRODUCTION

The 2,4-dichlorophenoxyacetic (2,4-D) and 4-chloro-2-methylphenoxyacetic acids (MCPA) were the first auxinic herbicides used in agriculture. In 1942, Zimmerman and Hitchcock showed that certain chlorinated phenoxyacetic acids, such as 2,4-D, were more active than the naturally occurring hormone indolylacetic acid (IAA) and they were not rapidly degraded in the plant. Due to the selectivity presented by this class of herbicides, the use of these substances innovated and revolutionized agriculture worldwide due to the control of broadleaf weeds. Thus, it was possible to reduce labor and increase cereal productivity (Kudsk & Streibig 2003, Barbosa 2004, Delaney et al. 2006, Silva & Silva 2007).

Several species of dicot weeds which are difficult to control using other herbicides are susceptible to 2,4-D. However, this susceptibility can also be altered. In bacteria found in the soil, for example, genes that are related to 2,4-D tolerance were discovered and their

transference to certain crops have triggered the development of resistant crops to this auxinic herbicide (Queiroz & Vidal 2014). Thus, due to the problems related to the resistance of weeds to conventional herbicides and the fact that they cause innumerable harmful effects to the environment and to humans, there is a demand for the development of novel herbicides.

One of the alternatives to obtain novel herbicides is the application of compounds originating from plant species. Compounds present in essential oils, such as eugenol, affect the development of other species because their allelopathic effects (Li et al. 2011, Stokłosa et al. 2012, Rowshan et al. 2014). In this way, they are candidates as active principle of herbicides, which are more environmentally friendly.

Eugenol is the major compound (approximately 85%) of clove essential oil (*Syzygium aromaticum* (L.) Merr. & L. M. Perry) and is present in cinnamon (*Cinnamomum zeylanicum* Blume), sassafras (*Ocotea odorifera* (Vell.) Rower) and myrrh (*Commiphora myrrha* (T. Nees) Engl.) (Lima et al. 2005, Affonso et al. 2012). Some studies have shown that eugenol and its derivatives act to inhibit seed germination and delay or inhibit the growth of several plant species (Pelissari et al. 2010, Miranda et al. 2015). Another potential compound is guaiacol (2-methoxyphenol), which is a volatile phenol derived from lignin pyrolysis during the processing and/or degradation of the latter in paper and cellulose industries (Mazumder et al. 2005, Ozagac et al. 2016). It is characterized as a natural phenol and can be found in the composition of essential oils which exhibits antiseptic activity (Pelissari et al. 2010).

The compounds of natural origin are considered promising in the obtention of novel herbicides. The introduction of existent functional groups in conventional herbicides to obtain semisynthetics ones can be promising in the design of new agrochemicals. The introduction of the -CH_2COOH group in the eugenol and guaiacol can be an alternative to enhance their herbicidal effects since this functional group is present in 2,4-D.

In view of what was mentioned before, in this work we synthesized phenoxyacetic acids (analogous to auxinic herbicides such as 2,4-D, but chlorine-free) from eugenol and guaiacol and evaluated their herbicidal potential. The synthesis of the compounds here studied can be found elsewhere (Oki & Horita 1961, Hoan et al. 2007). The effects of eugenol, guaiacol and their respective semisynthetic phenoxyacetic acids (Figure 1) were evaluated through bioassays on seed germination and initial seedling development of *Lactuca sativa* L. (eudicot) and *Sorghum bicolor* L. (monocot). The possible toxic effects were accessed from the mitotic cycle and from the DNA of the root meristematic cells of *L. sativa*, which is one of the species considered model for angiosperm assays.

MATERIALS AND METHODS

Plant material

Commercial seeds of the monocot *S. bicolor* L. (sorghum) var. Moench (BR seeds) and of the eudicot *L. sativa* L. (lettuce) var. Mônica (Feltrin) were used as plant models in the bioassays.

General experimental procedures

The phenols used in the reactions were the eugenol and guaiacol with the solvent acetone P.A.. The purification of the synthesized compounds was accomplished by diethyl ether P.A.. Both reagents were purchased from Sigma Aldrich®. Purified water by reverse osmosis was used in the reactions as well as in the preparation of the solutions used in the bioassays.

It was determined the melting points by using Quimis (Q340S) equipment. Mass spectra

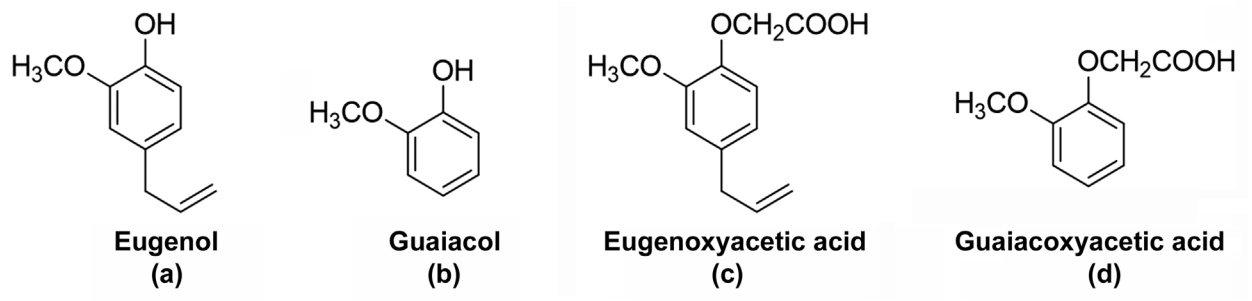

Figure 1. Chemical structure of the natural phenols eugenol (a) and guaiacol (b) and their semisynthetic derivatives eugenoxyacetic acid (c) and guaiacoxyacetic acid (d).

of eugenoxyacetic and guaiacoxyacetic acids were obtained by gas chromatography coupled to mass spectrometry (GC-MS) (Shimadzu, model QP Plus 2010) using the electron ionization mode of 70 eV. The mass spectra were recorded in ultra-high resolution and precision by using the mass spectrometer (model 9.4T Solarix, Bruker Daltonics, Germany). It was operated in negative electrospray ionization mode with Fourier transform cliclotron ion resonance mass spectrometry. The ESI (-) - FT-ICR MS spectra were acquired with the power resolution of m / Dm50% ca. 500000 where Dm50% is the maximum peak width at the maximum half height of m / z 400 and a mass accuracy <1 ppm. It provides an unambiguous view of molecular formula assignment for individually charged molecular ions such as $[M-H]^+$.

The Nuclear Magnetic Resonance (NMR) of ^1H and ^{13}C analysis were performed in a spectrometer from the Varian brand (model VNMRS 400). A magnetic field of 9.4 T by using a 5-mm probe BroadBand1H/19F/X at the temperature of 25 °C was applied. Deuterated chloroform ($CDCl_3$) was used as solvent.

Synthesis of the eugenoxyacetic and guaiacoxyacetic acids

The eugenoxyacetic (Figure 1c) and guaiacoxyacetic acids (Figure 1d) were similarly prepared. For the eugenoxyacetic acid, an initial solution of sodium chloroacetate (8.37 mmol; 0.783 g) was prepared by dissolving 2-chloroacetic acid (8.37 mmol; 0.791 g) in distilled water (8 mL) and acetone (2 mL). The resulting solution was then cooled in ice bath. Subsequently, sodium hydroxide (NaOH) was added under agitation to the solution until the pH was adjusted to be between 9 and 10. Then, by using a two-neck round-bottom flask, a mixture was prepared containing NaOH (7.37 mmol; 0.295 g), distilled water (40 mL), acetone (10 mL) and eugenol (6.70 mmol; 1.100 g). The mixture was agitated for 20 minutes at 100 °C. After heating, the sodium chloroacetate was added from drop by drop to the mixture. The reagent solution was kept under heating and reflux for 24 h. After cooling the solution up to room temperature, the pH value of the mixture was acidified to 1-2 using diluted hydrochloric acid (HCl) in ice bath (Yan et al. 2014 with adaptations, Alves et al. 2018). See figure 2.

The obtained solution was purified by liquid-liquid extraction with chemically active solvents. The material was dissolved in 30 mL of ethyl ether and poured into a beaker of 250 mL. It was cooled in an ice bath and a solution of saturated sodium bicarbonate ($NaHCO_3$) was added until reaching the alkaline pH. This mixture was transferred to a separation funnel where the organic and aqueous phases were separated. Further, the extraction was

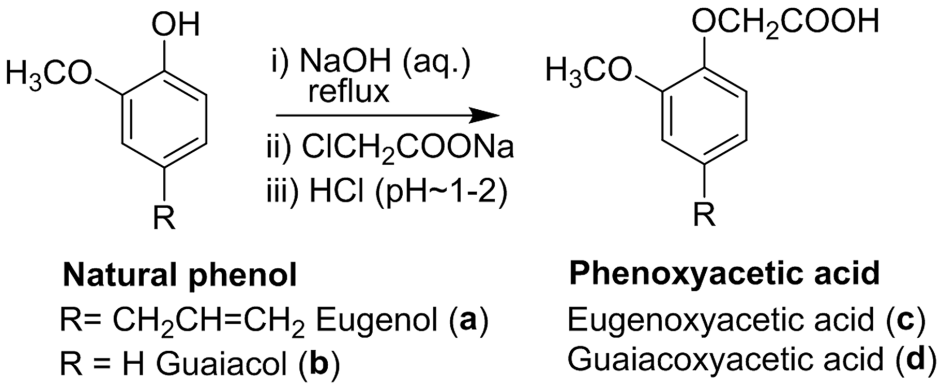

Figure 2. General scheme of the reactions used to obtain eugenoxyacetic acid (c) and guaiacoxyacetic acid (d) from the natural phenols eugenol and guaiacol, respectively.

performed using 30 mL of diethyl ether and the aqueous phase was again collected. The organic phase was properly discarded and the aqueous ones were combined in an Erlenmeyer flask, which was placed in ice bath and then acidified with concentrated HCl until reaching pH of approximately 1-2. The obtained material was again placed in separation funnel and washed with 2 x 20 mL of distilled water. The aqueous phase was discarded and the obtained organic phase was dried with anhydrous sodium sulfate, filtered, and the solvent evaporated.

Eugenoxyacetic acid (c) (light brown crystal); melting point (m.p.): 94-97 °C (literature: 95-97 °C; Abraham et al. 1984); ^{1}H NMR, δ_H (400 MHz, $CDCl_3$): 3.31 (d, m, CH_2-CH=); 3.85 (s, 3H, OCH_3); 4.63 (s, 2H, CH_2-COOH); 5.08 (m, 2H, =CH_2); 5.93 (m, 1H, CH_2-CH=); 6.65-6.81 (m, 3H, Ar-H).^{13}C NMR, δ_C (100 MHz, $CDCl_3$): 39.8 (CH_2-CH=); 55.8 (OCH_3); 67.2 (CH_2-COOH); 112.6 (C-6); 115.5 (C-3);115.9 (=CH_2); 120.8 (C-5); 135.3 (C-4); 137.2 (CH_2-CH=); 145.3 (C-1); 149.4 (C-2); 172.8 (CH_2-COOH). MS (EI) *m/z* (%): 223 ($C_{12}H_{14}O_4$, $[M + 1]^+$; 13); 222 ($[M]^+$, 100); 163 (70); 147 (12); 131 (20); 115 (17); 103 (43); 91 (28); 77 (19); 55 (10); 41 (20); 39 (8). ESI(-)-FT-ICR MS calcd. for $C_{12}H_{13}O_4$: 221.0892; found: 221.0819.

Guaiacoxyacetic acid (d) (light brown crystal); m.p.: 122-125 °C (literature: 123-126 °C; Sigma Aldrich catalog); ^{1}H NMR, δ_H (400 MHz, $CDCl_3$): 3.87 (s, 3H, OCH_3); 4.69 (s, 2H, CH_2-COOH); 6.89-7.04 (m, 4H, Ar-H); 9.69 (sl, 1H, OH). ^{13}C NMR, δ_C (100 MHz, $CDCl_3$): 55.9 (OCH_3); 67.3 (CH_2-COOH); 112.2 (C-3); 116.0 (C-6); 121.1 (C-4 or C-5); 123.5 (C-4 or C-5); 147.0 (C-1); 149.7 (C-2); 173.3 (CH_2-COOH). MS (EI) m/z (%): 183 ($C_9H_{10}O_4$, $[M + 1]^+$; 10); 182 ($[M]^+$, 100); 137 (63); 124 (20); 107 (57); 92 (37); 77 (56); 63 (33); 52 (25). ESI(-)-FT-ICR MS calcd. for $C_9H_9O_4$: 181.0579; found: 181.0508.

Bioassays

Four concentrations (3 mmol L^{-1}; 1.5 mmol L^{-1}; 0.75 mmol L^{-1}; 0.375 mmol L^{-1}) of eugenol, guaiacol, eugenoxyacetic acid, guaiacoxyacetic acid and herbicide 2,4-D were used as treatments in the macroscopic and microscopic bioassays. As negative control (C-) we used the solvent used in the solutions preparation: distilled water + acetone 2% (v v^{-1}) + Tween 80, 0.05% (v v^{-1}), which in previous tests showed to be similar to water. The commercial herbicide glyphosate was used as positive control (C+).

To evaluate the herbicide potential on germination and initial seedling development of *L. sativa* and *S. bicolor*, the seeds were directly exposed to treatments, as described by Pinheiro et al. (2015).

In order to evaluate the cytogenotoxic effects, lettuce roots were exposed to treatments with eugenol, guaiacol, eugenoxyacetic and guaiacoxyacetic acids and by fixing the controls (negative and positive). Only lettuce roots were used since they are considered a suitable model

for microscopic analysis (cytotoxic analysis and comet assay) to test the toxic effect of chemical compounds (Silveira et al. 2017). It is important to highlighted the importance of lettuce in the study and it presents high proliferative activity, rapid growth, high number of seeds, large chromosomes, high sensitivity to mutagenic and genotoxic compounds as well as easily manipulated roots (Andrade-Vieira et al. 2014, Aragão et al. 2015, 2017). The analysis of the cell cycle were performed according to the protocol described by Aragão et al. (2015), the variables mitotic index (MI) chromosome alterations (CA), nuclear alterations (NA). The frequency of each type of the observed CA was accessed.

The mutagenic effect of the compounds was evaluated by single-cell electrophoresis (comet) assay by using only the solution at a concentration of 3 $mmolL^{-1}$ of the tested compounds. The comet assay was carried out according to the protocol of Silveira et al. (2017).

A hundred random nucleoids were evaluated per slide and three slides per treatment, totaling 300 per treatment. Nucleoids were visually scored according to the intensity of fragmentation (tail length and nucleoid head size) in scores (0, 1, 2, 3 and 4). The arbitrary units (AU) were used as a parameter to determine the mutagenicity. They were obtained by multiplying the total nucleoids observed in each score by its respective score, according to Collins (2004). It resulted in the AU range between 0 (absence of damage) a 400 (maximum of observed damage).

The data obtained from the analyzes of phytotoxicity, cytotoxicity and mutagenicity were analyzed by the ANOVA. The mean values were compared to Dunnett's test (p<0.05). It has been used to compare treatments with controls (McHugh 2011). All analyzes were carried out using the statistical analysis program GENES VS 2015.5.0 (Cruz 2013).

Computational details and statistical analyzes

The molecular properties used as descriptor to perform the statistical analysis were calculated by using the Spartan '14 software. By taking into account the conformer distribution subroutine of the program, a conformational analysis with the semi-empirical method PM6 was performed (Shao et al. 2006, Stewart, 2007). Then, by using the most stable conformation for each compound, the geometry optimization and vibrational frequencies calculations were carried out at the theory level ωB97X-D/6-31G(d,p). Finally, the molecular properties were obtained from the final geometries in the same level of theory (Parr & Yang 1989, Chai & Head-Gordon 2008, Ditchfield et al. 1971).

The calculated molecular properties were obtained from theoretical methods and used as descriptors: Solvation Energy (E_{solv}), HOMO Energy (E_{HOMO}), LUMO Energy (E_{LUMO}), Band Gap energy (BG), Dipole Moment (μ), Molecular Volume (Vol.), Polar Surface Area (PSA) and Ovality (Oval.). They were used to compare the structural parameters of the phenols and their derivatives phenoxyacetic acids to the ones from the herbicide 2,4-D by means of Principal Component Analysis (PCA). The PCA was performed by using the Python.2.7.12 programming language with the Scikit-learn.0.20.3 Machine Learning & Statistical toolkit together with the graphical library Matplotlib.1.5.1.

RESULTS AND DISCUSSION

Synthesis of the phenoxyacetic acids

The obtention of eugenoxyacetic and guaiacoxyacetic acids from the eugenol and guaiacol phenols was performed since the group -CH_2COOH was presented in the structures of 2,4-D and MCPA auxin herbicides. The aim was to obtain semisynthetic compounds with

potentiated herbicidal action in relation to their respective precursors. The synthesized eugenoxyacetic and guaiacoxyacetic acids were properly characterized by mass spectrometry, NMR of ^{1}H and of ^{13}C. Their yields were, respectively, 53 % and 63%.

Studies that used as reagents other types of phenols and reactions with duration of 5 hours presented yields ranging from 52 to 85 % (Yan et al. 2014). Hoan et al. (2007) synthesizing eugenoxyacetic acids and a yield of 57% was achieved. This is very close to that those found in the current study.

The guaiacoxyacetic acid is commercialized, but no information was available about its yield or toxicity. In fact, there are more studies in the literature related to its degradation than to its obtention. This compound can be degraded by ruminant and anaerobic bacteria from the rumen that completely cleave the binding of beta-aryl ether, which is an important link between lignin monomers (Chen et al. 1985).

Macroscopic parameters

The herbicide potential of the natural phenols and their semisynthetic derivatives was evaluated by macroscopic tests, which consists of evaluating the action of the researched substance on the germination and initial development of the seedling of plant models or targeted weeds. Recently, for a better understanding of this herbicidal action, the microscopic tests have been carried out because they allow to verify the action on mitotic division as well as induction of chromosomal and nuclear alterations and cell death, characterizing the cytogenotoxicity, genotoxicity and mutagenicity of the studied substances (Fagodia et al. 2017, Alves et al. 2018, Santos et al. 2018).

Eugenol at the concentration of 3mmolL^{-1} inhibited 17.21% of the germination, 28.43% of the germination speed index (GSI), 80.64% of the root growth (RG) and 40.34% of the aerial growth (AG) of sorghum, as well as inhibited 100% of the development of lettuce, these inhibitions significant are according to Dunnett's test (p<0.05). Thus, it presented a phytotoxic effect for both the plant models tested (Figure 3). The bioherbicide effect of eugenol has been reported by several authors (Bainard et al. 2006, Ahuja et al. 2015, Miranda et al. 2015), moreover Vaid et al. (2010) studied and reported this effect for the eudicot weed *Bidens pilosa*, which had its germination completely inhibited after exposure to 5 µL of pure eugenol.

The eugenoxyacetic acid at 3 mmol L^{-1} was only phytotoxic for lettuce, inhibiting 18.58% of its germination, 40.17% of its GSI, 36.43% of its RG and 62.89% of its AG (Figure 3). This difference in the response between the plant models used can be explained by the chemical proximity of the semisynthetic molecule, eugenoxyacetic acid, to the synthetic 2,4-D molecule. Since the 2,4-D herbicide is selective, it inhibits the development of broadleaf plants only (Sbano et al. 2013).

Studies related to the toxicity of guaiacol are rare, although guaiacol is a natural phenol and undesirable residue of paper and cellulose industries (Mazumder et al. 2005, Ozagac et al. 2016). In the present study, guaiacol at 3 mmol L^{-1} was reduced to 82.49% the RG, 26.77% the sorghum GSI as well as 87.61% the germination, 94.03% the GSI, 98.23% the RG and 90.95% the AG from lettuce. Guaiacoxyacetic acid at 3 mmol L^{-1} inhibited 80.33% of germination, 88.90% of GSI, 97.93% of RG and 100% of AG of sorghum seedlings, and 100% of the same variables of lettuce. At the concentration of 1.5mmol L^{-1}, 44.38% of the GSI and 77.19% of the sorghum RG were inhibited, while all variables were significantly reduced in lettuce, with germination of 58.41%, GSI of 79.23%, RG of 86.58% and AG of 67.00%. The lettuce seedlings showed to be

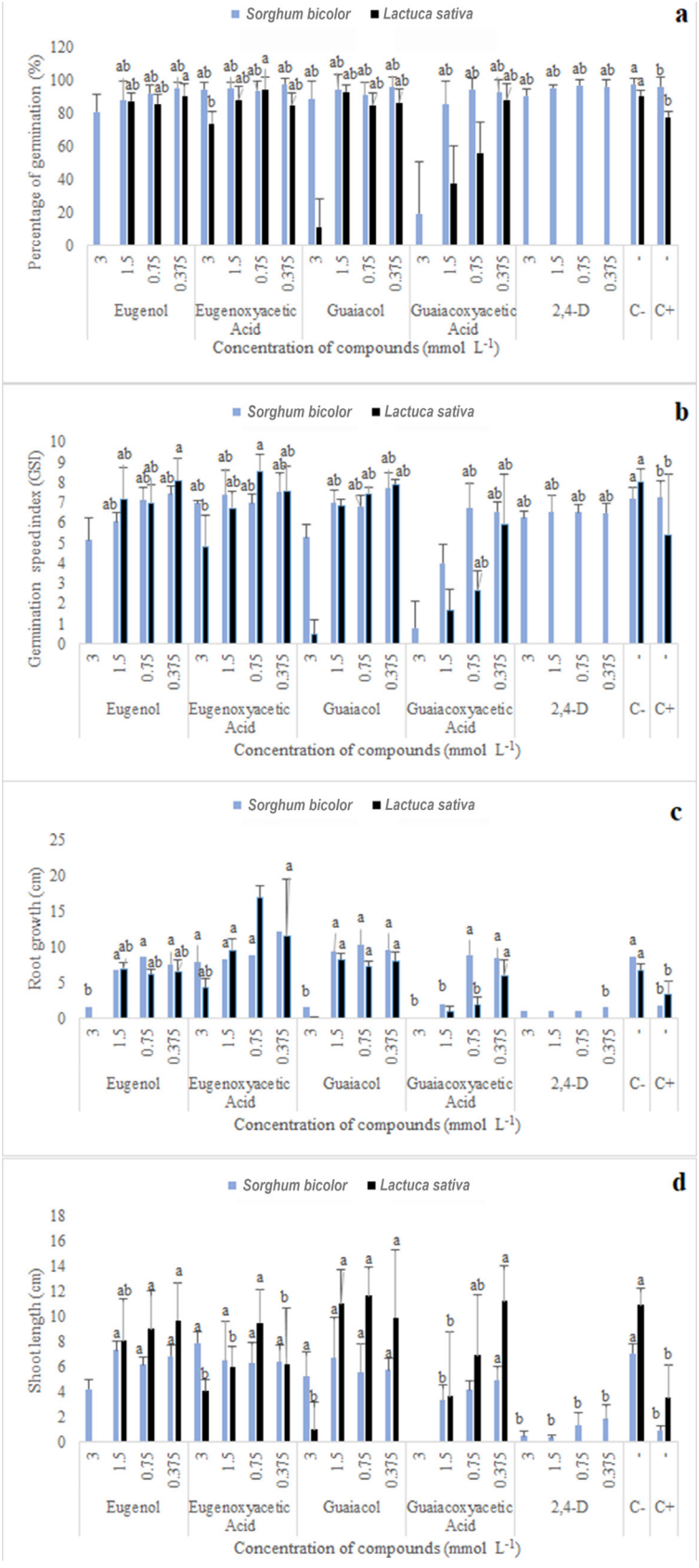

Figure 3. Phytotoxicity of the natural phenols eugenol and guaiacol, of their respective phenoxyacetic acids (eugenoxyacetic and guaiacoxyacetic acids) and of the herbicide 2,4-D at concentrations of 3, 1.5, 0.75 and 0.375 mmol L^{-1}, as well as of the negative control (C-), represented by the solvent (water, Tween 80 0.05% and acetone 2%) and positive control (C+) (commercial herbicide glyphosate), in the face of the initial development of *Sorgo bicolor* and *Lactuca sativa*. The data refer to: (a) percentage of germination, (b) germination speed index, (c) root growth and (d) aerial growth. Means in the bars followed by the letter *a* were equal to C- and the means in the bars followed by the letter *b* were equal to the C + according to Dunnett's test ($p<0.05$). Water was also used as a negative control in the experiment, but the result was statistically identical to the solvent. Thus, the water data were omitted.

more sensitive to guaiacoxyacetic acid because they were inhibited when treated with 0.75 mmol L^{-1}, in 38.05% in the germination, 66.91% in the GSI and 71.98% in the RG. Lettuce also presented 26.00% of GSI inhibition when treated with guaiacoxyacetic acid at concentration of 0.375mmol L^{-1} (Figure 3). Thus, guaiacoxyacetic acid showed to be more phytotoxic for both monocot and eudicot than the natural phenol guaiacol, since it inhibited the development of the plant models even in lower concentrations.

Kobayashi et al. (1994), demonstrated the antifungal and antibacterial action of guaiacol and derivatives of this molecule, which, in phytotoxicity tests, with other model species and at a concentration higher than that used in the present study, showed low inhibition. In the present study, guaiacol and guaiacoxyacetic acid presented phytotoxic effects on lettuce and sorghum.

Another important factor regarding the eugenoxyacetic acid is that at concentration of 0.375mmol L^{-1} it promoted a 39.52% increase in the root growth of sorghum when compared to the negative control. This response was also found for lettuce. However, in roots treated at the concentration of 0.75 mmol L^{-1} such induction was of 147.79% (Figure 3). This behavior is similar to that of 2,4-D, which is considered a synthetic auxin because it has an action similar to the indolylacetic acid (IAA). At low concentrations it can be used to stimulate root elongation (Swarup et al. 2001).

Tworkoski (2002), by studying the bioherbicidal effect of several essential oils in *Taraxacum officinale*, observed the phytotoxic effect in clove essential oil in which the major component is eugenol. After the test with *T. officinale*, the authors also developed experiments with weed which presented injuries: ions leakage (results in the death of the plants) which was treated with the essential oil at 5 and 10 % concentrations after seven days of exposure to the oil.

According to some reports, eugenol promotes inhibition of cellular respiration by acting on the ATPase enzyme. Gill & Holley (2006) concluded that eugenol at concentrations of 5 or 10 mM determined the inhibition of the ATPase enzyme. The authors also emphasized that this enzyme inhibition, under sublethal conditions, can be linked to the decrease on bacteria growth. Vaid et al. (2010) studied the effect of eugenol on eudicot weeds *Cassia occidentalis* and *B. pilosa*. They observed that both plants treated with eugenol showed a reduction in the initial growth rates, dry weight of the seedlings, concentration of chlorophylls and cellular respiration, which helped to support and explain the results found in the present study, evidencing the toxic effect of eugenol.

Eugenol also promotes the increase of reactive oxygen species. According to Ahuja et al. (2015), the exposure of roots to different concentrations of eugenol promoted the increase of oxygen peroxide, superoxide radical and hydroxyl radical. Due to the increase of reactive oxygen species it could be observed an increase in the amount of peroxidation of the membrane bioproducts. Nevertheless, the results found by the authors demonstrated that the antioxidant enzymatic machinery was not damaged. On the contrary, the quantity of this class of enzyme increased, which shows the plasticity of the plant activating its metabolic defense against the suffered injuries.

Microscopic parameters

The meristematic cells of the roots treated with guaiacol at concentration of 3 mmol L^{-1} and guaiacoxyacetic acid at 1,5mmol L^{-1} presented, respectively, 88.67 % and 28.53 % of reduction of the MI. In addition, an increase of the NA in approximately 11 and 8 times, respectively,

was observed when compared to the negative control. Cells treated with eugenoxyacetic acid at 1.5; 0.75 and 0.375mmol L^{-1} presented an increase of 23.06 %, 25.94 % and 26.84 %, respectively, in MI when compared to C-, and this increase was associated with an increase of approximately three times of the CA, and 8 times of the NA at 1.5 mmol L^{-1} (Figure 4).

The MI is a variable widely used in cytotoxicity tests because it is a plastic variable and can indicate the cytotoxicity by its increase or decrease (Leme & Marin-Morales 2009). The MI can increase due to two distinct forms of action of the cytotoxic agent. First, the increase of MI is accompanied by the increase in the number of CA. In other words, when the presence of more cells in the process of mitotic division occurs. However, such cells are not healthy, which results in a blockage of the cycle continuity (Pinheiro et al. 2015). Second, there is a significant increase in the MI of the cells treated with the cytotoxic agent compared to the C-. However such cellular proliferation occurs in a disordered way, which is harmful to the tissues and determines the formation of tumors. This makes the toxic agent carcinogenic (Leme & Marin-Morales 2009).

Cells treated with eugenol at concentration of 0.75 mmol L^{-1} presented 8 times more NA than the negative control (Figure 4). Despite the micronucleus (MN) observation, the most frequent NA in the treatments was expressed in the form of condensed nuclei. The process of cell death is cytologically characterized from the

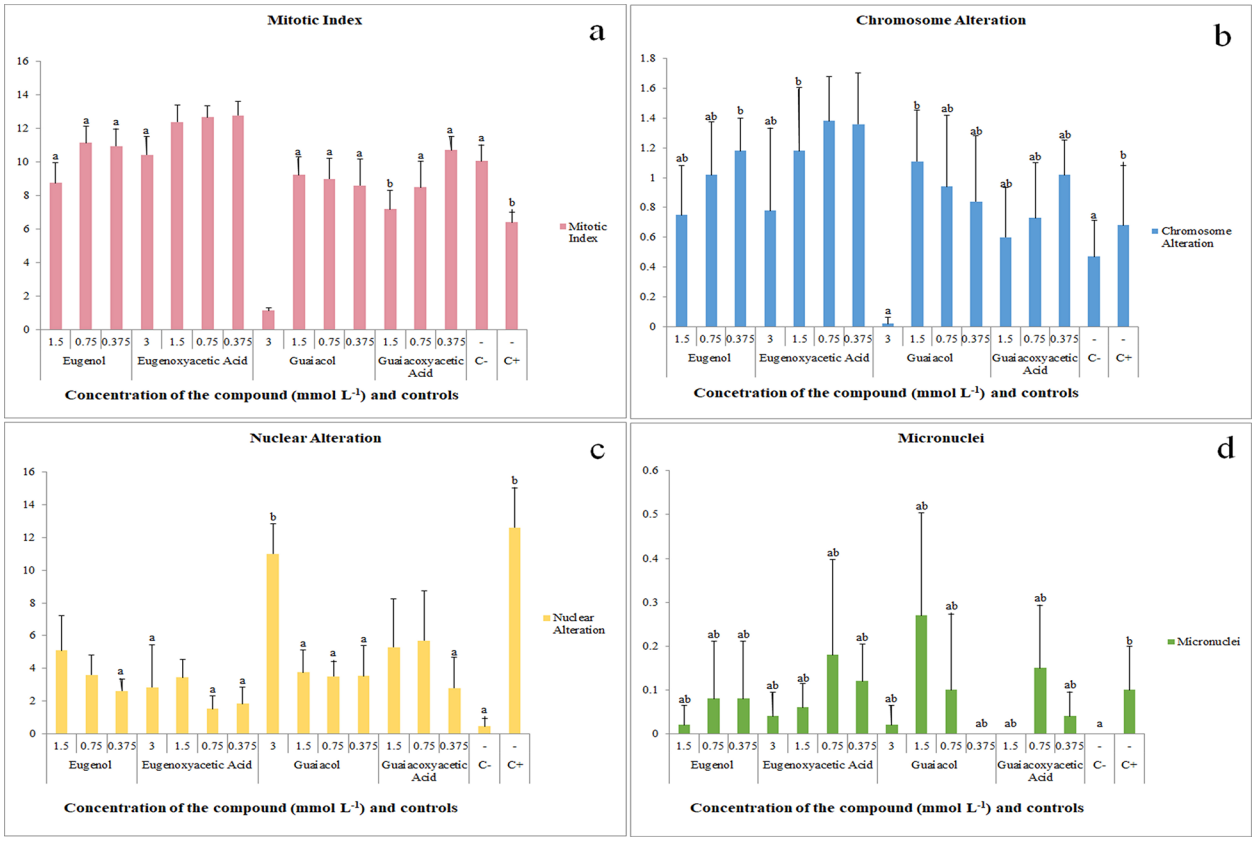

Figure 4. Parameters of the mitotic divisions of *Lactuca sativa* treated with different concentrations (mmolL^{-1}) of the phenols eugenol and guaiacol and their derivatives semisynthetics phenoxyacetic acid: (a) Mitotic index; (b) Chromosome alteration; (c) Nuclear alteration and (d) Micronuclei. The bars followed by letter *a* are statistically identical to the negative control (C-) and the means followed by letter *b* are statistically identical to the positive control (C+) according to Dunnett's test (p<0.05).

presence of nuclei that present pinocytic and strongly stained chromatin, which are called condensed nuclei. It is classified as a type of NA (Andrade-Vieira et al. 2011, Bernardes et al. 2015, Pinheiro et al. 2015). The condensed nuclei and the cell death are associated with the reduction on the roots growth since the division of the cells into the root meristem is essential for root development (Harashima & Schnittger 2010). Thus, roots treated with guaiacol, guaiacoxyacetic acid, eugenol, eugenoxyacetic acid and the synthetic herbicide glyphosate induced the process of cell death, which resulted, among the analyzed variables, in a mitodepressive activity. This process of cell death can be triggered by the recognition made by the cell itself of the damages caused by the external environment when exposed to the treatments in question (Pinheiro et al. 2015). It is important to notice that in the treatment with guaiacol at 3 mmol L^{-1} the amount of NA was equal to that of the positive control (glyphosate), demonstrating the toxicity of the test molecule and bioherbicidal activity.

This increase in cells under death process can be related to the toxic effects of eugenol, as described by Vaid et al. (2010). This effect was related to the alteration on ATPase enzyme and cellular respiration which decrease the energy supply in the cell. Therefore, the cell membranes are affected by the leakage of electrolytes, leading to a destabilization of the ionic potential of the cell. Babich et al. (1993) reported that eugenol caused peroxidation of the membrane of human and rat cells, resulting in the induction of the process of cell death.

Guaiacol can break down the bacteria cell wall, causing leakage of metabolites, besides inhibiting the biosynthesis of prostaglandins, giving guaiacol an anti-inflammatory effect (Kasugai et al. 1991). Thus, the cytotoxic activity of guaiacol may be related to its action on the cell walls that culminates in the loss of essential molecules, and to the compromise of metabolic processes such as nutrient transportation and cellular respiration.

Kasugai et al. (1991), in studying the cytotoxic effects of different phenolic compounds, tested guaiacol in cells of the RPC-C2A lineage (cell line obtained from rat dental pulp with elevated growth activity). The authors observed that the increase at the concentration of guaiacol in the cells resulted in a rise of the toxicity effects. The reason for the non-occurrence of a significant difference in NA is due to the not so strong effect at low concentrations nor by the change in the model organism. Thus, only the meristematic cells treated with guaiacol, at 3 mmol L^{-1}, showed increase in NA.

Markowitz et al. (1992) reported the cytotoxic effect of eugenol for mammalian cells. The cells died at two different conditions of exposure to the compound when they were exposed to eugenol at 10^{-2} mol L^{-1} in a short period and at 10^{-3}mol L^{-1} for a long period. Soloneski et al. (2016), by evaluating the genotoxic effect of glyphosate in *Rhinella arenarum*, reported a significant increase in the index of genetic damage. These results corroborated to the values of MI and the toxic effect of this substance, as observed in the present study, where a reduction of 36.28% in the number of cells in division was found.

The presence of CA (Figures 5 and 6) was also observed in the meristematic cells of the roots treated with eugenol and mainly eugenoxyacetic acid. This type of alteration can be caused by genotoxic agents that encountered the cells, causing damage to the mitotic division (Leme & Marin-Morales 2009). This effect has already been reported by Jeng et al. (1994) who investigated the effect of eugenol on human fibroblasts where the genotoxicity of eugenol in this cellular type was confirmed.

In the present study, the most frequent CA were adherent chromosomes (Figure 6c) and chromosome fragmentation (Figure 6d). Adherent chromosomes presented an increase of 5 times in cells treated with eugenol (0.375 mmol L^{-1}) and eugenoxyacetic acid (1.5 and 0.75 mmol L^{-1})

as well as 4 times in cells treated with guaiacol (1.5 and 0.375 mmol L^{-1}) and guaiacoxyacetic acid (0.375 mmol L^{-1}). Chromosome adhesion represents a severe toxic effect of the toxic agent since this alteration is often irreversible, determining the cell death (Fernandes et al. 2009). This demonstrates the mechanism of aneugenic activity of the toxic agent (Leme & Marin-Morales 2009).

The increase in chromosomal fragmentation was observed only in cells treated with the concentration of 0.375 mmol L^{-1} of eugenoxyacetic acid (5 times), when compared to the negative control. This increase allowed us to consider that such mutation is as an evidence of the chromosomal breaks. This is associated with the adherent chromosomes (Figure 6c) due to the clastogenic effect of these molecules (Andrade et al. 2008, Bernardes et al. 2015).

In order to determine the occurrence of primary damages in the DNA that induce single and double strands breakage, it was used the alkaline-alkaline comet assay (Lanier et al. 2015). It has demonstrated that eugenol, guaiacol, eugenoxyacetic acid and guaiacoxyacetic acid mainly induced type 1 damage, which can be repaired by the cell (Menke et al. 2001). Also, the results demonstrated that the only treatment that caused damage that is not amenable to repair (damages 3 and 4) was glyphosate, which is used as a positive control. Dinh et al. (2010) reported that eugenoxyacetic acid has been patented as a compound that can be used as a food preservation additive and is considered

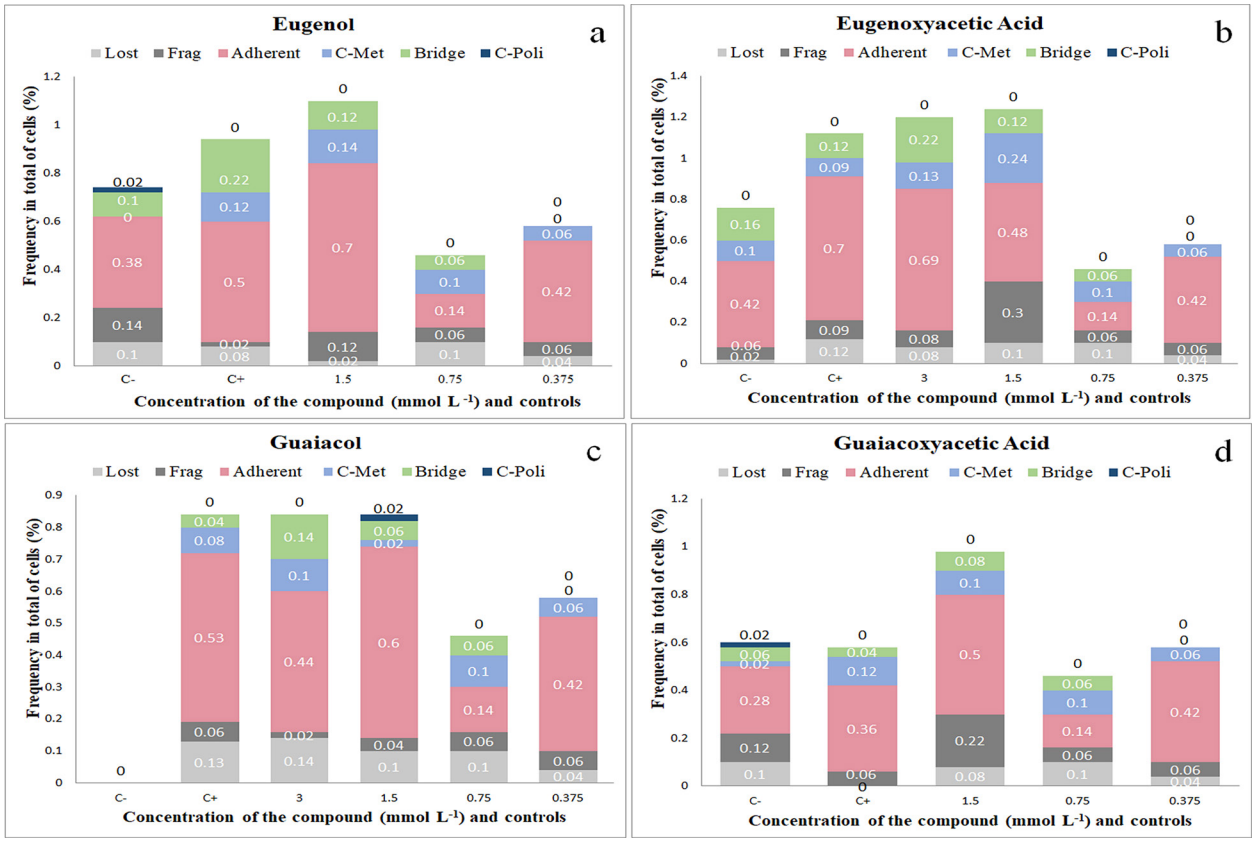

Figure 5. Chromosome alterations in meristematic cells of *Lactuca sativa* treated with different concentrations (mmolL^{-1}) of the phenols eugenol (a) and guaiacol (c) and their derivatives semisynthetics eugenoxyacetic acid (b) and guaiacoxyacetic acid (d). Where the acronyms refer to the frequency (by cell in division) of: Lost% = lost chromosomes; Frag% = chromosome fragments, Adherent% = adherent chromosomes, C-Met% = c-metaphases, Bridge% = chromosome bridges; C-Poli% = chromosome polyploidization.

non-toxic and odorless. Thus, although the molecules used as test have herbicidal action comparable to commercial herbicides, their toxicity is low and amenable to repair, indicating that their application may have ecologically positive effects.

Although most of the damages observed with eugenol and guaiacol treatment were type 1, an amount of AU significantly higher than the negative control and water was observed. In addition, AU values were statistically equal for the same treatments with the positive control, demonstrating their toxic potential (Figure 7).

Statistical analysis

It was used the Principal Component Analysis (PCA) in order to propose the molecular structural relationships by using the molecular properties as descriptors: Solvation Energy (E_{solv}), HOMO Energy (E_{HOMO}), LUMO Energy (E_{LUMO}), Band Gap energy (BG), Dipole Moment (μ), Molecular Volume (Vol.), Polar Surface Area (PSA) and Ovality (Oval.). (Appendix I).

The projections of the compounds 2,4-D (blue), eugenoxyacetic acid (green), guaiacoxiacetic acid (red), guaiacol (cyan), eugenol (purple) are shown in Figure 8. Based on PCA analysis, one could observe two groups. One is composed of the phenoxyacetic acids: 2,4-D, guaiacoxiacetic acid and eugenoxyacetic acid. The second correlates the eugenol and the guaiacol, two phenolic compounds. The results indicate that, even having different substituent group connected to the aromatic ring, their molecular properties were similar, as it can be observed in their structures in Figure 1.

Figure 9 presents the projection of the molecular properties in the first two main PCA components F1 (52.98 %) and F2 (33.38 %). As it can be observed, the molecular properties Volume (Vol.) and Ovality (Oval.) are (positively) correlated. The molecular properties E_{HOMO} and E_{LUMO} are correlated (negatively) with the molecular Dipole Moment (μ) since the angles between the vectors are about 180 °.

The properties that contributed the most for the F1 is the Solvation Energy (E_{solv}), E_{LUMO}, E_{HOMO}, PSA and Dipole Moment (μ). The properties Volume and Ovality presented the higher contribution to the second PCA component (F2).

CONCLUSION

Eugenol, guaiacol and their phenoxyacetic acids presented phytotoxic and cytotoxic activities. However, eugenoxyacetic acid was selective in the inhibition of lettuce which possesses a broadleaf, similar to what happens with the commercial herbicide 2,4-D, and it could be

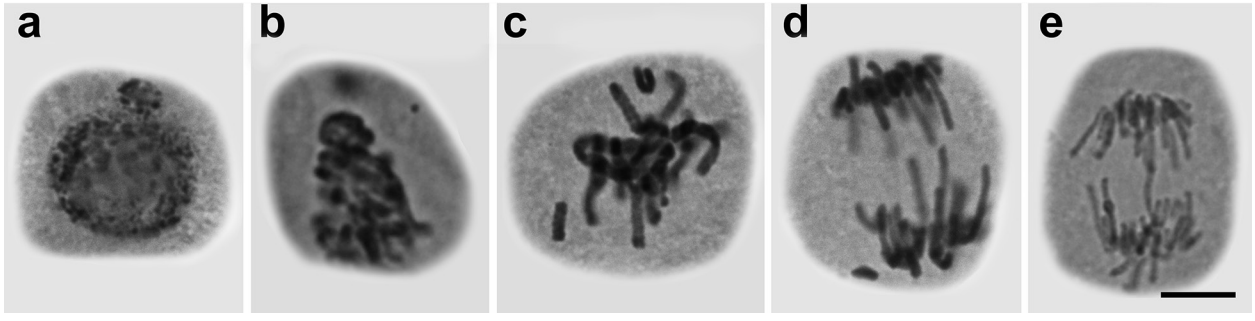

Figure 6. Meristematic cells of *Lactuca sativa* treated with eugenol, eugenoxyacetic acid, guaiacol, guaiacoxyacetic acid and glyphosate. Observed alterations: (a) interphase with micronucleus, (b) bud, (c) adherent with lost chromosomes, (d) anaphase with lost chromosomes, (e) anaphase bridge. Bar = 10 μm.

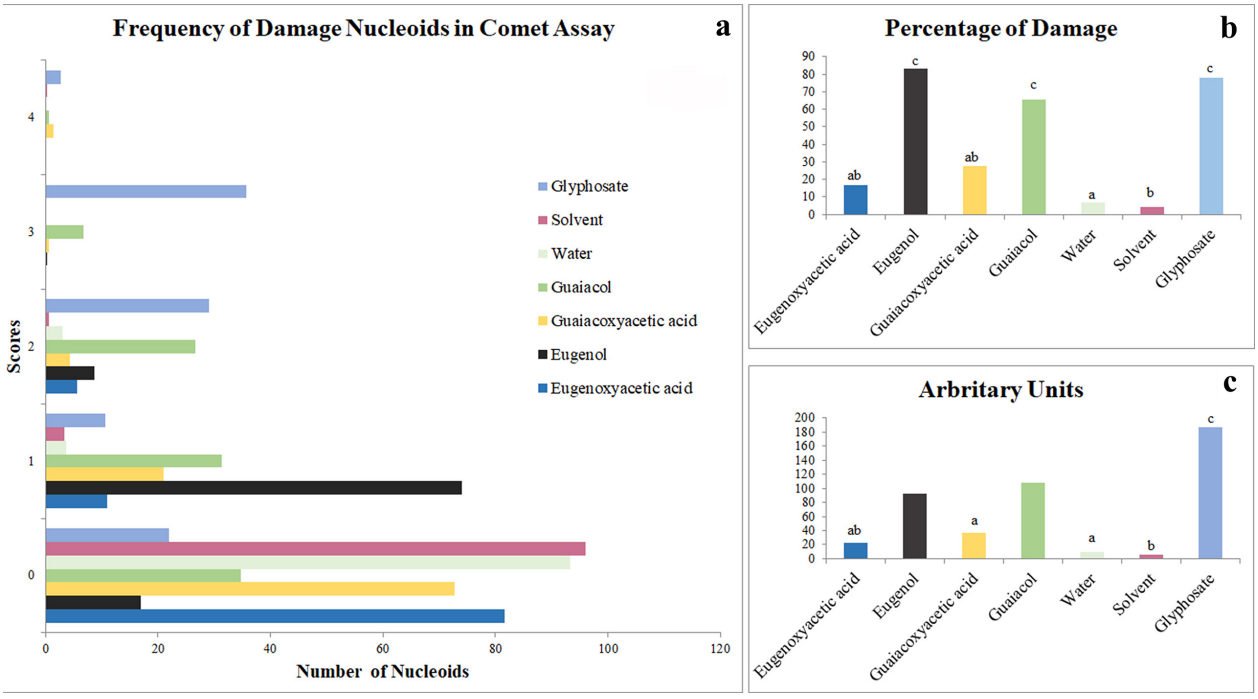

Figure 7. Comet assay in *Lactuca sativa* roots treated with the phenols eugenol and guaiacol and their derivatives semisynthetics phenoxyacetic acid. (a) Frequency of damage nucleoids in comet assay; (b) percentage of damage and (c) arbitrary units. Means in the bars followed by letter *a* are statistically equal to water, means followed by letter *b* are statistically equal to the solvent, averages followed by letter *c* are statistically equal to glyphosate, according to Dunnett's test ($p<0.05$).

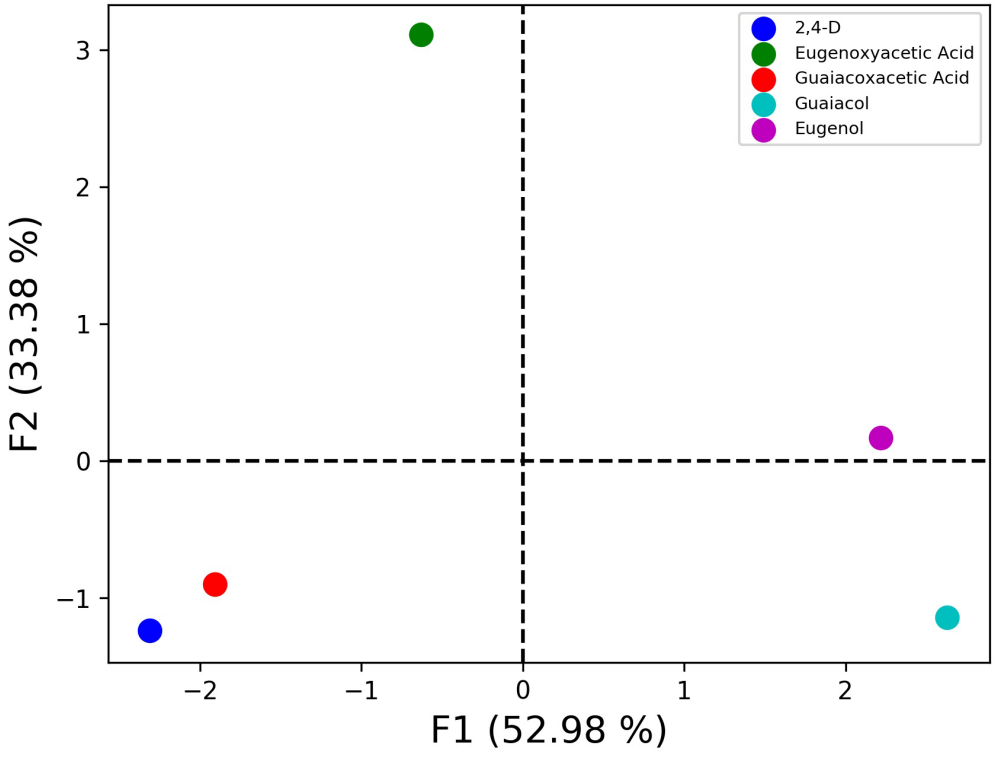

Figure 8. Correlation of the first (F1) and second (F2) PCA components of the studied compounds.

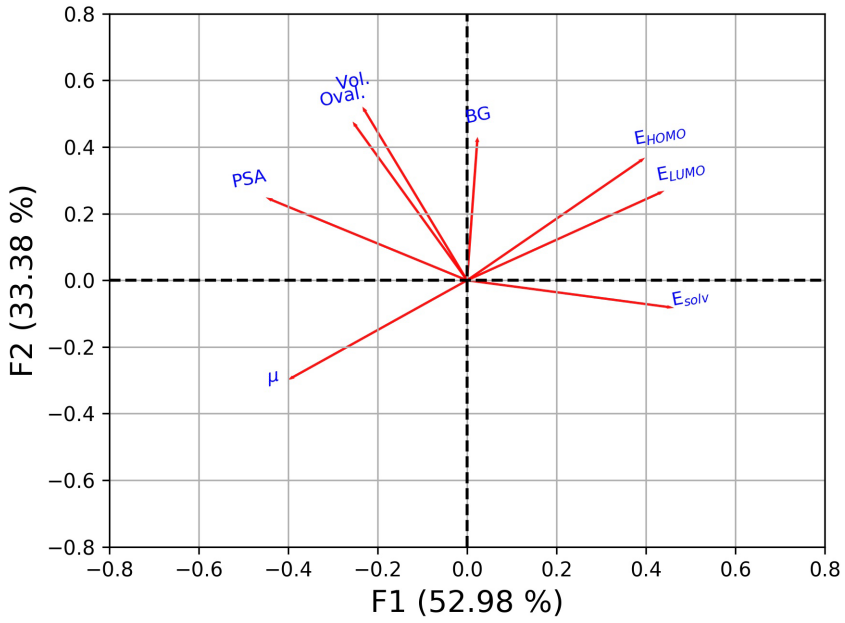

Figure 9. Dispersion graph of the molecular properties obtained from PCA as a function of the components 1 (F1) and 2 (F2).

used in the future as a selective semisynthetic herbicide.

Both eugenol and guaiacol presented aneugenic effect, demonstrating their action in the mitotic spindle. However, eugenoxyacetic acid also showed a clastogenic effect, promoting damage directly in the DNA of the organism.

The four molecules tested in the present study mainly induced type 1 damage in the lettuce and sorghum DNA and were able to be repaired by the cell. In contrast, glyphosate induced types 3 and 4 damages, which cannot be repaired. Thus, the natural phenols eugenol, guaiacol and their respective semisynthetics, eugenoxyacetic acid and guaiacoxyacetic acid, have the potential to be used as herbicides.

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APPENDIX I

Table I. Molecular properties obtained at the theory level ωB97X-D/6-31G(d,p)[a].

	E_{solv} (kJ/mol)	E_{HOMO} (eV)	E_{LUMO} (eV)	BG (eV)	μ (debye)	Vol. (Å^3)	PSA (Å^2)	Oval. (Å)
2.4-D	-29.62	-8.27	1.18	9.45	4.28	180.88	40.287	1.33
Eugenoxyacetic acid	-26.87	-7.44	2.05	9.99	2.76	230.89	44.134	1.40
Guaiacoxiacetic acid	-25.38	-8.31	1.47	9.78	5.18	180.27	40.103	1.30
Guaiacol	-16.90	-7.55	2.21	9.76	2.75	132.76	24.238	1.21
Eugenol	-12.61	-7.42	2.14	9.56	2.51	183.53	24.273	1.32

[a]Solvation Energy (E_{solv}, in kJmol^{-1}), HOMO Energy (E_{HOMO}, in eV), LUMO Energy (E_{LUMO}, in eV), Band Gap (BG, in eV), Dipole Moment (μ, in debye), Molecular Volume (Vol., in Å^3), Polar Surface Area (PSA, in Å^2), Ovality (Oval, in Å).

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The authors declare that they have no conflict of interest. Thammyres A. Alves and Patrícia F. Pinheiro contributed equally to this work. Thammyres A Alves: Conceptualization, methodology, investigation, data curation and writing - original draft. Patrícia F Pinheiro: Conceptualization, methodology, investigation, data curation and writing - original draft and financing. Milene M Praça-Fontes: Conceptualization, resources, visualization, formal analysis, writing - review and editing, supervision. Larissa F Andrade-Vieira: Conceptualization, visualization, writing – review. Maicon P Lourenço and Mateus R Lage: Conceptualization, investigation and data curation. Thayllon A Alves and Franceli A Cruz: methodology, investigation and writing. José W M Carneiro and Adésio Ferreira: Conceptualization, formal analysis and writing. Taís C B Soares: Conceptualization, resources, visualization, formal analysis, writing - review and editing, supervision and financing.