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Additional Information

Toxicological implications of amplifying the antibacterial activity of gallic acid by immobilisation on silica particles: a study on *C.elegans*.

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Abstract

Immobilisation of natural compounds on solid supports to amplify antimicrobial properties has reported successful results, but modifications to physico-chemical properties can also imply modifications from a toxicological viewpoint. This work aimed to study the immobilising process of gallic acid in the antibacterial activity of *L. innocua* and its toxicological properties *in vivo* using *Caenorhabditis elegans*. The experiment was based on obtaining the minimum bactericidal concentration for free and immobilised gallic acid by comparing lethality, locomotion behaviour, chemotaxis and thermal stress resistance on *C. elegans* at those concentrations. The results showed a lowering minimum bactericidal concentration and modifications to nematode responses. Increased lethality and velocity of movements was observed. Immobilisation increased the repellent effect of gallic acid with a negative chemotaxis index. Thermal stress resistance was also affected, with higher mortality for immobilised gallic acid compared to bare particles and free gallic acid. Thus despite evidencing a generalised increase in the toxicity of gallic acid *in vivo*, lowering the minimum bactericidal concentration allowed a bacterial reduction of 99% with less than one third of mortality for the nematodes exposed to free gallic acid.

1. Introduction

Emerging problems of the negative impact of some synthetic antimicrobials on consumer health, abuse of antimicrobial substances due to inadequate traditional food-conservation methods and a rise in antibiotic-resistant bacteria and fungi strains all render the development of new strategies to prevent food spoilage and contamination necessary (Pisoschi et al., 2018). One of the main approaches in this area is to use naturally-occurring antimicrobial chemicals and to focus on employing natural compounds from sources like plants, bacteria, animals and fungi to ensure food-product safety given their antimicrobial activity produced against given pathogens (Saleem, 2014).

Specifically for plant compounds, properties such as antioxidants, antimicrobials, anti-diabetics, anti-carcinogens, flavourings, beverages and repellents have been demonstrated for several herb and plant extracts, which represent wide applicability in food manufacturing (Hygreeva et al., 2014). Although the origin of these compounds is natural, observations generate requirements for specific systemic research to test the toxicity and mechanisms of action in order to devise a complete safety mode of use and regulations. Thus not only should original compounds be tested, but also their possible physico-chemical modified versions. Research about modifications to natural compounds has focused on the change in their properties, such as solubility, dispersion across food matrices, avoiding volatility, etc., to amplify the antimicrobial effect and to cushion the impact on products' organoleptic properties (Weiss et al., 2009).

Immobilisation of compounds on solid matrices offers higher potential in relation to the above-mentioned aim. This process provides increased antimicrobial capacity from a small amount of compound compared to its free version. This modification allows higher local compound concentrations and, thus, enhances membrane disruption mechanisms. Based on this approach, silica particles have been used as a solid matrix to be coated by certain natural compounds with successful results. Some of those results have been reported by Li and Wang (2013), who worked with lysozyme-coated silica nanoparticles, and showed the efficient amplification of antibacterial activity against *Escherichia coli*. Qi et al (2013) demonstrated how silica nanoparticles reduced

gram-positive bacteria by coatings with vancomycin. Pędziwiatr-Werbicka et al (2014) synthesised fatty acid-functionalised mesoporous silica particles with antimicrobial activity.

One of the groups with a potential for this aim is phenolic compounds, which form one of the most numerous and ubiquitously distributed groups of plant secondary metabolites commonly found in diverse dietary products, particularly vegetables, fruit, chocolate and beverages (Soobrattee et al., 2005). Natural phenolic products can exhibit a wide range of biological effects, which include antibacterial, antifungal, antiviral. Gallic acid (GA) is one of the main compounds in that group that possesses antimicrobial properties in human pathogens, such as *E. coli, L. monocitogenes, S. aureus*, etc., according to several reported experiments (Borges et al., 2013; Jayaraman et al., 2010). These antimicrobial properties have also been evidenced in *in vivo* systems such the nematode *C. elegans* by Singulani et al., (2017), where GA protected against *C. albicans* infection. Saul et al. (2011) reported improvements to the life span and thermal stress resistance of these nematodes by exposure to GA. Hence the immobilisation of GA onto particles could modify these properties, as reported by Abdel-Wahhab et al (2016), who evidenced enhanced antioxidant and antimicrobial activities *in vitro* and *in vivo* by immobilising GA onto chitosan and silica nanoparticles. Therefore, possible toxicological effects must be taken into account when making these modifications.

The aim of this work was to study the immobilising process of GA onto silica mesoporous particles in the antibacterial activity of *Listeria innocua* and its toxicological properties in vivo using *Caenorhabditis elegans*

2. Material and Methods

2.1. Reagents

The chemicals N-cetyltrimethylammonium bromide (CTABr), tetraethylorthosilicate (TEOS), NaOH, triethanolamine (TEAH3), (3-Aminopropyl)triethoxysilane (APTES), gallic acid (GA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-

hydroxysuccinimide (NHS) were supplied by Sigma-Aldrich (Madrid, Spain). Acetonitrile, ethanol and microbiological media grade were provided by Scharlab (Barcelona, Spain).

2.2. Synthesis of MCM-41 microparticles

The synthesis of microparticulated MCM-41 particles was carried out following the so-called "atrane route", in which CTABr was used as the structure-directing agent. For this synthesis, triethanolamine (TEAH3) (25.06 g, 168 mmol) was stirred at room temperature for 5 min before adding NaOH (12 mmol) in deionised water (2 mL). The reaction mixture was heated to 120°C for 20 min. Next the solution temperature was adjusted to 70°C. TEOS (10.6 mL, 45 mmol) was added and heated to 120°C for 1 h. The solution temperature was adjusted to 118°C and CTABr (4.68 g) was slowly added. The reaction mixture was cooled to 70°C and deionised water (80 mL) was added with vigorous stirring. A white suspension resulted after a few minutes. This mixture was left to age at 100°C for 24 h. The resulting powder was collected by filtration and washed with water and ethanol. Finally, the solid was dried at 70°C. The as-synthesised solid was calcined at 550°C in an oxidant atmosphere for 5 h to remove the template phase (Ruiz-Rico et al., 2016).

2.3. Immobilisation of gallic acid on MCM-41 microparticles

In a typical synthesis, 1 g of bare MCM-41 microparticles was suspended in 30 mL of acetonitrile and reacted with 2 mL (8.5 mmol) APTES with stirring at room temperature for 5 h. The functionalised solid was recovered by filtration, washed with water and ethanol, and dried overnight at 40°C. For GA immobilisation, the APTES-functionalised MCM-41 microparticles were dispersed in 30 mL acetonitrile. Then 0.3 g (1.75 mmol) of GA was reacted with 0.2 g (1.75 mmol) NHS and 0.4 g (2 mmol) of EDC in 10 mL acetonitrile at room temperature for 15 min before being added to the MCM-41 microparticles suspension (Figure 1). The mixture was stirred at room temperature for 24 h, followed by filtration and washing with water and ethanol.
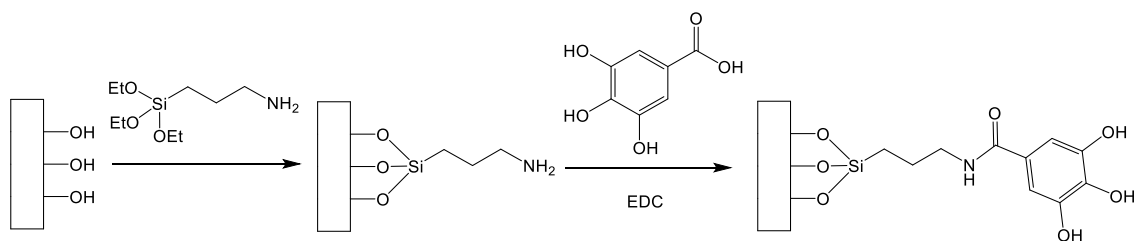
Figure 1. Representation of the synthesis procedure of the GA-immobilised silica MCM-41 microparticles (PGA).

2.4. Characterisation of bare and functionalised MCM-41 microparticles

Bare (P) and immobilised silica supports (PGA) were characterised to establish their zeta-potential, particle size distribution and degree of functionalisation. To determine the zeta-potential of the materials, a Zetasizer Nano ZS (Malvern Instruments, UK) was used. Samples were dispersed in water at a concentration of 1 mg/mL. Before taking each measurement, samples were sonicated for 2 min to preclude aggregation. The zeta-potential was calculated from the particle mobility values by applying the Smoluchowski model. The average of three recordings was reported as the zeta-potential. The particle size distribution of P and PGA was determined by a Malvern Mastersizer 2000 device (Malvern Instruments, UK). Water was the employed dispersion medium and each sample was measured in triplicate. To facilitate particle dispersion, samples were sonicated for 2 min to ensure the stabilisation of the particle size distribution. The degree of GA immobilisation of the PGA was determined by a thermo-gravimetric analysis (TGA) and an elemental analysis. The TGA were carried out on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Switzerland) in an oxidant atmosphere (air 80 mL/min) with a heating programme that consisted of heating steps at 5°C per minute from 25ºC to 1°C.

2.5. Microbial susceptibility assays

The *in vitro* antibacterial activity of both GA and PGA was evaluated against *L. innocua* using the macrodilution broth susceptibility test method according to CLSI guidelines (Clsi, 2013) *L. innocua* was chosen as a safe surrogate of the relevant food-borne microorganism *L. monocytogenes* to establish the potential of GA as a food preservative. The *L. innocua* strain (CECT 910) was obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). To prepare the inoculum, the cells from a colony of the microorganism grown on plate count agar (PCA) were transferred to 10 mL of tryptic soy broth (TSB) and were incubated at 37°C for 24 h to obtain an inoculum with a density of approximately 1 x 108 cells/mL of broth.

Increasing GA and PGA concentrations were evaluated by adding different amounts of the compound or the solid to 15 mL of TSB in Erlenmeyer flasks until the minimum bactericidal concentration (MBC) was obtained, which corresponded to the lowest antimicrobial concentration to give rise to a reduction of 99.9% in the initial bacterial inoculum. Bare particles (P) were also tested at equivalent concentrations following the PGA results. Flasks were inoculated with 10 μL of inoculum to provide an initial cell density of approximately 105 CFU/mL, and were incubated with orbital stirring (150 rpm) at 37°C for 24 h. After incubation, serial dilutions of samples were plated on PCA and were incubated at 37°C for 48 h. Viable cell numbers were determined with a detection limit of 5 CFU/mL. These values were logarithmically transformed and expressed as log CFU/mL. All the treatments were done in triplicate. Positive and negative controls were included in all the assays. PGA was expressed as mg/mL immobilised GA from the TGA analysis to make a better comparison with GA.

2.6. *C. elegans* preparation

Wild-type *Cahenorabditis elegans* Bristol strain N2 was employed as the biological model. The nematode population was reproduced on nematode growth medium (NGM) plates seeded with *E. coli* OP50 at 20°C (Brenner, 1974). Animals were synchronised by the standard bleach method (Stiernagle, 2006). The age-synchronised L4 individuals were used to perform the experiment.

2.7. Studies on *C. elegans*

2.7.1. Lethality

Lethality (M) was evaluated by the proportion of nematode survival after 24 h of exposure to increasing concentrations of GA, PGA and P following the results of the above-explained microbial susceptibility assays. Those concentrations were increased to determine lethal concentration 50 (LC_{50}). All exposures were carried out in the liquid medium K-medium (32 mM KCl, 51 mM NaCl) following the protocols of Zhou et al., (2016). Exposure was done in 3.4 mL well plates (Corning® 3738 Costar® 24-Well Flat Bottom) at 20°C in an incubator in the presence of food. One hundred nematodes were exposed per concentration. Untreated nematodes were included as the control group. After treatment, nematodes were counted as live or dead under a stereomicroscope by gently probing with a platinum needle. Responses were plotted on concentration-percentage mortality curves.

2.7.2. Locomotion behaviour analysis

The locomotion behaviour of nematodes was studied based on the features extracted from the analyses of the tracking videos captured after the lethality assays. A 1-minute video was run of each live nematode after 24 h of exposure. The device comprised a web cam controlled by a computer (Logitech C920 HD Pro Webcam, Logitech Europe S.A.), which was situated perpendicularly at 3.5 cm onto a white polarised light source, generated by a TFT-LCD 7" screen (NHD-7.0 OLED, Newhaven Display Intl.). Both elements were placed inside a light-isolated chamber (20x20x20 cm). Videos were captured on 300-µl microwells (V96 MicroWell® Clear Plates), where nematodes were individually placed after being selected from the lethality assay. Microwell plates were located between the camera and the light source for the recording process. Videos were captured at 30 fps in the RGB format at a resolution of 1080x720 pixels.

The behavioural analysis was performed based on the properties of movement during locomotion of each recorded nematode by means of discretising movements and extracting image descriptors

for the later analysis. The behavioural analysis was divided into four parameters, which collected information about locomotion metrics based on the displacement properties.

The objective of this block of descriptors was to collect the maximum information about displacement in terms of the amount of movement per treatment. The descriptors which collected that information were:

- m: summation of the total displacement in pixels

- t: time used to complete M

- V: velocity of movement during the recorded minute, calculated from M and t

- A_m: effective area of movement, calculated as:

$$Am = \left(P_x^{max} - P_x^{min}\right) \cdot \left(P_y^{max} - P_y^{min}\right) \qquad (1)$$

2.7.3. Chemotactic Index

The objective of this test was to know the effect of immobilising GA on the response of nematodes in attraction or repulsion terms. Chemotaxis assays were run following those described by Margie et al., (2013). Assays were carried out on 55 mm plates containing NGM. Each plate was divided into four equal quadrants and a circumference of radius 0.5 cm around the central crossing point was marked as the test origin. A point in each quadrant was marked to ensure that sites were equidistant (2 cm) from the origin and from one another. The top left and bottom right quadrants were the quadrants used to test the analysed compounds. The top right and bottom left quadrants were the controls. Firstly, 1 μl of 0.25M sodium azide and 1 μl of GA, PGA or P were added to the points of the test quadrants, while only sodium azide was added for the control quadrants. Immediately afterwards, 2 μl of the K-medium with nematodes (about 60 worms) were pipetted to the origin circumference. Plates were incubated at 20°C for 2 h and then the number of worms in each quadrant was scored. To calculate the Chemotaxis index (CI), Equation 2 was used:

$$CI = \frac{nT - nC}{N} \qquad (2)$$

where nT is number of nematodes in the quadrants with the tested compound, nC is the number of nematodes in the control quadrants and N is the total of the scored nematodes. The used concentrations were in accordance with the results of the above-explained microbial susceptibility assays. Each concentration was tested in triplicate.

2.7.4. Thermal stress resistance

The effect of immobilising GA on the thermal stress resistance of *C. elegans* was tested following the considerations reported by Zevian and Yanowitz (2014). The L4-synchronysed nematodes were exposed in K-medium with the presence of food at 20°C in 3.4 mL well plates (Corning® 3738 Costar® 24-Well Flat Bottom) for 6 h at increasing concentrations of the GA, PGA and P, following the results of the above-explained microbial susceptibility assays. Nematode mortality was scored every 30 min until 100% mortality registered for the control nematodes. The untreated nematodes were included as the control group. The results were plotted on curves, and expressed as the percentage of mortality against time and concentration. Each concentration was tested in triplicate using at least 60 nematodes per replica.

2.7.5. Quantifying the immobilisation effect at the MBC

The effect of the immobilisation process of GA at the MBC was measured by comparing the results from the different assays done on *C. elegans* between MBC GA and MBC PGA. A comparison was made by calculating the increments between both concentrations for all the parameters measured in *C. elegans*. Figure 2 depicts an example of the concept used to interpret these parameters.
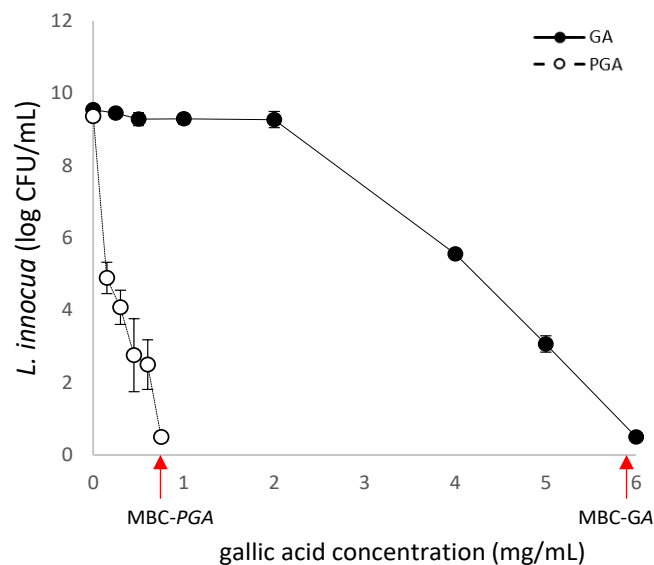
Figure 2. Microbial count (log CFU/mL) of L. innocua after incubation with gallic acid GA, immobilised gallic acid PGA. Red arrows mark the minimum bactericide concentration MBC. Results expressed as mg/mL of gallic acid. Bars mean standard deviations, n=3.

2.7.6. Statistical analysis

The results of particle characterising and the data from the *C. elegans* studies were studied by one-way variance (ANOVA). The *C. elegans* studies were focused at MBC-GA and MBC-PGA data. In the cases in which the effect was significant (P-value < 0.05), the average was compared by Fisher's least significant difference (LSD).

3. Results

3.1. Synthesising and characterising the GA-functionalised silica particles

The characterisation results, including particle size distribution, zeta-potential and GA content of the bare and functionalised materials, are shown in Table 1. P and PGA displayed a size distribution within the microscale size range with non-significant differences between samples. As regards the zeta-potential, P fell within ca. -30 mV due to the presence of silanol groups on the support's surface. The zeta-potential of the PGA particles significantly differed from the non-

modified silica particles (p<0.05) because of the covalent grafting of the GA-alkoxysilane derivative. Finally, the GA content attached to the silica particles was determined by elemental and thermogravimetric analyses, which resulted in ca. 0.06 g GA/g SiO_2. This value was used to calculate the amount of the support required to evaluate the equivalent concentrations of the free and immobilised GA.

Table 1. Characterisation parameters of the bare and GA-functionalised silica particles: particle size distribution ($d_{0.5}$), zeta-potential and content (α) in grams of GA acid per gram of SiO_2.

	Size $d_{0.5}$ (μm)	Zeta-potential (mV)	α_{GA} (g/g SiO_2)
P	0.785±0.014[a]	-27.6±2.0[a]	-
PGA	0.627±0.161[a]	-12.7±5.3[b]	0.0601

Different letters in rows indicate significant differences between samples (p<0.05)

3.2. Antimicrobial activity of the free and immobilised GA

The *L. innocua* counts after the 24-hour treatment with GA and PGA are shown in Figure 2. PGA was represented as mg/mL of the immobilised GA from the TGA analysis for a better comparison with GA. The influence of P on bacterial viability was also evaluated. GA showed anti-listerial activity, with total microbial growth inhibition being accomplished at a concentration of 6 mg GA/mL. After immobilisation, the MBC drastically lowered (0.75 mg GA/mL), which demonstrates the enhancement of the inhibitory properties of the phenolic compound due to covalent grafting on the siliceous support. Conversely, the incubation of *L. innocua* in the presence of equivalent P concentrations had no effect on bacterium growth (data not shown). These GA concentrations were the basis for exposing nematodes: 0.25, 0.5, 1, 2, 4, 5 and 6 mg/mL for GA; 0.5, 1. 2 and 4 mg/mL for PGA.

3.3. Effect of free and immobilised GA on *C. elegans*

3.3.1 Lethality

The dose-response curves are represented in Figure 3. Curves showed a relation between the GA concentration (Figure 3A) and P (Figure 3B) vs. nematode mortality M (%). PGA was represented

as mg/mL of immobilised GA from the TGA analysis to make a better comparison with GA. The three tested compounds brought about a significant increase in nematode mortality, with observed differences among them. Note that concentrations increased to 10 mg/mL and 2 mg/mL for GA and PGA, respectively, to reach LC_{50}. GA had a mortality of 10% at 2.5 mg/mL, and LC_{50} was observed at 9.2 mg/mL. For PGA, evolution displayed minor differences with GA up to 1.25 mg/mL, from which point PGA displayed an increasing slope, with significantly large differences in mortality (around 21%), although LC_{50} did not rise. In this case, LC_{50} was not observed because of the reduced proportion of GA obtained in the functionalised particles. This could be solved by increasing the immobilisation yield during synthesis by thus providing a higher GA concentration per functionalised particle mass unit. So the theoretical LC_{50} was calculated by extrapolating a linear adjustment on the PGA curve to cross 50% mortality. Figure 3 shows that by following that curve (red lines), the obtained theoretical LC_{50} was 5 mg/mL, which is lower than that observed for GA. Note that in both cases, the MBC came before LC_{50}, and MBC-PGA gave the least statistical significance. P had less impact on mortality with around 10% from 20 mg/mL (the equivalent concentrations of the solid matrix used for PGA), with no variation noted for the next concentrations. The LC_{50} value was not reached in this case. This effect denotes the importance of the relative size of a given biological system for particle toxicity as the presence of P alone produced a mortality baseline in nematodes which was not observed in *Listeria innocua*. This fact made the differentiation between the effects of the solid particle itself and the immobilised GA difficult on the mortality observed for PGA by taking only this analysis into account.
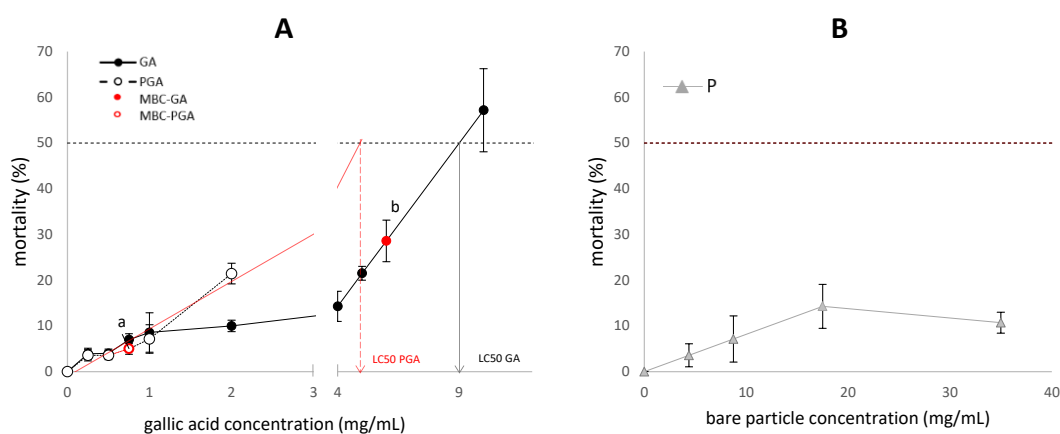
Figure 3. Lethality assay. A: Dose-response curves for the concentrations of GA and PGA; B: Dose-response curves for P. The dotted black line indicates 50% mortality. The red line shows the extrapolation to 50% mortality from the PGA curve. Vertical lines mark LC50. Bars represent standard deviation. Different letters indicate significant differences between values at MBC-GA and MBC-PGA (p<0.05).

The results revealed that, unlike *Listeria innocua*, nematode mortality caused by the immobilisation process was not modified at concentrations below 1.25 mg/mL, although toxicity seemed to become increasingly significant from that concentration. The relatively high LC_{50} for GA agreed with that previously reported in the *C. elegans* literature, which shows reduced toxicity and protective effects for chemical and biological contaminants, as well as improved life span. This fact has also been observed in acute toxicity in other *in vivo* biological systems. In assays done with rabbits, the reported LD50 was 5 g of GA/Kg/24h orally. The same value has been described for rats, but was subcutaneously administered (Greenstein, 2014). Techer et al., (2015) reported a low LC_{50} of 0.7 mg of GA/mL for zebra fish, but after 96 h of exposure. In that case, no deaths were recorded after the first 24 h of exposure. So it would seem that the empowerment of GA immobilisation in terms of nematode mortality remained, albeit on different scales compared to the tested bacteria. By taking into account the observed MBC, immobilising GA increased the bactericidal capacity at those concentrations, with a mild effect on nematode mortality. The increased mortality between MBC GA and MBC PGA is included in Table 2, which marks -85.7%.

Table 2. Increments between data from the C.elegans studies at MBC-GA and MBC-PGA:

	MBC	M	m	Am	V	CI	Th
Δ(%)	-87.5	-85.7	740.0	59.2	99.3	10.3	455.6

M: lethality; m: summation of the total displacement in pixels; Am: effective area of movement; V: velocity of movement; CI: chemotaxis index; Th: thermal stress resistance.

3.3.2. Locomotion analysis

The locomotion analysis results after 24 h of exposure are provided in Figure 4. The variables from the locomotion metrics, e.g. summation of total displacement (M), effective area of movement (Am) and velocity of movement (V), are represented vs. concentration in Figure 4A, 4B and 4C, respectively. The MBC for both the GA presentations displayed major differences between them for all the parameters, while MBC GA gave similar values to the control mainly for Am and V. Moreover, the increments between both MBC values (Table 2) showed an increase in all the parameters with the immobilisation process. The highest was m, multiplied by 7, followed by V with a 99.2% increase and Am with a 59.2% increase.

These differences were observed in the common tendency, namely a significant increase in all the parameters compared to the control nematodes (red dotted line), principally at low concentrations, although the tendency was for values to lower following increasing concentrations. P (Figure 4E, 4F and 4G) had the least effect on those parameters, principally with M. This evidenced that the immobilising process generated differences in the effects of particles, which were not easy to observe in the previous mortality assay.

GA and PGA showed similar evolution at the low concentration for M and Am, and in the same way as that observed in the lethality plots. For V, this effect was not conclusive. Exposure to both GA and particles produced greater nematode displacement in both distance and area terms at low concentrations. These results meant that the presence of particles had a stimulant effect, also because GA was both free and immobilised. Moreover when concentrations increased, locomotion reduced, probably because of the harmful effect of compounds on the physical capacities of nematodes, principally for GA and PGA. PGA showed a strong reduction for Am at 2 mg/mL, which evidenced an increase in the immobilised GA effects also on the displacement properties of nematodes. The observed enhanced activity with GA matched those previously described in several reports about the effects of other polyphenols and plant extracts on

nematodes. These detected activities include increased velocity displacement, pharynx pumping, body bends, etc. (Saul et al., 2011; Yuan et al., 2018).
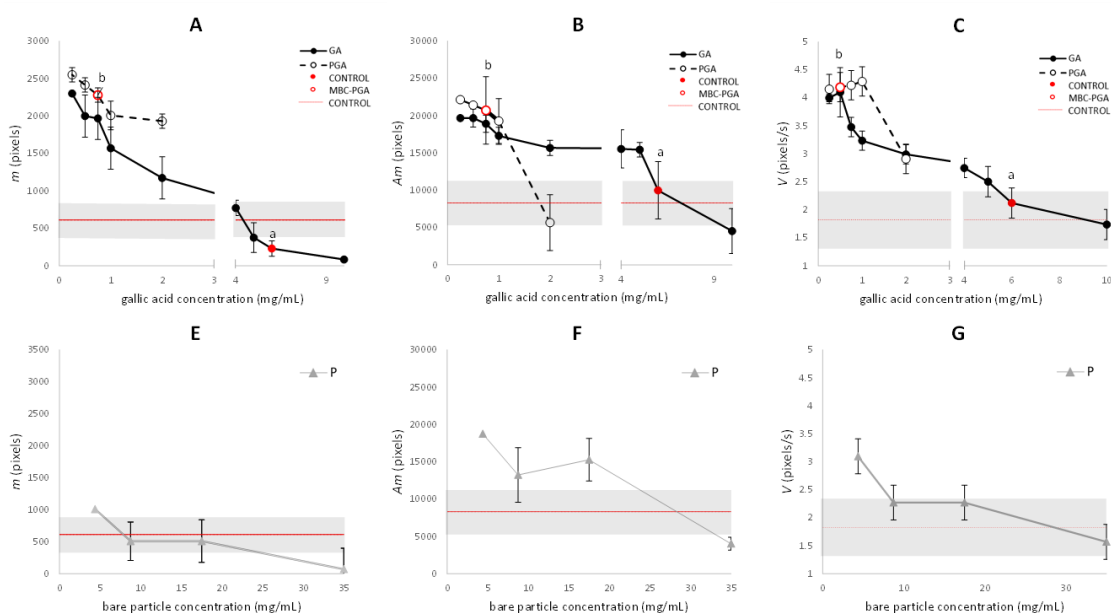

Figure 4. Locomotion analysis. Results for GA and PGA: A: summation of total displacement in pixels (m); B: velocity of movement (V); C: effective area of movement (Am). E, F and G represent the same parameters for P. Control: red lines. Red dots and circumferences: MBC GA and MBC-PGA, respectively. Bars represent standard deviation. Different letters indicate significant differences between values at MBC-GA and MBC-PGA (p<0.05).

3.3.3. Chemotaxis index

In this case, the MBC concentrations did not show any significant differences in the CI for both GA presentations (Figure 5), but the calculated increment marked 10% (Table 2). Moreover, *C. elegans* displayed a significant behavioural response because of the presence of P, PGA and GA. This meant that all compounds with some differences repelled nematodes. P had a CI value of around -0.1 from the concentration of 5 mg/mL, and this value continued until the maximum concentration. Likewise, GA had no repellent effect at concentrations below 2 mg/mL, and its CI value rose to -0.15 at 10 mg/mL. With PGA, the repellent effect was observed from 0.3 mg/mL of GA, with a marked slope until a CI value -0.37 for 2 mg/mL GA. Thus GA had a significant

repellent effect on nematodes in the free state. This effect inversely worked in the study by Saul et al., (2011), who described an attractant effect for GA. This difference could be because those authors provided GA by means of enriched bacteria, but not in an isolated form, and the presence of food had an attractant effect (Margie et al., 2013). Immobilisation increased that effect at all the concentrations, with CI values more than doubling for them all.
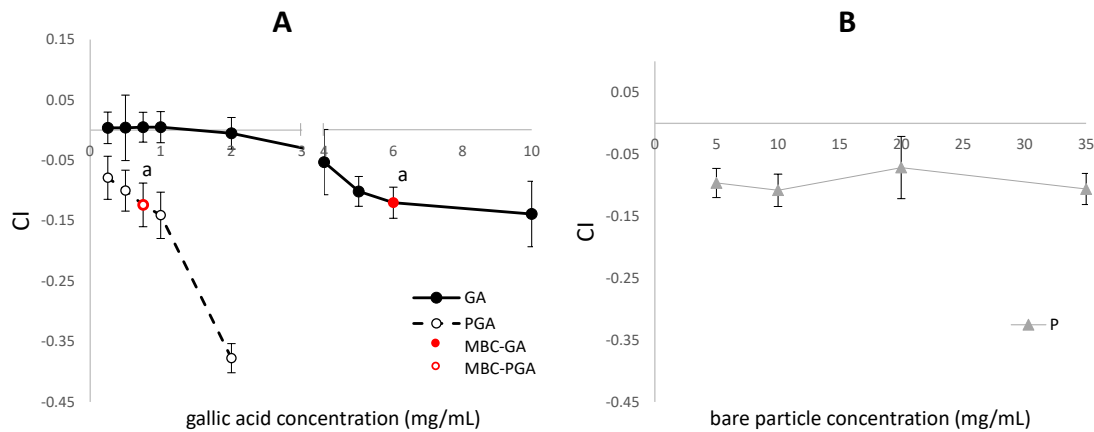

Figure 5. Chemotaxis Index (CI). A: GA and PGA results; B: P results. Bars represent standard deviation. Different letters indicate significant differences between values at MBC-GA and MBC-PGA (p<0.05).

3.3.4. Thermal stress resistance

The thermal stress resistance assay results are shown in Figure 6. The effects of GA, PGA and P are represented in Figure 6A, 6B and 6C, respectively. Heat shock had no effect on survival until around 110 minutes for the control nematodes, with 100% mortality appearing at around 180 min. This behaviour was modified with the presence of both the free and immobilised GA and bare particles, but in different senses. GA brought about a reduction in mortality of more than 50% at 180 min, while PGA increased mortality from 80 min for the minimum concentration (0.3 mg/mL) and from 20 min for the others. With P, mortality increased from 20 min to 20 and 40 mg/mL, while no effect was observed for the 5 and 10 mg/mL concentrations. In this case,

immobilisation led to increased toxicity, which diminished the thermal resistance of nematodes. MBC PGA multiplied MBC GA mortality by 4 at 180 min (Table 2).

Accordingly, the protecting effect shown by GA seemed to be lost with immobilisation and increased toxicity under these conditions. The results showed a protective effect of GA on thermal stress, but increased the single toxic attributes of both particles and GA when combined. The effect of GA agrees with previously reported results of the effects of polyphenols on the thermotolerance of *C. elegans* (Wilson et al., 2006). In a study about some effects of polyphenols on *C. elegans* properties, Saul et al., (2011) reported an increase in the thermal stress resistance of nematodes when GA was administered. These authors reported an increase to 160% survival at 35ºC after 8 h for the GA concentration of 1.36 mg/mL. In our case, this effect displayed changes because of immobilisation in particles. The difference between GA and PGA could be explained by the internalisation of the free compound by the nematode's organism. The protection effects derived from the GA properties inside tissues and cells, which was not possible for PGA because of immobilisation. Then amplified toxic effects took place because that process was maintained and increased due to stressful heat conditions.
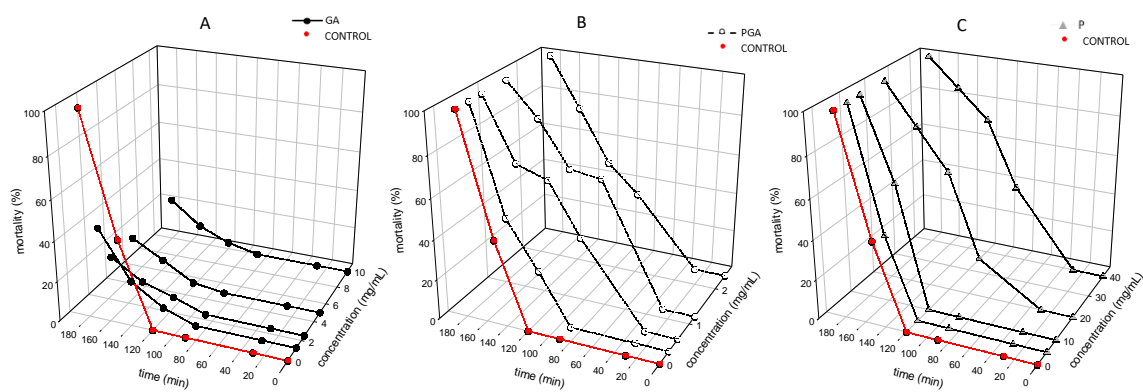

Figure 6. Thermal stress resistance. A: GA; B: PGA; C: P. Different letters indicate significant differences between values at MBC-GA and MBC-PGA (p<0.05).

3.4. Discussion

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The characterisation of PGA confirmed the immobilisation of the phenolic compound, and microparticles were obtained within a similar size range to the evaluated bacterium, in which the antimicrobial effect was due to the cell envelope and the bioactive molecule attached to the particle's surface coming into contact. This premise was confirmed by the bacterial susceptibility assays, which resulted in the inhibition of the microorganism after incubation with both free and immobilised GA, but not with bare particles.

The anti-listerial activity shown by GA was similar to the results obtained in previous studies with an MBC of ca. 6 mg/mL (Borges et al., 2013, 2012). The antimicrobial activity of phenolic compounds involves several modes of action, including the destabilisation and permeabilisation of the cytoplasmatic membrane, protein denaturation and nucleic acid synthesis inhibition. Nevertheless, GA showed poor antimicrobial activity than the other biocidal molecules, which justifies our attempt to improve inhibitory properties by immobilisation. PGA displayed greatly enhanced antimicrobial activity compared to the free molecule, which resulted in complete *L. innocua* inhibition after treatment with GA concentrations below 1 mg/mL. The enhanced antimicrobial activity of naturally-occurring antimicrobial compounds immobilised on silica particles has been recently described. Vico et al. (2016) reported silica nanoparticles functionalised with GA that resulted in enhanced stability and increased antimicrobial activity against *Paenibacillus* larvae. These authors suggested that an improvement in GA antimicrobial activity could be due to GA internalisation being favoured by nanoparticles, which allows higher intracellular local GA concentrations to enhance membrane disruption mechanisms. However in our case, the use of microparticles suggests that the antimicrobial effect may be displayed on the surroundings of the external cell envelope. Similarly, Ruiz-Rico et al. (2017) demonstrated the maintenance and improvement of the antimicrobial activity of essential oil components covalently immobilised onto the surface of silica supports with different particle sizes, and with distinct textural properties and chemical reactivity.

The toxic phenomena seemed similar in nematodes. However, note that GA has been previously reported to be a beneficial compound for *C. elegans*, which leads to life span extension and

thermal stress protection. In this case, GA and PGA showed significant differences for some assays, perhaps because of the compound's internalising. GA diluted in the medium could be absorbed by nematodes to tissues to then produce chemical activity which, depending on the concentration, can have either favourable or unfavourable effects for the system. Moreover, immobilisation blocked the possible compound absorption to nematode tissues and then high local concentrations were maintained, which reduced any protective effects and increased possible toxic ones. So the observed toxicity could be assumed from two points of view due to the immobilisation process: chemical activity in cells because of the internalising of excessive free compounds and the effect of a higher local concentration. The lethality assay showed this difference at 2.5 mg/mL, where PGA started a different evolution because that GA concentration produced high mortality when immobilised. In the behaviour study, this effect was observed at the same concentration for the reduction in Am from PGA. It agrees with the observed negative CI for PGA. The higher local compound concentration produced a repellent effect that generated movements in a small area. The postural spectra differences moved in the same way. The PGA spectrum presented fewer differences to the control than GA, probably because the immobilised compound could not affect that property, unlike internalised GA.

The thermal stress assay also matched that hypothesis. GA internalisation conferred nematodes protection by reducing mortality, as mentioned above. In this case, the exposure time sufficed to avoid lethality, but enhanced the protective effect. This protection was not possible for PGA because the immobilised compound only came into contact with nematode surfaces. The effect in that case was negative given the synergic effect of the high local compound concentrations and heat. Membrane damage could be amplified when particles come into contact with tissues at high temperatures, probably for the same reasons as bacteria damage on the cell scale.

4. Conclusion

Amplifying the antimicrobial effect of GA by immobilisation on silica particles led to significant changes in the toxicological response of *C. elegans* compared to the same free compound. PGA

increased mortality and its theoretical LC_{50} was half that of GA. The locomotion behaviour of all the parameters was also affected. Thus we conclude that stress increased as a result of combining rising total displacement, high velocity and the effective area, compared to lesser equivalent GA concentrations. Moreover, immobilising GA eliminated the protective effect of this compound under thermal stress conditions, with higher toxicity shown than for the bare particles in these circumstances. Thus the immobilisation process amplified the effects of GA for both *L. innocua* and *C. elegans,* but on different scales, which implies that a the reduction in the MBC reduced nematode mortality from 28% to 6% with the same reduction of 99.9% in the initial bacterial inoculum. Therefore, modifying GA by immobilisation on solid supports implies significant alterations to toxicological parameters *in vivo* which have to be taken into account when the aim of this technology is to develop new alternative antimicrobials.

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6. Bibliography

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