Inhibition of bacterial growth on marble stone of 18th century by treatment of nanoencapsulated essential oils

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Abstract

Controlling of cultural heritage biodeterioration is a serious problem in the world. Chemical biocides, used to kill unwanted microorganism, can represent a risk for human health and environment, and interfere with the restoration material. Natural biocides could represent a valid alternative to conventional ones. In this study we report the use of nanocapsules suspensions (NCs) loaded with *Origanum vulgare* and *Thymus capitatus* essential oils (EOs) to contrast the development of bacterial growth of two microrganisms *(Escherichia coli* and *Kokuria rhizophila)* on the marble stone from 18th century church altar. No structural change was observed on the stone after treatment with the aqueous suspension containing nanoparticles as evidenced by SEM-EDX analysis. The nanostructurate systems (EO-NCs) were able to inhibit the bacterial grow on the stone pretreated with bacterial inoculum as showed by agar discs contact test. Nanocapsules, based on biodegradable and biocompatible polymer (poly(ε-caprolactone)), loaded with thyme EO are more efficient than nanocapsules loaded with oregano EO. The obtained results evidenced the potential of these natural biocides in the treatment of biodeteriorated cultural heritage.

Keywords:

Biodeterioration, natural biocides, nanoencapsulated essential oils, antimicrobial activity, stone monument.

1. Introduction

Biodeterioration is one of the main causes of the stone material degradation of cultural heritage (Charola et al., 2011). It consists of undesirable changes in the properties of the material caused by the biological activity of living organisms (Hueck, 2001). The magnitude of the phenomenon depends on the heritage artefact nature, and the climatic and environmental conditions to which it is exposed. Microorganisms such as bacteria, lichens, fungi, and protozoa involved in the biodegradation processes can cause different types of damage on the stone monuments ranging from aesthetic (forming crusts and patinas on the surface), mechanical (by generating serious fractures) to chemical (due to the production of metabolites) (Caneva et al., 2008; Urzì et al., 2010; Pangallo et al., 2013; Pinheiro et al., 2019).

Preventive and restoration interventions are frequently need to counteract the development of microorganisms capable of causing serious damage on stone monuments. In these interventions, conservators and restorers very often use commercial product containing organic and inorganic biocide substances with high toxicological impact on environment and human health (Allsopp and Allsopp, 1983).

In this context, natural products could represent a valid alternative to conventional chemical biocides (Pinna et al. 2012; Fidanza and Caneva, 2019). In particular, essential oils (EOs) from plants, "generally recognized as safe" (GRAS) compounds, are environmental friendly and rich in components known for their antibacterial properties ((Reineccius, 2016; Chouhan et al., 2017). Their mechanism of action is multitarget and for this reason, EOs are also effective for fighting the multi drug resistant bacteria (Chávez-González et al., 2016). However, the color, high volatility, insolubility in water and sensitivity to oxygen, light, and heat could make them difficult to use in cultural heritage remediation.

The nanotechnologies, advantageous in solving issues related to medicine, agriculture and environment, in recent years have found interesting applications in conservation-restauration of the cultural heritage. Nanomaterials have been used successfully in several restoration work-shops in Europe and in Mexico for the consolidation of mural paintings, lime mortars, stone, paper, canvas, and wood deacidification (Hansen et al., 2006; Giorgi et al. 2010, Chelazzi et al., 2013, Dei and Salvadori, 2006; Baglioni et al. 2013, Castillo et al., 2019). Nanomaterials to be used in the restoration are already on the market. As an example Nanorestore Plus® (Csgi - UniFI) based on nanoparticle of calcium hydroxide dispersed in nanolime solution (http://www.csgi.unifi.it/products/plus.html).

Nanoencapsulation is a technique that allows to protect the sensitive bioactive compounds from unfavorable environmental conditions, improving compatibility masking color and flavor, increasing solubility and physical stability, and decreasing volatility (Pisoschi et al., 2018). In addition, the nanoscale dimension provides unique physicochemical properties including high surface to volume ratio and enhanced reactivity with biological systems (Maryam et al. 2015). Therefore, the

nanoencapsulation of EOs could be a valid strategy to realize novel nanomaterial non-toxic and ecofriendly to be used in cultural heritage field.
The aims of this study is the evaluation of antimicrobial activity of nanocarrier systems, based on biodegradable and biocompatible polymer, loaded with natural compounds (*Origanum vulgare* (Or) and *Thymus capitatus* (Th) essential oils), to the protection of marble stones from bacterial colonization. Antibacterial activity of these potential natural biocides against two bacterial strains, *Escherichia coli* (Gram negative) and *Kocuria rhizophila* (Gram positive), were performed on 18th century marble sample from an altar in restoration at the San Francesco Borgia church in Catania (Italy).

2. Materials and methods

2.1. Sampling and physical analyses of marble stone sample.

A sample of Sant'Agata red marble, spontaneous detached from a side altar was used as sample material. It was kindly provided by the "Soprintendenza per i Beni Culturali e Ambientali (Catania, Italy)" to carry out the research.
The stone sample has a triangular shape with a surface area of about 80 cm^2 and a thickness of 3.5 cm. Stone surface were observed by light stereoscopic microscope Leica WILD M8, zoom (6 – 50 fold magnification). Stone fragments, obtained by scratching with tip of a spatula from rifts, were analyzed by scanning electron microscope SEM - FEI Quanta 200 FEG, equipped with an EDS X-ray spectrometer - Inca Oxford 250. The fragments were fixed by carbon adhesive discs onto aluminum stubs, and observed in low vacuum mode (0.6 Torr).

2.2. EO-NC morphology

The morphology of EO-NCs, prepared by nanoprecipitation methods (Granata et al., 2018a), were assessed by scanning electron microscope (SEM FEI Quanta 200 FEG), equipped with an EDS X-ray spectrometer (Inca Oxford 250) . The EO-NC suspension was diluted 1:200; a microdrop was placed onto stub and left to evaporate under a laminar flow hood. Before observations, samples were coated with a homogeneous layer (18 ± 0.2 nm) of Au–Pd using Emitech mod. K575X and observed in high vacuum mode using a secondary electron detector (accelerating voltage 30.00 kV) and 100000× magnification.

2.3. Antibacterial evaluation

The antimicrobial activity of EOs and EO-NCs were assayed against the Gram-positive strain *Kocuria rhizophila* (DSM 348) and the Gram-negative *Escherichia coli* (DSM 498), purchased from Deutsche Samlug von Mikroorganismen und Zellkulturen GmbH (DSMZ Germany).
The EOs from sicilian aromatic plants were obtained by hydrodistillation and characterized by GC and GC-MS (details are given in Supplementary Data, Granata et al., 2018a, Napoli and Ruberto, 2012).
The EO-NCs were prepared by nanoprecipitation methods and characterized as

previously reported (Granata et al., 2018a, 2018b). Brief experimental details and physicochemical features (particle size, polydispersity index (PDI), zeta potential (ζ)), dimensional stability and essential oil retention (%) over time are given in Supplementary Data.

2.3.1. Determination of minimum inhibitory concentration (MIC) of EOs

MIC testing of EOs was performed by using the broth dilution methods. The *K. rhizophila* was grown in Nutrient Broth at temperature of 35°C for 48 h while the *E. coli* in Luria-Bertani (LB) medium at temperature of 37°C for 24 h.

EOs, serially diluted with the appropriate medium, were added to 1 mL of bacterial inoculum (10^5 CFU/mL) to obtain a concentration range between 800 and 50 µg.

Kanamycin and streptomycin sulfates were used as positive controls for *E. coli* and *K. rhizophila*, respectively. The broth solutions were incubated and MIC value (lowest concentration of antimicrobial agent that inhibited the growth) was determined by measuring of the optical density (λ 600 nm). All the experiments were carried out in triplicate.

2.3.2. Antimicrobial activity of the EO-NCs

ASTM standard Test Method E 2149-01 (ASTM, 2001) under dynamic contact condition was employed to determine antimicrobial activity of Or-NCs and Th-NCs (Silvestre et al., 2013; Straccia et al, 2014). Bacterial cells of *K. rhizophila* and *E. coli*, grown in the appropriate medium, were recovered by centrifugation at 15344 × g and washed with a sterile buffer solution (RS) and suspended in the same buffer in order to obtain a working bacterial suspension of about 10^5 CFU/mL. 565 µL of the Or-NCs or 270 µL of the Th-NCs were added to 5 mL of working bacterial suspensions. The samples were kept at room temperature on a wrist-action shaker. 100 µL of each sample, after fixed contacts time (0, 6 and 24 h), were spread onto petri plates and incubated as required for each bacterial strain. The surviving cell number was determined by a standard plate count method. Working bacterial suspension without and with empty NCs were used as negative controls. All the assay was replicate three times.

2.3.3 Antimicrobial activity on marble sample

Stone sample was sterilized, by autoclaving (121°C × 20 min), and was kept under laminar flow about 1 hour (Romano et al., 2019). Stone's area was about 80 cm^2. On the stone surface were identified 4 sectors of 3 × 3 cm^2 each. 10 µL of working bacterial suspension (10^6 CFU), were spread in each sectors. After absorption/evaporation of bacterial suspension, 100 µL of Or-NCs and Th-NCs were added in two different sectors; in addition, 100 µL of NCs and 100 µL of RS were added to the other remaining sectors. After complete absorption/evaporation, the treated areas were covered by LB or nutrient agar discs (diameter 3 cm) in order to transfer microorganisms from stone

to agar discs, via direct contact for 5 min. The conditions were standardized applying 0.02 kg/cm^2 of constant pressure for 10 s. This ensures the absence of air bubbles and perfect adhesion of the agar disc on the surface stone.
Media agar plates were incubated at 37°C for 24 h (*E. coli*) or at 35°C for 48 h (*K. rhizophila*) and colony counts were carried out. The stone was cleaned with running water, dried at room temperature and autoclaved before performing another experiment. The test was performed three times and average colony count of duplicate plates was used to calculate the CFU/mL.

3. Results and discussion

3.1. Sampling site and preliminary physical analyses of marble sample.

The church of San Francesco Borgia in Catania is an example of typical "Barocco" style of Val di Noto area (Sud-Est of Sicily). This area is included in the UNESCO world heritage list. The church was rebuilt in the XVIII century after the huge 1693 Sicily earthquake. It has a basilica plan with three naves and side altars (Fig. 1a and Fig. 1b). The church is owned by the city of Catania and today is home to cultural events that attract a high number of visitors.
Red marble sample from San Francesco Borgia church side altar was taken as model material to test the antimicrobial activity of nanocapsules, loaded with oregano and thyme essential oil (Fig. 1c). The altar from which the stone specimen spontaneously detached is located in an environment with high humidity. This resulted in serious structural damage to the altar, which has not undergone any restoration in the past. The marble sample showed an irregular profile and the presence of some rifts on the upper and lateral surface as highlighted by the stereomicroscope visual observation.
Marble fragments taken from the rifts on the upper and lateral surface of the stone, were analyzed by SEM-EDS. The upper surface fragment is mainly constituted from calcium, oxygen, carbon, silicon, magnesium, aluminium, sulphur elements (Fig. S1a). Such as expected, oxygen (O) and calcium (Ca), were the most abundant elements present, confirming that the marble sample is composed primarily of the mineral calcite ($CaCO_3$) (Lindawati et al., 2019). After treatment by EO-NCs, the stone sample was cleaned with running water, dried at room temperature and autoclaved. A fragment was taken from the upper surface and examined by stereomicroscope and SEM-EDS analyses (Fig. S1b). No significant change in color, structure, chemical composition was observed on the stone after the treatments .
Moreover, the time request by the stone to adsorb bacterial inoculum and EO-NCs suspension was the same before and after the treatments, that agrees with no change on stone surface.
A fragment from the lateral surface of the stone (1.5 cm below the surface) was also analyzed by SEM-EDS. The spectrum EDS showed that it was composed mainly of silicon, calcium and contained particles rich in aluminium, sulphur, iron and magnesium (Fig. S2).

3.2. Evaluation of antibacterial activity of EO-NCs

Nanocapsules based on biodegradable and biocompatible polymer (poly(ε-caprolactone)) were prepared with procedures reported in previously works that provided robust and scalable nanosystems (Ephrem et al., 2014; dos Santos et al., 2015; Granata et al. 2018a, b).

Polymeric nanocapsules loaded with Or and Th essential reported by Granata et al. (2018a), were here used as antimicrobial agents on the 18th century marble sample. In particular, Or-NCs and Th-NCs possess diameter in nanometric range (about 170 nm), monomodal distribution, spherical morphology, high encapsulation efficiency, stability in the time and low release of bioactive (Fig. 2, Table S1, Fig. S3, S4). The nanocapsules showed also antibacterial activity mainly due to the presence of EO bioactive components (carvacrol and thymol). These characteristics makes these systems potentially interesting to combat the biodeterioration of stone material exposed to different environmental conditions.

Two bacteria, *E. coli* and *K. rhizophila,* were selected to assay the antimicrobial activity of EO-NCs on the heritage marble sample. *E. coli*, a Gram negative bacteria, is a model microorganism for testing various antimicrobial agents (Carrillo-Gonzales et al., 2016; Mati et al., 2014); *K. rhizophila*, a Gram positive bacteria, belongs to the genus *Kocuria* envolved in some bioderioration processes of stone monuments (Savvides et al. 2014; Cappitelli et al. 2007, Sáiz-Jiménez, C. and Laiz, L. 2000; Heyrman et al. 1999). At first, the minimum inhibitory concentration (MIC) of EOs against *E. coli* and *K. rhizophila* were determined by microdilution assay. Or-EO exhibited activity against *E. coli* and *K. rhizophila* with MIC values of 0.6 and 0.5 mg mL^{-1} respectively, whereas Th-EO showed a higher effectiveness with MIC value of 0.3 mg mL^{-1} for both microorgamism. The MIC of Th-EO were lower than the pure Or-EO (Table S2).

The EO MICs values were useful to indicate the concentration range of the nanocapsule suspensions to be used in the ASTM E2149 antimicrobial test.

This test allows to evaluate the effectiveness of antimicrobial agents under dynamic conditions, at room temperature and different contact times (Catanzano et al. 2015). The results of this assay are shown in Fig. 3. In particular, at 6 h of contact Or-NCs and Th-NCs were capable of reducing the bacterial concentration of three and four orders of magnitude for both microorganisms. After 24 h of contact, no viable bacterial cells were observed. Empty EO-NCs did not show any effect on the bacterial concentration. Strains incubated in buffer (RS), with and without empty NCs (negative control), were not inactivated after the fixed incubation time. These results highlight higher antimicrobial activity for the nanocapsules loaded with thyme essential oil than those loaded with oregano.

Finally, the antimicrobial activity of EO-NCs, was evaluated on original marble stone surface treated with bacterial inoculum and suspension of EO-NCs. In Fig. 4a the different sectors selected on the stone to perform the test and in Fig. 4b the agar discs in contact with the stone are indicated. The results obtained after the stone treatment by Or-NCs, showed a strong grow reduction for both microbial strains. Considering that chemical biocides are often more active against gram-positive than gram-negative

bacteria, the broad spectrum of action of these nanosystems (Or-NCs and Th-NCs) suggests potential higher efficacy in treatment of biodeteriorated cultural heritage (Maillard, 2002). No bacterial growth for both microorganisms was observed after Th-NC treatment. For *E. coli* the photos of the agar plates incubated at 37°C for 24 h, which have been in contact with the pretreated stone for 5 min, are shown in Fig. 4c. The comparison between the plate 3 and 4, related to the treatment with Or-NCs and Th-NCs respectively, showed a higher antimicrobial activity of the Th-NC suspension. This phenomenon could be attributable to different quantity of bioactive oxygenated monoterpenes present in the EOs. In particular the Th-EO is characterized by a high content of carvacrol (73%, carvacrol-chemotype) while Or-EO by high amount of thymol (43%, thymol-chemotype), as evidenced by GC and GC-MS analysis (Table S3, Fig S5). It is known that carvacrol and thymol interact with bacterial cell wall, increasing fluidity and permeability, interfering with ATP production and pH homeostasis, until to cause structural and functional damages to cytoplasmic membrane (Rai et al., 2017). In principle, this makes them potentially able to counteract bacterial development in a consortium of different microorganisms responsible for biodeterioration process of artefacts of artistic and archeological interest (Faleiro and Miguel, 2013,).

All these studies suggest the efficacy of nanocapsules containing Or-EO and Th-EO in treatment of the 18th century marble stone. These polymeric nanocapsules protect EOs from degradation phenomena, increase solubility and biological activity, ensure controlled release even at different temperatures (Granata et al., 2018a).

In light of all these features, the realized nanosystems could be used in addition to consolidants and water-repellent to reduce microbial recolonization in analogy with other examples reported in the literature (Pinna et al. 2019).

4. Conclusion

This study reports the first example of the application of eco-friendly polymeric nanocapsules, loaded with oregano and thyme essential oils, which are able to inhibit the growth of gram negative and gram positive microorganisms, (*E. coli* or *K. rhizophila*), on stone cultural heritage material pretreated with bacterial inoculum. The encouraging results obtained suggest these nanosystems could represent a valid alternative to chemical biocides. They could be used in association with commercial products for restoration (e.g. consolidants) to enhance their potentialities.

Conflicts of interest

The authors of this manuscript declare no conflict of interest regarding the manuscript submission in the journal of “International Biodeterioration and Biodegradation”.

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Appendix A. Supplementary data

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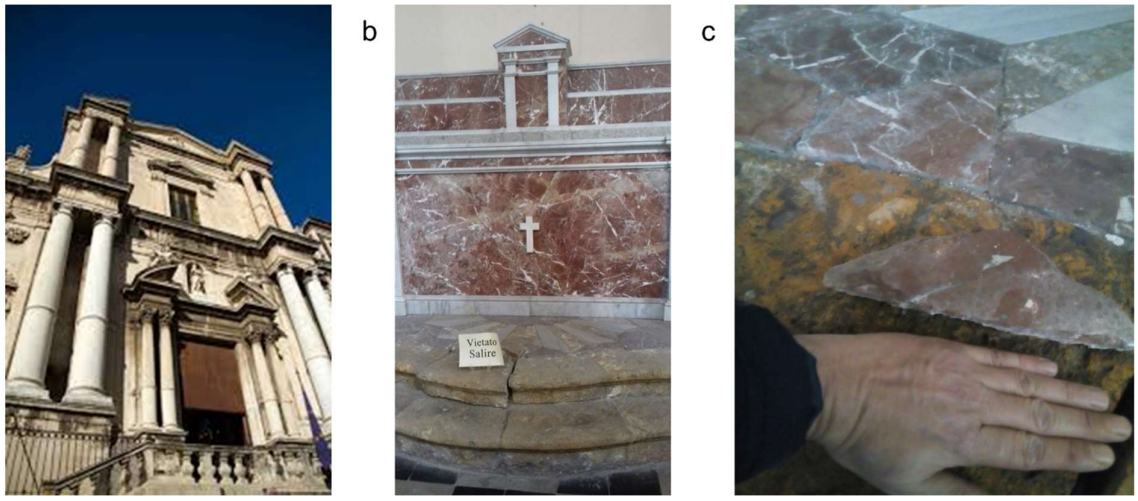


Fig. 1. Photographs: a) the exterior of the San Francesco Borgia church in Catania; b) side altar; c) red marble sample.

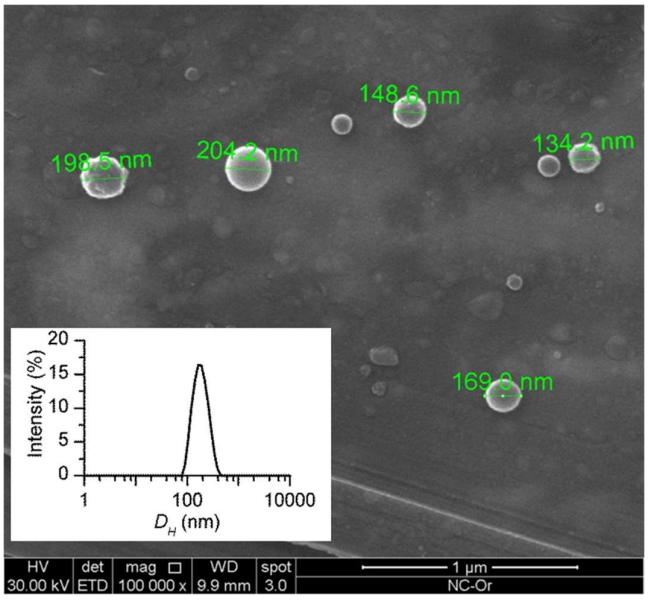
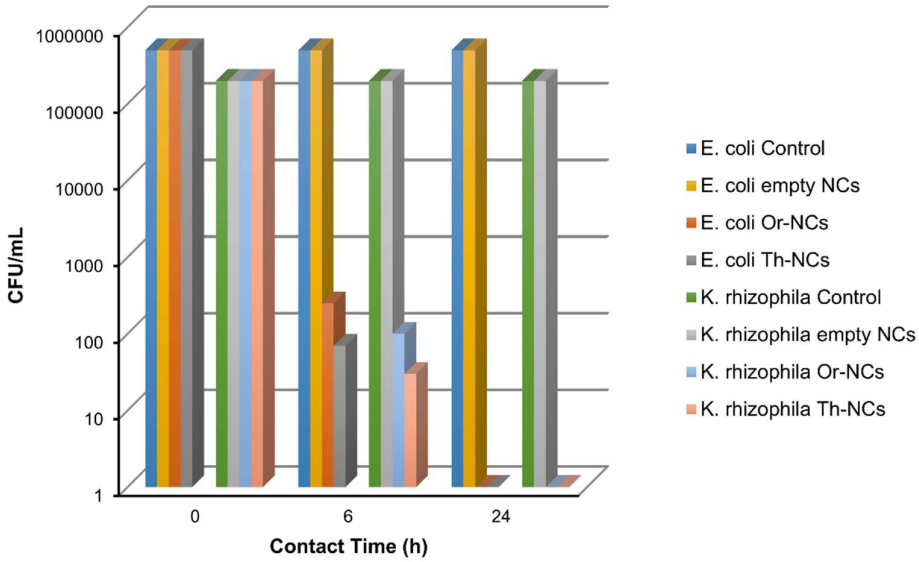


Fig. 2. SEM image at high magnification (100000 X) of Or-NC suspension. The inset shows the intensity weighted distribution of the hydrodynamic particle diameter (D_H) of Or-NC suspension.



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510 **Fig. 3.** CFU/mL reduction of *E. coli* and *K. rhizophila* by treatment with EO-NCs suspension at different contact time (h).
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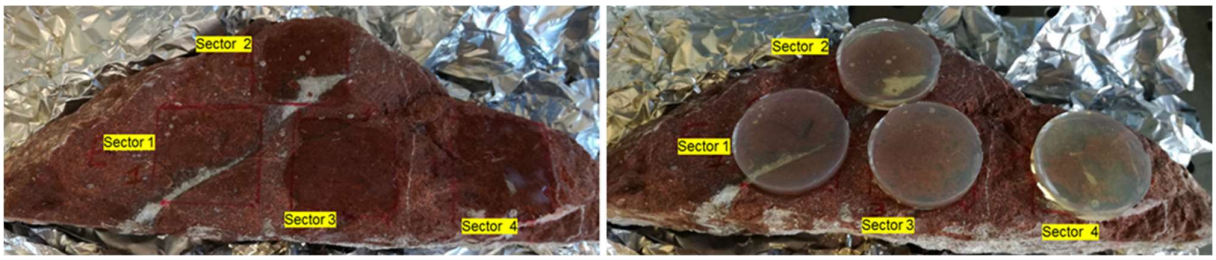
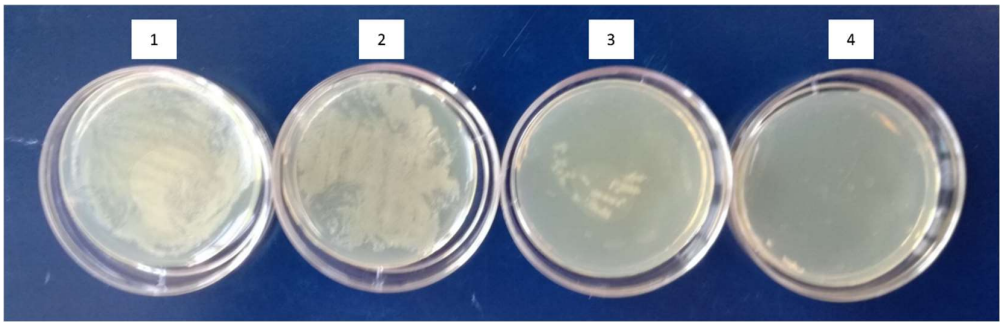
512 

513 

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518 **Fig. 4a-c.** Stone sectors (a). Agar discs in contact with the stone treated with inoculum of *E. coli.* Sectors. 1: 10 µL of bacterial
519 inoculum + 100 µL of buffer solution (RS); sector 2: 10 µL of bacterial inoculum + 100 µL of empty NC suspension; sector
520 3: 10 µL of bacterial inoculum + 100 µL of Or-NC suspension; sector 4: 10 µL of bacterial inoculum + 100 µL of Th-NC
521 suspension (b). Photo of the agar plates after incubation at 37°C for 24 h and contact time of 5 min on treated stone. 1 *E. coli*
522 inoculum + buffer solution (RS). 2 *E. coli* inoculum + empty NC suspension; 3. *E. coli* inoculum + Or-NC suspension; 4. *E.*
523 *coli* inoculum + Th-NC suspension (c).