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Dual role of Tannic acid and pyrogallol incorporated in plaster of Paris: morphology modification and release for antimicrobial properties.

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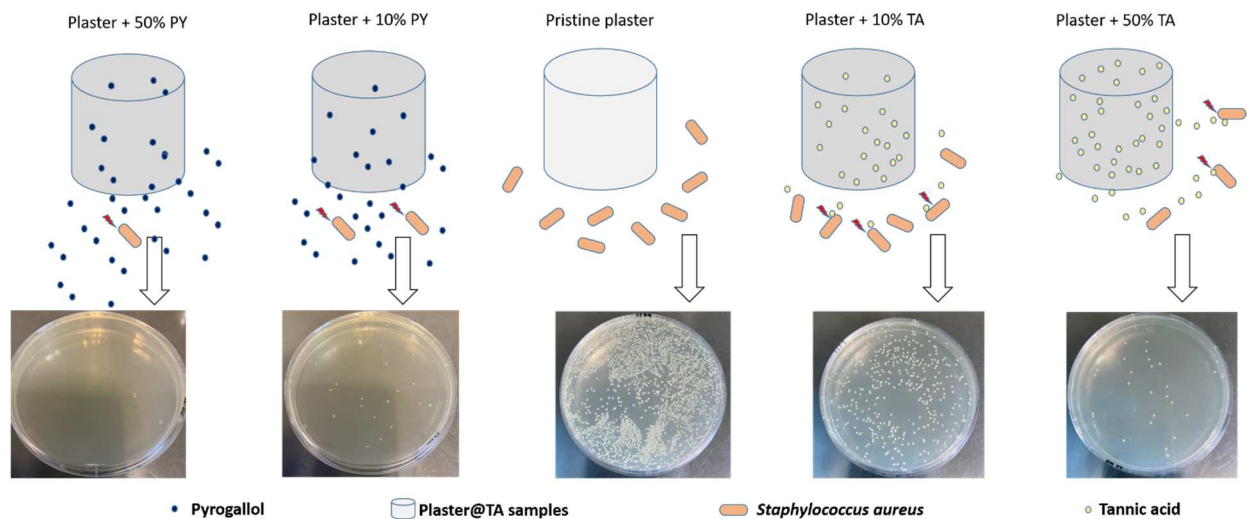
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Abstract:

The design of bioactive plasters is of major interest for the amelioration of dental and bone cements. In this article, a one pot and environmentally friendly strategy based on the addition of a cheap polyphenol-tannic acid (TA) or the main phenolic constituent of TA, namely pyrogallol (PY)- able to interact with calcium sulfate is proposed. Tannic acid and pyrogallol not only modify the morphology of the obtained plaster+TA/PY composites but a part of it is released and provides strong-up to twenty fold- antibacterial effect against *Staphylococcus aureus*. It is shown that the higher antibacterial efficiency of PY is related to a greater release compared to TA even if in solution the antibacterial effect of PY is lower than that of TA when reported on the basis of the molar concentration in PY units.

Keywords: plaster, tannic acid, antimicrobial composites, *Staphylococcus aureus*, morphology change.

Graphical abstract



1. Introduction

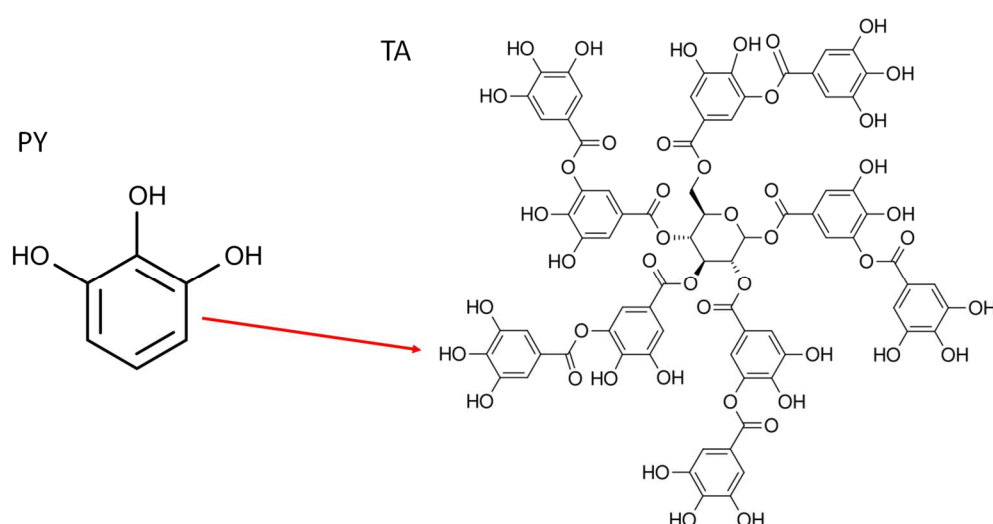
In biomaterials science, plaster of Paris, obtained through the controlled hydration of calcium sulfate hemihydrate, is used as a material in the immobilization of bone fractures [1] and bone repair materials [2-6]. It is biocompatible, stable, easy to use, inexpensive, bioresorbable and sterilizable [7, 8]. Overall plaster of Paris is an excellent material for medical applications [9].

In the past, plaster was used as a dental material to repair crestal perforations. On its own, plaster does not present any antimicrobial activity; however, several studies analyzed the effect of antibiotic loading in plaster for bone infection treatments. They demonstrated that plaster can be used as a filling and a bone grafting material [10-12]. Therefore, the addition of antibacterial and antibiotic agents to the plaster of Paris becomes an interesting and easy approach. Some antibiotics were used in the calcium sulfate formulations as gentamicin, vancomycin and tobramycin [13, 14]. However, antibiotics are known to become progressively less efficient due to adaptation of the targeted bacteria. Hence, the use of natural molecules synthesized by plants as potential antibacterial agents becomes of the highest interest. Tannic acid (TA) is a natural polyphenol that can be found in and extracted from red wine and various plants, tealeaves and fruits [15-17]. It has been incorporated within many materials due to its antioxidant properties, anticancer and antibacterial activity [18, 19]. Reitzer *et al.* [20] added TA to formaldehyde crosslinked gelatin gels and proved the antibacterial activity of the obtained material against *Staphylococcus aureus* (*S. aureus*), at the origin of many infections in the human body [21] and against *Escherichia coli*. *Staphylococcus aureus* (Gram-positive) is one of the major causative bacteria of osteomyelitis and bone infections [22, 23]. Several studies [24, 25] used some antibiotic agents (ampicillin, tetracycline, gentamicin...) in order to modify the plaster of Paris to treat the periprosthetic joint and wound infections. They demonstrated that TA is incorporated in the gelatin gels under two distinct populations, one free and the other one stabilized by the gelatin network as expected on the basis of hydrogen bonds and hydrophobic interactions between both components [20]. The weakly bound fraction of TA can be released from the gel and serves as an antimicrobial agent.

When considering the structure of TA, it appears as a glucose unit ending with ten exposed pyrogallol molecules (Scheme 1). The degradation of TA molecule involves

some steps containing the formation of glucose, gallic acid, resorcinol and pyrogallol [26]. Pyrogallol (PY) (1,2,3-trihydroxybenzene) is a polyphenol that is known for its antimicrobial properties [27, 28]. The obtained results supported that the presence of its hydroxyl groups is responsible for its antibacterial activity [27].

The aim of this article is to compare PY and TA as organic additives to improve the antimicrobial properties of plaster without reducing its mechanical properties. Considering the need to produce antibacterial sealing materials, we investigated the incorporation of TA and PY in plaster or Paris. It is of interest to know if either individually added PY units or PY units linked on a central glucose unit would be more efficient with this respect. We focus our study on the antimicrobial properties of those new plasters and the consequences on the mechanical properties to insure their potential use as sealing material. Morphological changes (using scanning electron microscopy), compressive strength, compositional changes (using energy dispersive X ray analysis), and release kinetics of the active compounds from the plaster@TA and plaster@PY composites were investigated. The experimental variable was the weight percentage of TA/PY in the initial formulation, the amount of water being held constant. *In vitro* antibacterial activity of the cements was evaluated after 24 and 72h of TA/PY release for an intermediate composition in TA/PY as well as for the most concentrated composite.



Scheme 1: Structure of PY and TA. The arrow is aimed to represent a typical PY ending group on TA.

2. Materials and Methods

2.1. Materials

Calcium sulfate hemihydrate (“Fugii Rock EP Classic”, GC Europe N.V., Leuven, Belgium) was stored in a closed vessel before its use, to avoid uncontrolled hydration and setting. It has been modified with the addition of different weight percentages of TA (ref. 403040, Sigma Aldrich, Saint-Quentin-Fallavier, France, molecular mass: 1702.0 g.mol⁻¹) or PY (ref. P0381-250G, Sigma Aldrich, Saint-Quentin-Fallavier, France, molecular mass: 126.1 g.mol⁻¹) to the water used to set the plaster. The antioxidant activity of the prepared plaster based samples was evaluated using the discoloration of 2,2-diphenyl-1-picrylhydrazyl (DPPH, ref. D9132 from Sigma-Aldrich).

2.2. Sample preparation

The different compositions of the composites, which were made from the plaster modified with PY or TA, are described in Table 1. The experimental procedure to prepare the plaster and its composites is illustrated in the Supplementary data file (Scheme S1). All solutions of TA or PY were made from MilliQ water ($\rho=18.2$ m Ω .cm, Millipore, Molsheim, France) just before setting of the plaster. Note that the % of TA and PY corresponds to the weight of polyphenol relative to the weight of water used to set a constant weight of calcium sulfate hemihydrate. All groups of samples, called plaster@TA and/or plaster@PY blends in the following, were mixed according to the manufacturer’s instructions, keeping the same amount (g) of CaSO₄, ½ H₂O in the composition: namely a liquid/powder ratio (20 mL/100 g). Different dimensions of cylindrical teflon molds were used to make the plaster@TA and/or plaster@PY samples for scanning electron microscopy observations (SEM), compressive strength analysis and for the different antibacterial tests, including agar diffusion and direct contact tests (3.0 mm in diameter and 3.6 mm in height). Larger samples were made for x-ray diffraction analysis and water sorption tests (20.0 mm in diameter and 3.0 mm in height). Finally, molds of 15.0 mm in diameter and 8.0 mm in height containing 5.0 g of plaster with the different percentages of TA or PY and plaster were used for the release of TA/PY from the plaster in water. All these tests were performed after

the setting time of 40 min at ambient temperature following the manufacturer's instructions.

Samples	Pristine plaster	Plaster + 5%TA or PY	Plaster + 10%TA or PY	Plaster + 15 % TA or PY	Plaster + 30%TA or PY	Plaster + 50 % TA or PY
Calcium sulfate hemihydrate (g)	5.0	5.0	5.0	5.0	5.0	5.0
Water (mL)	1.0	1.0	1.0	1.0	1.0	1.0
Tannic acid (g)	0.0	0.05	0.10	0.15	0.30	0.50
Pyrogallol (g)	0.0	0.05	0.10	0.15	0.30	0.50

Table 1: Proportions (w/w % with respect to water) of TA or PY used in the preparation of plaster@TA and plaster@PY composites.

2.3. Scanning electron microscopy (SEM) and Energy dispersive x-ray (EDX) analysis

Plaster's samples were mounted on aluminium SEM stubs. The samples were sputter-coated with gold-palladium alloys (20/80) using a Hummer JR sputtering device (Technics, CA, USA). Later on, the morphology (magnification: 5000X) was analyzed using a Quanta 250 FEG scanning electron microscope (FEI Company, Eindhoven, The Netherlands) functioning with a 10kV acceleration voltage of the electrons.

The chemical compositions of the plaster@TA and plaster@PY samples were analyzed in a semi quantitative manner using the same device.

2.4. Compressive strength

The plaster powder with water or water with tannic acid (10%, 30% and 50%) or pyrogallol (10%, 30% and 50%) ($n=15$ for each tested group) were removed from their molds and checked under 10X magnification using a Zumax microscope (Zumax medical, Suzhou New District, China) for the presence of an eventual void or a fractured edge. All damaged samples were discarded before further mechanical

analysis. Then, the samples were stored into a closed box until testing. The samples were tested through uni-axial compression tests to determine the stiffness of the cement and the maximum load before rupture. Those tests were performed on the universal electromechanical tensile Instron 3345 (Norwood, MA, USA) device instrumented with a 1kN cell force (Class 0.5 following ISO 7500-1) and with a displacement sensor. The tests were performed at constant crosshead speed of 0.5 mm.min⁻¹. The engineer compressive strength was calculated in megapascals (MPa) according to the formula: $\sigma_c = 4P / \pi D^2$; where P is the recorded load during the test and D is the initial sample diameter. A One Way Analysis of Variance test was applied using Sigma plot (release 11.2, Systat Software, Inc., San Jose, CA, USA) to determine whether significant differences existed in the compressive strength values between the different composites. In all tests, a statistical significance level of $\alpha = 0.05$ was adopted.

2.5. X ray diffraction

The XRD measurements were carried out on a D8 Discover Bruker powder diffractometer in the Bragg-Brentano geometry. The diffractometer was equipped with a front monochromator (CuK α 1 wavelength $\lambda = 0.154\ 056$ nm). The samples were ground to powder to reduce preferential orientation of the crystallites observed in pressed samples. The diffraction patterns were refined by the Rietveld method using the Fullprof software [29]. The background, modelled as a polynomial function based on ten experimental points, was refined, as well as the zero shift. Preferential orientation was taken into account using a March model. The atomic positions of the crystal structures were kept fixed. The lattice parameters, crystallite sizes, isotropic Debye-Waller factors were refined for each phase, as well as their scale factor. This allowed to quantify the ratio of the different crystallized phases.

2.6. Water sorption tests by the plaster@TA and the plaster@PY composites

Three samples of pristine plaster, plaster with 10% TA and/or PY and plaster with 50% TA and or PY were used. They were dried in an oven at 37 °C for 24 h to reach the natural hydration of plaster, 2 water molecules per calcium sulfate unit. At higher temperatures the calcium sulfate dihydrate may transform in calcium sulfate hemihydrate.

Then, a contact angle measurement device (Attention Theta, Biolin Scientific, Göteborg, Sweden) was used to measure the adsorption time of a 5 μL drop of distilled water onto the cement surface. A drop of distilled water was dropped via a 0.7 mm diameter needle on the surface of each sample. A video was recorded (10 frames per second) by a horizontal camera in order to track the profile of the water drop and the absorption time of the water drop until its engulfment into the volume of the cement. This measurement is a qualitative indication of the cement's hydrophilicity.

Static contact angles could not be measured because of the absence of a steady state wetting.

2.7. Release kinetics of TA and of PY in water and pH measurement with of water in contact with the composites

Stock solutions of TA (0.1 $\text{mg}\cdot\text{mL}^{-1}$) and PY (0.1 $\text{mg}\cdot\text{mL}^{-1}$) were prepared in distilled water. These solutions were diluted gradually between 2 and 100 times with distilled water. Their absorption spectra were then measured using a double beam mC^2 spectrophotometer (SAFAS, Monaco) to establish a calibration curve allowing the quantification of TA and PY release from the plaster@TA and the plaster@PY composites. The measurement cuvette (Quartz, 1 cm path length) was filled with the TA or PY solutions, whereas the reference cuvette was filled with distilled water.

Two samples of each plaster@TA and plaster@PY material (containing 5.0 g of plaster with the different percentages of TA or PY and plaster) were immersed in 50 mL of distilled water using a glass bottle. After 1, 2, 4, 18, 24 and 72 h, 1 mL of each supernatant solution above the composites was taken (after vigorous shaking) using a pipette. Then, the absorption spectrum of 1 mL of each diluted solution was measured in the wavelength range between 200 and 700 nm. The diluted solution of the plaster of Paris without TA or PY, was used in the reference cell. After each measurement, 1 mL of distilled water was added into each glass bottle to maintain a constant volume above the plaster@TA and plaster@PY composites.

The pH measurements were performed at $24 \pm 2^\circ\text{C}$ after incubation of the composites with water, in the same conditions as for the release experiments, for 24h and 72h. Before each pH measurement, the pH meter was calibrated with standard solutions at pH 4, 7 and 10 (Hanna Instruments, Lingolsheim, France).

2.8. Antimicrobial activity

2.8.1 Bacterial strain

Staphylococcus aureus (*S. aureus*, ATCC 25923) was cultured according to the manufacturer's instructions using Muller-Hinton broth "MHB" (Le Pont de Claix, France). In all the following tests, the turbidity of MHB containing *S. aureus* (bacterial medium) was adjusted to $OD_{600(nm)}=0.5$.

2.8.2. Agar diffusion tests

The agar diffusion test were performed similarly to ref. [30] using six identical agar filled petri dishes containing 25 mL of Muller-Hinton agar. Three plates were used to evaluate the anti-bacterial activity of the different plaster@TA and plaster@PY composites. 100 μ L of the bacterial medium were spread homogeneously onto the petri dishes. Four wells in each petri dish, 3.0 mm in diameter and 3.0 mm in depth, were made with an adapted punch by removing the agar. The first three wells were filled with the prepared samples (part: 2.2.) of different plaster@TA and plaster@PY composites (pristine plaster, plaster + 10% TA or PY and plaster + 50% TA or PY) and the fourth wasn't filled to serve as a positive control. For the other three plates, the first well was filled with 21 μ L of 20% Chlorohexidine (CHX) (ref.18472510, Sigma Aldrich, Saint-Quentin-Fallavier, France). The second and third wells were filled with 21 μ L of 10% TA and 50% TA; 10% PY and 50% PY without plaster. The fourth well wasn't filled in order to serve as a control. All the agar petri dishes were incubated at 37°C for 24h under aerobic conditions. The same operator measured the inhibition zones after 24 h of incubation. The inhibition zones were determined as half the inhibition zone diameter minus the well diameter. The inhibition zone was measured in millimeters using a digital caliper (Dexter, Elkhart, Indiana, United States) and was recorded for each plaster@TA and plaster@PY composite. The experiment was repeated three times.

2.8.3. Direct contact tests

Each sample plaster@TA and plaster@PY (n=3) was put in a 2 mL Eppendorf tube (Trefflab, Degersheim, Switzerland). One milliliter of the bacterial medium was injected in each eppendorf. In the positive control, the bacterial medium was put into the Eppendorf tube without any composite. In the negative control, the Eppendorf tube was filled with 21 μ L of 20% Chlorohexidine. After that, the Eppendorf tubes

were incubated for 24h and then for 72h at 37°C under constant stirring. After each incubation period, ten microliters from each Eppendorf tube were assessed by 10-fold serial dilution up to 10^5 in MHB and then 100 μL of the dilution was spread homogeneously onto a plate. The plates were incubated at 37°C for 24h. After incubation, colonies on the plate were counted and their CFU/mL were determined.

2.8.4. Direct contact tests of bacteria in solution in the presence of TA and PY

These tests were performed in order to compare the antibacterial activity of TA ($1702.0 \text{ g}\cdot\text{mol}^{-1}$) and PY ($126.1 \text{ g}\cdot\text{mol}^{-1}$) as a function of the molar concentration in pyrogallol units. To express the molar concentration of TA in terms of PY units, its molar concentration was multiplied by 10 owing to its structure (Scheme 1). A TA solution was prepared by mixing 50 mg of TA powder with 50 mL of distilled water as well as a PY solution with 3.70 mg of PY powder with 50 mL of distilled water. Three dilutions (x10, x20 and x40) of the initial concentration were performed in order to analyze the antibacterial activity of both polyphenol solutions. In triplicate experiments, one hundred microliter of the initial concentration and each diluted solution were added to 900 μL of the bacterial medium which was adjusted to $\text{OD}_{600(\text{nm})} = 0.5$. After that, the Eppendorf tubes were incubated for 24h at 37°C under constant stirring. After the incubation period, 10 μL from each Eppendorf tube were assessed by 10-fold serial dilution up to 10^5 in MHB and then 100 μL of the dilution was spread homogeneously onto a plate. The plates were incubated at 37°C for 24h. After incubation, colonies on the plate were counted and their $\text{CFU}\cdot\text{mL}^{-1}$ were determined.

2.8.5 Biofilm accumulation of *S. aureus*

Three samples of each composite (pristine plaster, plaster + 10% TA or PY and plaster + 50% TA or PY) were compacted in teflon molds (8.0 mm in diameter and 1.0 mm in thickness) with glass slides underneath to provide a flat surface. After the setting time, each disc was placed in a well of a 24-well plate. 20 μL of MHB containing *S. aureus* ($\text{OD}_{600(\text{nm})} = 0.3$) was applied for each well which contained a specimen [31]. After 1h of incubation at 37 °C, 2 mL of MHB were added to each well. After 24h of incubation, the discs were rinsed gently with distilled water for 10s, then the samples were fixed by using a solution of 0.05 M glutaraldehyde in 4% cacodylate buffer for 8 h. After that, the samples were rinsed by using a 4 %

cacodylate buffer for three times, 5 min each. The samples were dehydrated in graded series of ethanol (35%, 50%, 70% and 95%) for 3 min each. After the graded series of ethanol solution, the samples were dried by using a chemical drying agent, Hexamethyldisilazane (HMDS). The samples were transferred from 95% ethanol into 1:1 solution of HMDS for 10 min, then transferred into 100% HMDS for two times, 10 min each. All HMDS steps need to be carried out in the fume hood overnight, owing to the high toxicity of this reagent.

After the dehydration and drying steps, the discs were sputter-coated with gold-palladium alloys (20/80) and observed by a SEM to determine the density, morphology and accumulation capacity of *S.aureus* biofilms on the different composites.

2.9 Antioxidant activity

One sample of each plaster@TA and plaster@PY material (containing 5.0 g of plaster with the different percentages of TA or PY and plaster) were immersed in 15 mL of DPPH (10^{-4} mol/L in 70 % ethanol). After 5 min, 1 mL of each supernatant solution above the composites was taken (after vigorous shaking) using a pipette. Then, the absorption spectrum of 1 mL of each solution was measured in the wavelength range between 300 and 600 nm. The solution which was in contact with the plaster of Paris without TA or PY, was used in the reference cell. Digital pictures of all DPPH solutions above the samples were also taken after 1 min of contact.

2.10. Stability of the plaster in the presence of water

Four specimens for each tested material were prepared as reported by Kharouf *et al.* [30] and analyzed by using the procedure described in ISO 6876:2001. Stainless steel ring molds (internal diameter of 20 ± 0.1 mm and a height of 1.6 ± 0.1 mm) were first cleaned with acetone in an ultrasound bath for 15 min and then weighed three times (accuracy ± 0.0001 g; Ohaus, Pioneer[®] Analytical, Nänikon, Switzerland) before sealer injection, and the mean value was recorded. They were placed on a glass plate, and the sealers were plugged into the molds avoiding air entrapment. After 2h, the filled molds were weighed again 3 times (accuracy ± 0.0001 g) before aging immersion periods. After the two immersion periods (24 and 72h) in 50 mL of distilled water, the specimens were removed from distilled water and dried at 37 °C for 24 h. Thereafter, the weight of each specimen was assessed three times, and the

mean was recorded as the final weight. The difference of mass between the initial weight (before immersion) and the final weight was recorded as a percentage to determine the solubility percentage of each sealer.

3. Results

3.1. Scanning electron microscopy (SEM) and Energy dispersive x-ray (EDX) analysis

The addition of TA or PY to water had a marked influence on the morphology of the obtained plaster (Fig. 1). A remarkable morphological changes was observed for the plaster + 50% PY samples. A pseudo-fibrillar aspect was detected on the surface and the plaster particles were covered by this structure. This pseudo-fibrillar structure was eliminated after the immersion of the sample in water for 24h (Fig S1 Supplementary data) already suggesting that part of the incorporated polyphenol may be released in water.

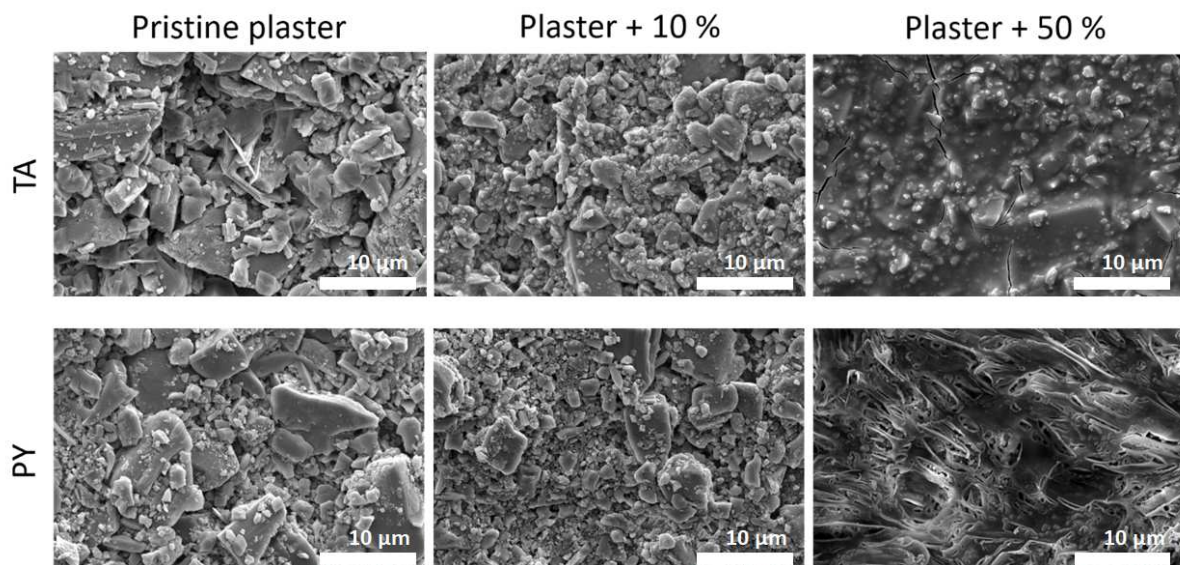


Fig. 1: Scanning electron micrographs of plaster set in the absence of TA or PY and for the plaster + 10% of TA or PY and plaster + 50 % of TA or PY formulations.

The incorporation of TA or PY in the blends led also, as expected, to a marked increase in the carbon content -the chemical signature of TA and PY with respect to plaster- with a concomitant decrease of the Ca and S (due to sulfate anions) content (Table 1).

Elements\samples	Pristine plaster	Plaster + 10%		Plaster + 50%	
		TA	PY	TA	PY
C (%)	5.6±0.6	10.4±4.2	10.6±2.3	30.1±7.4	26.5±5.5
Ca (%)	25.6±0.9	25.0±2.4	24.7±1.3	15.2±3.6	18.0±1.9
S (%)	20.2±1.5	20.4±1.7	22.0±4.5	12.4±1.3	15.6±2.7

Table 2: Relative composition of the obtained plaster samples as measured by means of EDX.

3.2 Compressive strength

Fig. 2 shows the compressive strength values of the pristine plaster and plaster@TA/PY (10, 30 and 50%). The compressive strength values showed no statistically significant difference between the pristine plaster values (58 ± 5 MPa) and plaster + 30%TA (48 ± 8 MPa) ($P > 0.05$). However, statistically significant lower compressive strength values were observed for plaster + 10%TA (11 ± 3 MPa), plaster + 50%TA (34 ± 7 MPa) and all plaster@PY composites compared to the pristine plaster samples ($P < 0.05$) (Fig.2).

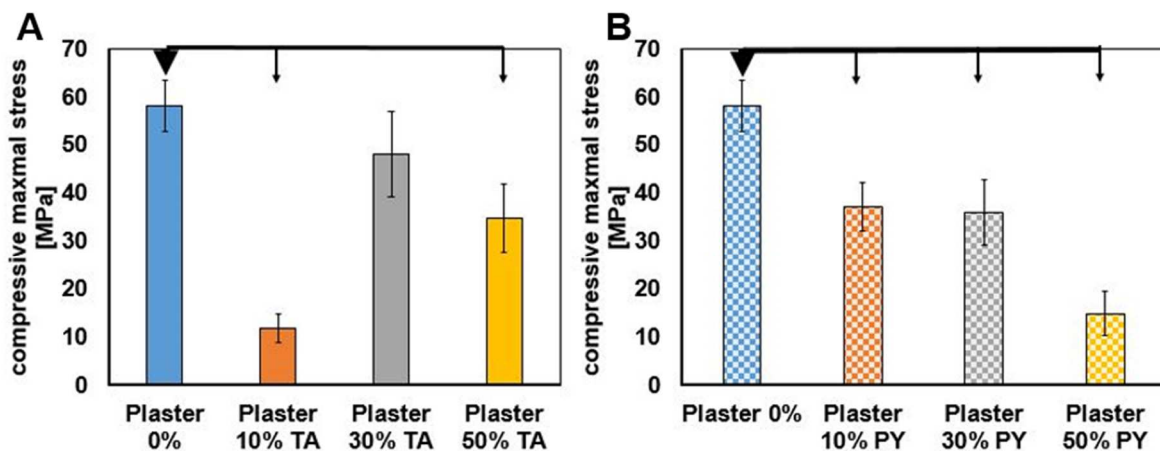


Fig. 2: Evolution of the maximal stress under compression for the pristine plaster, the plaster@TA (part A) and the plaster@PY (part B) composites. ($P < 0.05$ Bold arrows).

The minimal compressive stress value obtained for the plaster 10% TA composite is surprising but reproducible (over 15 independent experiments) and could be due to the interaction between TA and Ca^{2+} cations inducing a phase change. To validate or

to invalidate this assumption we performed some X-Ray diffraction experiments on all the investigated materials.

3.3. X ray diffraction

The X-ray diffraction pattern of pristine plaster can be indexed with two calcium sulfate hydrate phases from the ICDD database: Bassanite $2\text{CaSO}_4\cdot\text{H}_2\text{O}$ (ICDD 04-011-1765) and Gypsum $\text{CaSO}_4\cdot 2\text{H}_2\text{O}$, (ICDD 04-010-9409). No peak remains unindexed indicating that there are no other crystallized phases whatever the content in added TA (Fig S2, S3 and S4 in the supplementary data file).

The diffractograms of the plaster@TA composites are very similar to those of the pristine plaster, and the same conclusions concerning the absence of any other phase than Bassanite or Gypsum, or amorphous phase can be withdrawn (Fig. S5 in the supplementary data file). Each of these XRD patterns has been refined and the ratio of Gypsum and Bassanite is constant within the error bar at a ratio around 1 to 3 (Fig. S5 in supplementary data).

One cannot therefore explain the weaker compressive strength observed for the plaster-10%TA composite (Fig. 2A) by a peculiar modification of its structural properties with respect to the pristine plaster or plaster with other compositions in TA. One can note that the samples containing PY present XRD patterns with additional peaks (Fig. 3 and FigS3 in the supplementary data file). All those additional peaks can be indexed with the ICDD PDF file 00-008-0611 [32]. Hofer *et al.* [32] were not able to propose a crystal lattice and neither could we. The presence of an unknown crystal structure leads to unreliable quantification, therefore such an analysis was not performed on the plaster@PY samples.

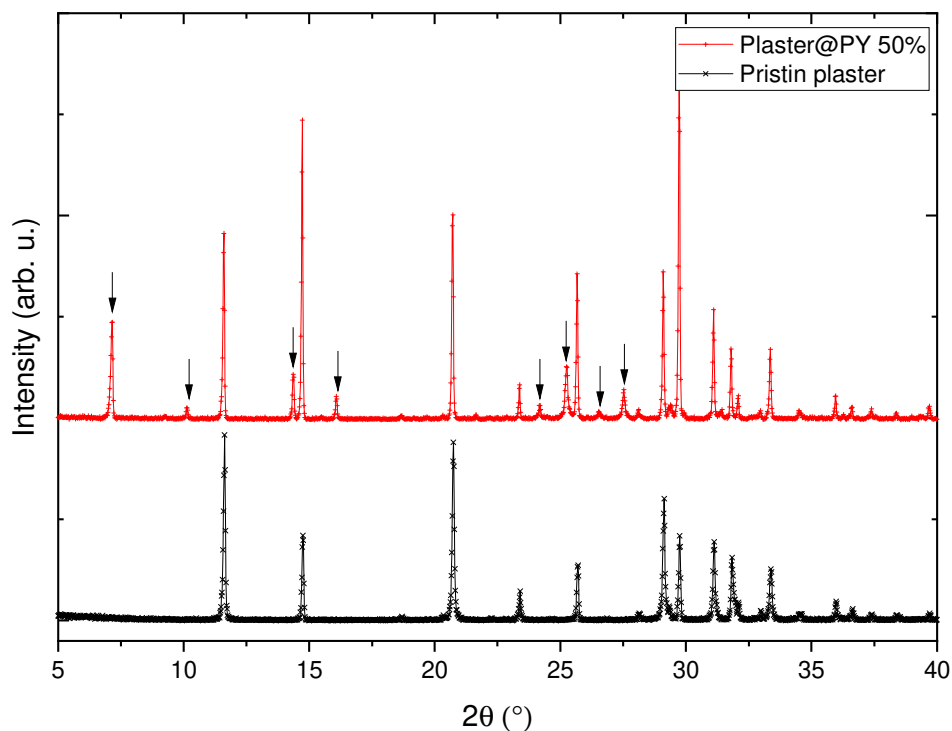


Fig. 3: XRD patterns of the pristine plaster (black) and plaster@PY50% (red). The black arrows emphasize the peaks only present in the PY composite, and which can be indexed with the ICDD 00-008-0611 PDF file.

3.4. Water sorption tests by the plaster@TA and plaster@PY composites

Even if the composition and morphology of the obtained plaster is markedly affected by the addition of TA and PY, the overall hydrophilicity of the material is not affected since a 5 μL droplet of water is engulfed by the plaster in less than 1 s up to 50 % (w/w) of TA and/or PY with respect to the used water (Fig. S6 in the Supplementary data file). The TA and PY independent hydrophilicity of the plaster is of main importance for its biological applications in particular for the release of weakly bound polyphenols which could then provide the composite plasters with antimicrobial properties.

3.5. Release of TA/PY from the plaster composites and pH changes of the water in contact with the composites

To investigate the release of TA or PY from the plaster blends, we first established a calibration curve by dissolving different concentrations of TA and PY (relying on weighing with an accuracy better than 0.5 mg) in water and measuring the

UV-vis spectra of the obtained solutions. Water, with a slightly acidic pH was chosen as a medium because the auto-oxidation of TA and of PY (but to a lower extent) are limited in these conditions. An absorption peak is observed at $\lambda=274$ nm for the TA solutions as described in the literature [33]; and at $\lambda=267$ nm for the PY solutions. The absorbance versus concentration curves displayed a linear relationship up to 0.05 mg.mL^{-1} with a linear correlation coefficient higher than 0.995 for both polyphenols (data not shown). Based on this calibration curve, we had to set-up an experimental design to measure the release of TA and of PY from the plaster@TA/PY blends in order not to reach concentrations higher than 0.05 mg.mL^{-1} . Indeed, for samples containing 50 mg of TA (Table 1), if all the polyphenol would be released in 50 mL of water, one would reach a final concentration of 1 mg.mL^{-1} in TA, far above the linearity limit of the obtained calibration curve. To that aim, we diluted the supernatant in contact with the plaster@TA or plaster@PY composites by a factor of 20 in water (as explained in the Materials and Methods section). In all cases, the absorbance never reached values higher than 2.4 (i.e about $0.05 \text{ mg.mL}^{-1} \times 48.9 \text{ mL.mg}^{-1}$). The release kinetics was pretty fast, with a first initial burst of release occurring after 1h of contact with water (data not shown).

The results show that the more TA is incorporated initially in the blend, the higher is the steady state concentration of TA released in water (Fig. 4). In all cases, only a pretty small fraction, less than about 6%, of the initially added TA is released in water. This means that the incorporated TA in plaster has a dual role: a large fraction of it is strongly bound to the plaster or incorporated in its structure (without changing the crystalline phases), and a lower fraction of TA fills either the pores of the structure or is only weakly adsorbed on the surface of the plaster particles, which are smaller upon an increase in incorporated TA (Fig. 1). This weakly bound or pore filling TA remains available for subsequent release and biological applications. This dual role of TA in its composites with plaster is similar to the role played by the same polyphenol when blended with gelatin [34]. Concerning PY, a much larger fraction than for TA, about 50%, is released in water after 72 h in an almost independent manner on the fraction of initially incorporated polyphenol (Fig. 4). This suggests also that there are two populations of incorporated PY, but that the strongly bound fraction, not released in water after 72h, is smaller. These findings point to a stronger interaction between TA and plaster than between PY and plaster. This is not so surprising knowing the much larger size of TA compared to PY (Scheme 1) allowing

more interaction points with the surface of the calcium sulfate particles, perhaps in a cooperative manner considering TA as a decamer of PY at the first level of approximation.

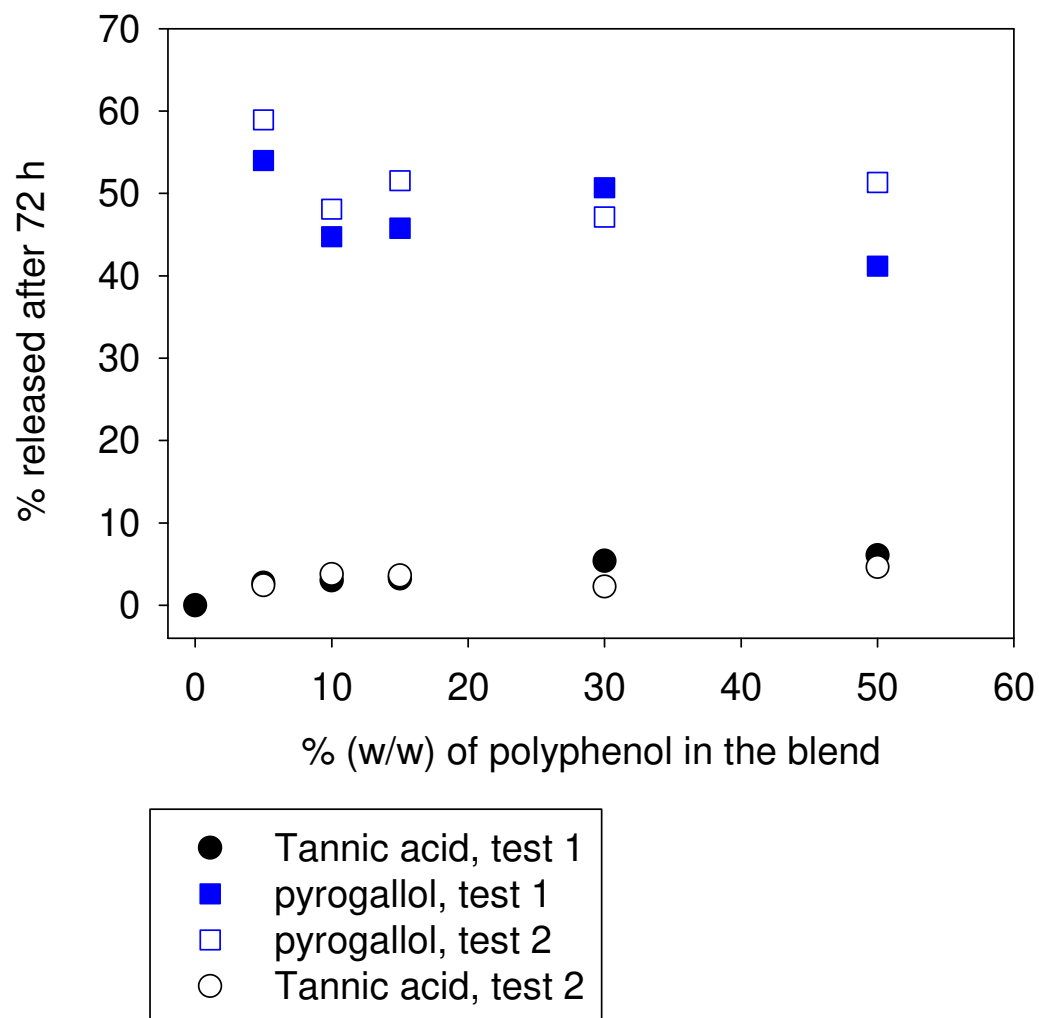


Fig. 4: Percentage of TA and PY released with respect to the TA/PY initially included in the blend after 72 of contact with water. Two independent experiments were performed in each case as indicated in the inset.

The release of TA acid, as a weak acid, [33] induced a slight, decrease in the pH of water in contact with the plaster@TA blends. This reduction in pH was more pronounced than in the absence of added TA (Fig. S7 in the supplementary data file). In contrast the pH change of the water in contact with the plaster + PY blends was much more limited (Fig. S7 in the supplementary data file) This finding, a rather important pH decrease when TA is released in water and a much lower pH decrease when a higher amount of PY is released may be rationalized on the basis of the pKa

values of both polyphenols. TA is characterized by an average pKa value of 8.5 [35] (only an approximation because it is a polyacid) whereas the two pKa values of PY amount to 9.02 and 11.2 [36]. This shows that PY is a weaker acid than TA and explains hence a smaller reduction in pH during its release in water put in contact with the composite.

3.6. Antimicrobial activity

The release of part of the TA or PY from the prepared composites allowed for an antibacterial activity against *Staphylococcus aureus*. The tests were performed both with Agar diffusion and direct contact tests.

3.6.1. Agar diffusion tests

The pristine plaster does not allow to create an inhibition zone on Agar gels, however the composite doped with 10 %TA (with respect to the water amount added) already presents a large inhibition zone which increases when the highest doped composite is considered (Fig. S8-A). This is in probable relation with the higher concentration of TA released from the plaster + 50 % TA composite than for the plaster + 10 % TA composite (Table 2 and Fig.4). Note that the inhibition zones, as well as part of the gel appear brown in the case of the plaster @TA/PY composites (Fig. S8-A and Fig. S8-C) as well as for the TA/PY containing solutions (Fig. S8-B and Fig. S8-D). This is related to oxidation of the polyphenols in the growth media. Concerning the PY composites, the inhibition zones were more important than for the TA composites (Fig. S8-5C versus Fig. S8-5A and Table 2).

Sample	CHX					
	TA 10%	PY 10%	TA 50%	PY 50%		
Inhibition zone (mm)	0	3±0.5	9±0.7	5±0.5	12±0.5	7±0.7

Table 3: Size of the inhibition zones (mm) against *Staphylococcus aureus* as a function of the TA and PY contents in the composites.

To confirm these encouraging but qualitative data, we also performed some direct contact tests.

3.6.2. Direct contact tests

The pristine plaster allowed for the same bacterial development as the positive control (Fig. 5). The composite doped with 10 % of TA (w/w with respect to water) allowed to kill about half of the *Staphylococcus aureus* bacteria after 24 and 72 h. When considering a TA percentage of 50 %, the inhibition of bacterial growth is 20 fold compared to the results of pristine plaster in Fig.5. The plaster@10% PY composite was more effective (kills 70% of the bacteria) than the same fraction of TA incorporated in plaster after 24h. In addition, the plaster@50% PY killed more than 98% of the bacteria after 24h and no bacterial colonization was observed after 72h.

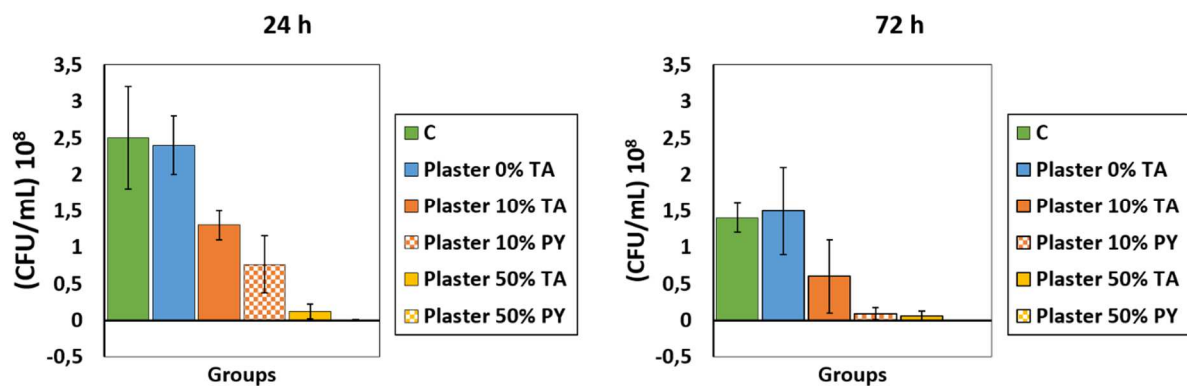


Fig. 5: Number of CFU of *Staphylococcus aureus* in the presence of pristine plaster and plaster @10% TA, plaster @ 50% TA; and plaster@10% PY and plaster@50% PY after 24 and 72h of culture. C represents the control experiment, without any material.

3.6.3. Biofilm of *S. aureus* morphology and accumulation

The surface of the composite samples was observed by SEM at 10.000X magnification. After 24h of incubation with the bacteria at 37°C, we can observe a dense packing of bacteria on pristine plaster discs whilst, plaster @ 10% TA/PY and plaster @ 50% TA/PY display less accumulated bacteria and no compacted biofilm was observed on plaster@50% TA and on plaster@50% PY (Fig. S9).

3.6.4. Direct contact tests of bacteria in solution in the presence of TA and PY

When the number of colony-forming units (CFU) of *Staphylococcus aureus* in suspension is measured as a function of the molar concentration in PY units (Fig. 6) it appears that PY is by far much less efficient than TA with respect to its

antimicrobial activity against *S. aureus*. This strongly suggests that the 10 PY units on TA act cooperatively in its antimicrobial activity.

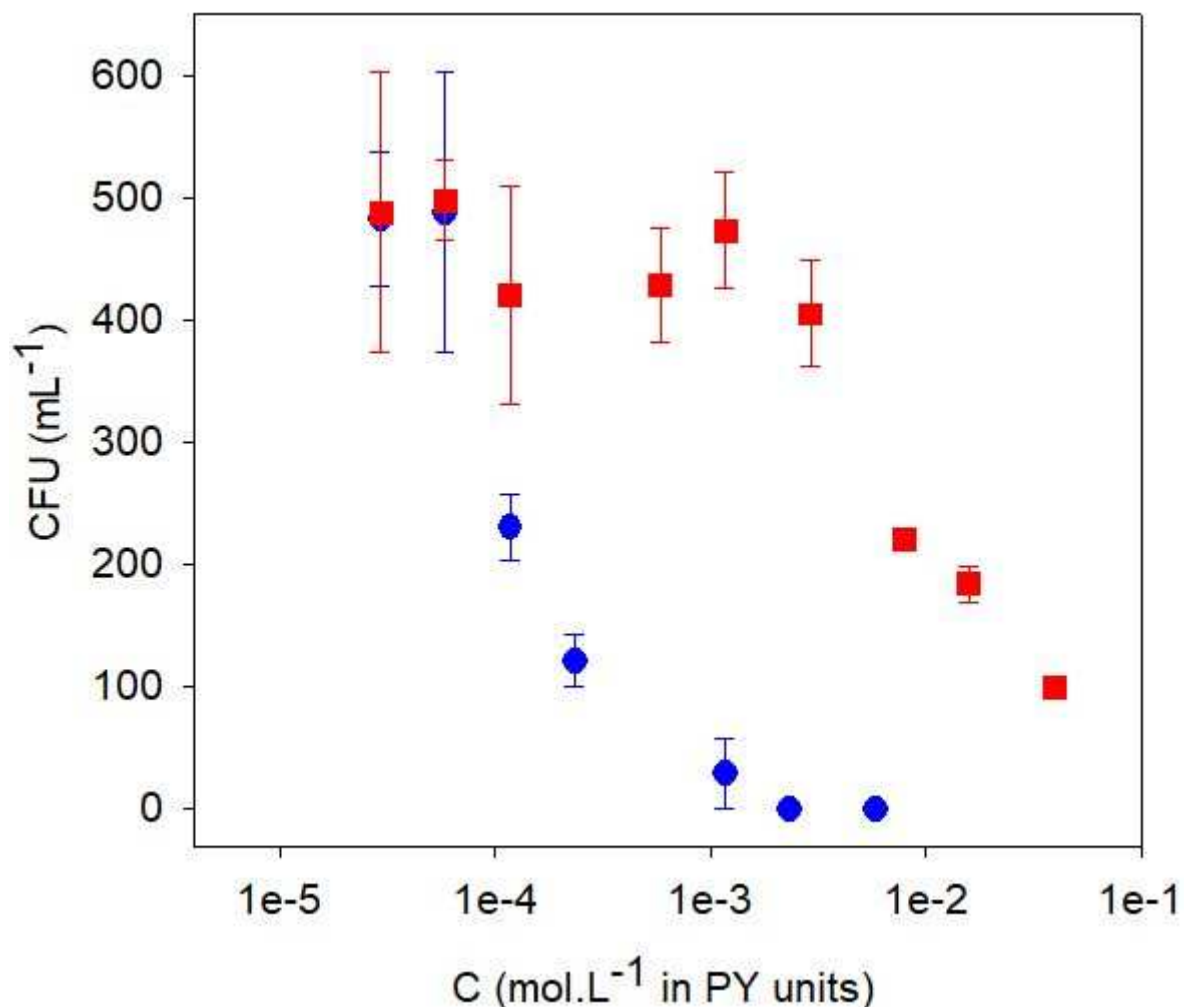


Fig. 6: Number of CFU as a function of the molar concentration in PY units (one TA molecule is equivalent to ten PY owing to its structure, Scheme 1). The blue circles correspond to TA and the red squares correspond to PY.

The fact that the plaster @ PY composites appear more efficient in the inhibition of bacterial growth (Fig. S8 and Fig. 5) has then to be related to a higher release of PY from the blends as compared with TA (Fig. 4).

3.7 Antioxidant activity.

As expected the pristine plaster sample had no effect on the color of the DPPH solution, hence no antioxidant activity (Fig. S10). But the incorporation of either PY or TA provided this additional property to the composite sample (Fig. S10).

After 1 min of contact between the DPPH solution and the composite samples, there seems to be a slightly larger effect of TA than of PY (Table 4) even if the release of TA is lower than that of PY at all time and for all weight fractions of the polyphenol (Fig. 4) in probable relation to an intrinsic higher fraction of antioxidant moieties (the hydroxyl groups on the aromatic rings) on TA. Note also that an increased fraction of the incorporated molecule, PY or TA, seems to have a beneficial effect (Table 4).

	10%TA	30%TA	50%TA	10%PY	30%PY	50%PY
525 (nm)	-0.79	-0.83	-0.89	-0.75	-0.76	-0.79

Table 4: Antioxidant activity of the plaster @ PY and plaster @ TA composites.

3.8 Stability of the plaster and plaster composites in water

For medical applications, even if plaster is aimed to provide a progressive calcium release and to be replaced by bone, it is important to control its resorption. In particular here, it is important to evaluate the influence of PY and TA on its solubility in water. We found that pristine plaster undergoes a slight mass increase after 24 and 72h. The same holds true for the composite plasters after prolonged exposure to water and is mainly due to water infiltration in the pores. However, after 24h, the PY and TA doped plasters undergo a slight reduction in mass which is attributed to a release of the active molecule in water. Nevertheless, in all cases, satisfactory stability of the material in contact with water is ensured for at least 72h.

Samples	24h			72h		
	Unmodified plaster	Plaster+ 50%PY	Plaster+ 50%TA	Unmodified plaster	Plaster+ 50%PY	Plaster+ 50%TA
Solubility (%)	+1.21±0.15	-3.11±0.15	-1.51±0.1	+1.23±0.02	-4.3±0.14	-2.32±0.11

Table 5: Solubility of plaster and of plaster incorporating 5% of either PY or TA in contact with water for 24 or 72 h.

4. Discussion

In the present study, plaster of Paris was modified with different weight fractions of PY and TA. It was not possible to prepare composites in which the TA or PY fractions were higher than 50% with respect to the mass of water because in such samples the amount of available water was not sufficient anymore to set the plaster which then stayed in a powdered form. In all cases, however the setting of the plaster

was not markedly influenced by the addition of either TA or PY, as long as its mass percentage with respect to water was not higher than 50%. For higher mass fractions, the plaster did remain in a powdered form most probably due to the highly hydrophilic nature of TA or PY which solubilization reduced the amount of available water for setting the plaster.

The incorporation of TA or PY in water used to prepare plaster of Paris allows a change in the plaster morphology, namely a perceptible reduction in the grain size. The ability of TA to modify the morphology of the plaster may well be related to the chelating ability of TA with respect to Ca^{2+} cations [37]. We assume that PY plays a similar role but in a less pronounced manner. The incorporation of PY or TA in the plaster of Paris not only changes the morphology of the material but may also change its porosity and its pore size distribution, a property which affects the mechanical properties and its ability to release the incorporated organic molecules. We thought to investigate an eventual change in pore size distribution upon incorporation of PY or TA using nitrogen sorption and data analysis using the Brunauer-Emmett-Teller method (BET). This is however not possible with a highly hydrated material like calcium sulfate dihydrate because the BET method requires intensive degassing of the material before performing nitrogen adsorption. Such a degassing process will induce dehydration and phase change from calcium sulfate dihydrate to calcium sulfate hemihydrate with a concomitant change in morphology and pore size distribution. We could have thought also to determine the average porosity by buoyancy, but this is not possible owing to the release of PY and TA in the water and even in other solvents. Hence the influence of porosity and pore size distribution on the properties of the composite plasters investigated herein remains an open question.

Even if the hydrophilicity of the plaster-based composites is not affected, they incorporate TA and part of it, less than 6%, is released in the presence of water. The release of PY from the plaster@PY composites in water is much more important, up to 50% of the initially incorporated organic material, and implies a weaker bond of this smaller polyphenol on the surface of plaster. This implies that most of the used TA takes part as a strong structural component of the obtained composite. The PY incorporated as a structural component appears much weaker. The smaller fraction of released TA compared to PY is certainly not related to steric constraints because even if TA is significantly larger than PY, this increase in size should not inhibit the

release of those molecules owing to the large pores (not quantified) of plaster (Fig. 1). But, interestingly, for TA percentages higher than 10 % (with respect to the mass of water), the slow amount of released TA is sufficient to strongly inhibit the proliferation of *Staphylococcus aureus*. The inhibitory effect of PY is even much stronger; this is not due to a higher intrinsic inhibitory effect of PY but to a higher release.

Plaster modified with TA and PY presented an antibacterial activity in agar diffusion tests and colored part of the gel in brown. This coloration is easily explained by a partial oxidation of TA/PY at physiological pH values at which the agar diffusion tests were performed. This important antibacterial efficiency and the strong coloration could be explained by the important liberation of PY from the composites (Fig. 4). Regarding the direct contact test, both composites demonstrated an antibacterial activity after 24 and 72h against the growth of *Staphylococcus aureus*. Cynthia *et al.* [27] and Kyaw *et al.* [38] determined the MIC of TA and PY against *S.aureus*. They reported that TA (MIC = 256 $\mu\text{g.mL}^{-1}$, namely $1.50 \times 10^{-4} \text{ mol.L}^{-1}$) had more antibacterial activity than PY (MIC = 512 $\mu\text{g.mL}^{-1}$, namely $4.1 \times 10^{-3} \text{ mol.L}^{-1}$). In accordance, our study shows that TA in solution was more effective against *S.aureus* than PY in solution when compared in their molar concentration (Fig. 6). The MIC values determined in this investigation are furthermore very close to the published data [27, 38].

Our article shows that TA and PY release spontaneously from an inorganic porous material. It has been shown previously that TA can be released from TA crosslinked capsules with a similar release profile as the one found in this investigation [39, 40]. The released PY and TA also provide some antioxidant properties to the as-prepared composite materials.

The observed discontinuity in the compression resistance of the plaster with the increase in incorporated TA (Fig. 2A) may well be due to a different role played by the polyphenol. At low TA mass fraction (plaster + 10%TA) more TA could be irreversibly bound to the inorganic material than for plaster +30%TA and plaster 50%TA. But for both composite samples with 30% and 50% TA, the absolute amount of incorporated polyphenol is much higher and part of it may be adsorbed on the surface of the inorganic particles inducing some increased interactions between them, for instance via hydrogen bonds. Those bonds could strengthen the material and reduce the probability of crack propagation. Anyway, for all TA containing

samples, above 10 % TA, the compressive strength decreases with an increase in the mass fraction of TA, as expected upon the addition of an organic filler to an inorganic material. The TA containing plasters display hence a reproducible discontinuity in their compressive strength at 10 % in TA. Such a discontinuity is not found when PY is added to the plaster (Fig. 2B). In the future it may be of interest to investigate if the presence of PY or TA may change the behavior of plaster under compression tests or confined compression tests to reach the ductile behavior

We wondered if the addition of TA in plaster of Paris could induce some phase changes in particular in the presence of 10% TA, which could explain the weaker compressive strength found at this particular concentration. To that aim, we systematically investigated the plaster@TA or plaster@PY blends by means of X-ray diffraction.

Anyway, the results obtained in this report are qualitatively comparable with those obtained previously in the case of gelatin@TA composites, namely that the polyphenol plays a double role: a structural additive and a partly releasable agent providing some additional biological activity to the as formulated biomaterial [34].

Further studies are required to evaluate the antibacterial activity of these composites when there are in contact with other strains of bacteria. Also, additional studies on different calcium based cements such as, mineral trioxide aggregate and calcium-silicate cements are necessary to confirm these latter outcomes. From a mechanical point of view, we found that the compressive strength of the plaster @ TA 10% composite displayed a marked reproducible but unexplained minimum. In the case of the PY containing composites, the compressive strength decreased monotonously with higher fraction of incorporated organic molecules, as expected.

5. Conclusions

It has been shown that the incorporation, in a one pot process, of TA or PY in plaster of Paris formulations allows to change the size distribution of the plaster particles up to a maximal load of 50% in polyphenol with respect to the amount of water without affecting the hydrophilicity of the plaster. A large part of the incorporated amount of TA serves as structural component of the obtained composite, but a small part of it- up to 6 %- can be released in the ambient medium and is sufficient to inhibit the proliferation of *Staphylococcus aureus* in 20-fold manner after 72h. Concerning PY, a much higher release in water allows for an even better antimicrobial activity when

compared to TA. This is however not due to a higher intrinsic antimicrobial activity of PY. Indeed, when compared on a molecular basis of the number of provided PY units (10 per TA molecule) both polyphenols are almost undistinguishable in their inhibitory effect against *S. aureus*.

Overall, this article shows that the incorporation of either PY or TA in a one pot preparation manner provides some antimicrobial and antioxidant properties through a release of the active molecules but without an important reduction in the compression resistance and without an alteration of the chemical stability of the plaster.

The findings presented in this report may be interesting in providing bone sealing materials with antibacterial potential at a low cost owing to the high availability and low prize of TA and PY

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Credit authorship contribution statement

Conceptualization: Vincent Ball, Youssef Haikel and Naji Kharouf; Methodology: Vincent Ball, Naji Kharouf, Florent Meyer, Nadia Bahlouli and Nathalie Viart; Investigation: Sophie Helle, Naji Kharouf, Jihed Zghal, Hamdi Jmaland Mark Lenertz; Writing Original Draft: Naji Kharouf, Vincent Ball and Mark Lenertz; Writing Review & Editing: Davide Mancino, Florent Meyer, Vincent Ball, Davide Mancino and Natalie Viart; Funding Acquisition: Youssef Haikel and Vincent Ball; Supervision: Vincent Ball and Youssef Haikel.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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