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ZnO nanoparticles impregnated with *Hibiscus sabdariffa L.* extract: Characterization and antimicrobial activity.

Nanopartículas de ZnO impregnadas con extracto de *Hibiscus sabdariffa L.*: Caracterización y actividad antimicrobiana.

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ABSTRACT

Zinc oxide can be a vehicle for the encapsulation of bioactive compounds obtained from *Hibiscus sabdaridffa L* by-products due to its mesoporous structure. The nanoparticles obtained with zinc nitrate showed an average particle size of 120 mn, when impregnated with ultrasound-assisted extract of Jamaican by-product did not show morphological alterations, however; it was observed that there is a modification of the ZnO energy bands attributed to the interactions between ZnO and the extract molecules. T1.0 % and T1.5 % presented higher inhibition on *Listeria Monocytogenes, Staphylococcus aureus, Escherichia coli*, and *Staphylococcus mutans* compared to T0.5 % and EUSJ. The results show that the synthesis process and the impregnation of Jamaican by-product extracts have interesting antibacterial properties associated with the potentiating effect of ZnO and extract, which can be a starting point for further studies on some applications or other types of microorganisms.

KEY WORDS: Zinc oxide, by-products of *Hibiscus sabdariffa* L, Ultrasound, Antimicrobial.

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RESUMEN

El óxido de zinc puede ser un vehículo para la encapsulación de compuestos bioactivos obtenidos a partir de subproductos de *Hibiscus sabdaridffa L*. atribuido a su estructura mesoporosa. Las nanopartículas obtenidas con nitrato de zinc mostraron un tamaño medio de partícula de 120 mn, cuando se impregnaron con extracto de subproducto de Jamaica asistido por ultrasonidos no mostraron alteraciones morfológicas, sin embargo; se observó que existe una modificación de las bandas de energía del ZnO atribuida a las interacciones entre el ZnO y las moléculas del extracto. T1.0 % y T1.5 % presentaron mayor inhibición sobre *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* y *Staphylococcus mutans* en comparación con T0.5 % y EUSJ. Los resultados muestran que el proceso de síntesis y la impregnación de los extractos de subproductos jamaicanos presentan interesantes propiedades antibacterianas asociadas al efecto potenciador del ZnO y del extracto, lo que puede constituir un punto de partida para futuros estudios sobre algunas aplicaciones u otros tipos de microorganismos.

PALABRAS CLAVE: Óxido de zinc, subproductos de *Hibiscus sabdariffa*, subproductos y antimicrobianos.

Introduction

The *Hibiscus* (*Hibiscus sabdariffa L.*) is an annual plant cultivated in tropical and subtropical climates around the world. The plant material quality is determined by several factors: local growing conditions, harvesting time, post-harvest handling, and, above all, the drying stage. In Mexico, it is grown mainly in the states of Guerrero, Oaxaca, Michoacán, and Nayarit; the main producer is the state of Guerrero, which contributes more than half of total production. *Hibiscus* is one of the plants with the presence of antimicrobial compounds in dried calyxes (Betrán-Debón *et al*., 2010; Ahmadpoor-Dehkordi, 2018; Sindi *et al*., 014). It is an annual, shrubby, and fast-growing plant. It has an average height of 2 to 3.5 m. It is cradle-shaped, palmately veined, covered with leaves starting at the crown, as well as on the branches, and supports large axils with flowers (Duarte-Valenzuela *et al*., 2016).

Phenols

Phenols are a large family with more than 4,500 members. Within the phenol group are phenolic acids and the large family of flavonoids among others. Flavonoids are found in fruits, vegetables, seeds, and flowers. They play an important role in plant biology; thus, they respond to light and control the levels of auxins that regulate plant growth and differentiation. The

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importance of phenols lies in the fact that they provide mechanical support to plants, contribute to the coloration of flowers and fruits, protect against pathogens and herbivores, and are highly effective in protecting tissues against ultraviolet radiation (Ochoa-Velasco *et al*., 2018).

The main bioactive compounds identified in *Hibiscus sabdariffa* calyxes are delphinidin-3-glucoside, sambubioside, cyanidin-3-sambubioside, flavonoids (gossypetin, hibiscetin) with their respective glycosides, protocatechuic acid, eugenol, sterols such as β-sitosterol, and ergoesterol (Duarte-Valenzuela *et al*., 2016). Several studies demonstrate the presence of polyphenols in *Hibiscus sabdariffa L.* calyx extracts, such as flavonoids and anthocyanins. This group of compounds reports multiple biological effects, such as antioxidant, anti-inflammatory, antithrombotic, antimicrobial, anti-lipid peroxidation, anti-allergic, anti-carcinogenic, vasodilators, as well as the ability to neutralize reactive nitrogen species.

Antimicrobial Activity

Antimicrobial activity has been evidenced when evaluated against bacterial species such as *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Salmonella paratyphi, Listeria monocytogenes, Shigella flexneri* and *Vibrio cholerae* (Borrás-Linares *et al*., 2015; Gómez-Aldapa *et al*., 2017). Therefore, *Hibiscus* calyx extracts are considered to represent an alternative as a food additive that could prolong the shelf life of foods such as meat products (Patel, 2014).

By-products of *Hibiscus sabdariffa* L

Dietary fiber and antioxidants are two components of functional foods and ingredients that are usually studied separately in research and industry. However, as previously described (Saura-Calixto *et al*., 2007; Vitaglione *et al*., 2008; Goñi *et al*., 2009), a part of the bioactive compounds present in plant samples, whether antioxidants or not, are associated with the components of dietary fiber, as a consequence of the ability of some of them to form complexes with proteins and polysaccharides.

Specifically, in the case of polyphenols, a considerable part of them may be associated with the insoluble fiber fraction, mainly compounds with a higher degree of polymerization such as condensed tannins (proanthocyanidins) and hydrolyzable tannins. Polyphenols of lower molecular weight such as some flavonoids, phenolic acids, dimers, and trimers of proanthocyanidins are usually associated with the soluble fiber fraction (Sayago-Ayerdi *et al*., 2010).

Ultrasound-assisted extraction

The extraction of bioactive compounds by ultrasound is a clean, simple, fast, and green method compared to conventional methods. In addition to its high reproducibility in a short time, easy to handle and decreases in the use of solvents compared to other methods. The ultrasound waves cause the mechanical rupture of the cell wall releasing the bioactive components, in turn the

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local heating of the solvent increases the diffusion of the extract, thus improving the mass transfer through the solid-liquid interface. The mechanical effects of sonication induce further dissolution of the solvent in the cell walls and membranes, facilitating the release of cell contents and improving mass transfer (Rojas *et al*., 2019).

Ultrasound Applications (US)

The applications include extractions in herbs, oils, proteins, and bioactive components of plants (Robles-Ozuna & Ochoa-Martínez, 2012). Among these applications, the use of US, at 25KHz and 150W of power, has been evaluated in the preparation of different bakery products, and when compared to traditional methods, better sensory qualification and physicochemical analysis were observed, than products prepared by traditional technologies; mainly improving texture, water activity, viscosity, volatile compounds and color (Pingret *et al*.,2014). Another field in which the US has been applied is physicochemical characterization. This has been since the composition, structure, and physical state of many foods can be determined based on the velocity and attenuation coefficient of low-intensity ultrasonic waves propagated through the food (Robles-Ozuna *et al*., 2012).

Encapsulation

The encapsulation techniques are an alternative when trying to protect these microorganisms from the effect of environmental agents that may affect their viability during processing, storage, consumption, and passage through the gastrointestinal tract by allowing them to maintain their viability and functionality over time, reducing cell damage by retaining the cells inside encapsulating materials that generate their isolation (Rodríguez *et al*., 2016).

Zinc oxide as an encapsulating matrix

Zinc oxide (ZnO) is a semiconductor material of the II-VI family, the difference in electronegativities between zinc and oxygen produces a high degree of ionicity in its bond, making it one of the most ionic compounds of the family (Pérez-Larios *et al*., 2012), This causes a considerable repulsion between its charge clouds, making its most stable crystalline structure hexagonal, in this structure the atoms are far apart to compensate for these repulsions, so each atom of zinc is surrounded by four oxygen atoms; this crystalline structure belongs to the space group P6mc(C46v), this can be described by a combination of alternating planes of oxygen atoms and planes of zinc atoms, its pressure is and temperature are: a = 3. 253 A and c = 5.213 A. ZnO it´s a semiconductor and piezoelectric material used in the manufacture of optoelectronic devices, sensors, transducers, and related to biomedicine. Due to its absorption in the ultraviolet region, it is added in particulate form to cosmetic creams and sunscreens for skin protection (Di Mauro *et al*., 2017).

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ZnO antimicrobial activity

The synthesized zinc oxide nanoparticles (NP's-ZnO) also demonstrated potential as antimicrobial agents. Such property is due to three main mechanisms of action: (a) generation of ROS; (b) release of Zn2+ ions and (c) accumulation of NP's-ZnO in the cell membrane. The release of ROS in aqueous solutions is triggered by light (both ultraviolet and visible) that generates electron-hole pairs on the surface of ZnO. In the valence shell of the surface, atoms cause the formation of hydroxyl radicals, singlet oxygen or superoxide anion, and even hydrogen peroxide. On the other hand, Hirota *et al*. (2010) noted that even under dark conditions NP's-ZnO can stop the growth of *E. coli* and *S. aureus* which they attribute mainly to the release of superoxide anion or alternative modes of action of ZnO. The reactive oxygen species generated cause leakage of intracellular contents due to oxidative damage to bacterial cell membrane proteins and lipids, leading to eventual cell death as shown in Figure 1. In addition, it has been determined that such oxidative stress has a weak mutagenic potential in microorganisms. (Di Mauro *et al*., 2017).

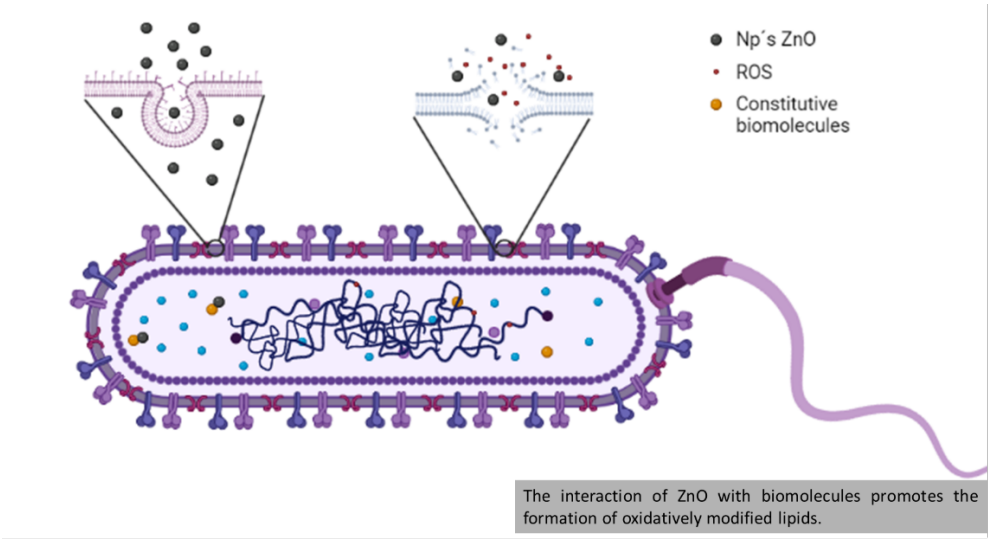

Figure 1. Scheme of proposed pathways for bacterial inhibition by ZnO nanoparticles.

Therefore, in this work, a mixture was made through the impregnation of mesoporous materials and the extract of the by-product of *Hibiscus sabdarifa L.* assisted by ultrasound, to evaluate its possible synergistic activity and its potential antibacterial effect.

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Material and Methods

Nanoparticle synthesis: zinc nitrate, polyethylene glycol (PEG), ethanol, ammonium hydroxide (NH_4OH). Biological material: *Listeria monocytogenes* (*L. monocytogenes* ATCC 15313), *Staphylococcus aureus* (*S. aureus* ATCC 33862), *Salmonella paratyphi* (*S. paratyphi* ATCC 9150), *Streptococcus mutans* (*S. mutans* oral cavity isolate) and *Escherichia coli* (*E. coli* ATCC 8789). Vegetal material: *Hibiscus sabdariffa L.* dehydrated purchased locally in Nayarit.

Extract of by-products of *Hibiscus sabdariffa* L

A conventional aqueous extraction was performed with 250g of dehydrated Jamaica flower in 2 L of purified water at 90 °C for 15 min. The calyx obtained was dried at 35 °C/10 h in an air-circulating oven (Scorpion Scientific, A-52055, Mexico). The dried material was ground in a food processor (NutriBullet, NBR-0804B, Los Angeles, CA, USA), and sieved (0.5 µm) to obtain a homogeneous contact surface. A solution was prepared in distilled water with 7.5 % of the calyx powder, ultrasonicated (Hielscher, UP400S), under the conditions of 55 % amplitude, cycle: 0 .7 for 15 min. The extract was centrifuged (600 rpm at 4 °C/10 min), and the supernatant was identified as an ultrasound-assisted extract of the Jamaica by-product (EUSJ).

Synthesis of ZnO

The nanoparticles were synthesized by the sol-gel method (Razura-Carmona *et al*., 2022a), and were obtained using zinc nitrate as a precursor to zinc oxide (ZnO). Used 2 g of PEG, and 10 g of organic salt were homogenized within 140 mL of ethanol, the solution was adjusted to pH = 7 with NH_4OH, then the solution was heated with reflux (70 °C/3 h). The gel formed was dried (70 °C/24 h), The product was ground until a fine powder was obtained with a mortar, then the obtained xerogel was annealed at 500 °C/5 h under a controlled atmosphere with a heat ramp of 1 °C/min and we ended up grinding again in a mortar until obtaining fine dust.

Impregnation

ZnO nanoparticles were activated in dry heat (110 °C/2 h), incorporated into EUSJ at concentrations (P/V) of 0.5 % (T0.5 %), 1 % (T1.0 %), and 1.5% (T1.5 %) with homogenization for 16 h at 25 °C, then the treatments were freeze-dried (-40 °C, 0.125 mBar; Labconco, FreeZone 6; Kansas, MO, USA) (Rodríguez-Barajas *et al*., 2022).

Optical characterization

The treatments and control were recorded at a wavelength of 4000 to 400 cm^{-1} and the absorption spectra were obtained with a UV-vis spectrophotometer (Shimadzu UV-2600, Tokyo, Japan) with an integration sphere for diffuse reflectance studies. From the plot, the energy of the explosive gap was calculated using the Plank equation (1).

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$$Eg = 1239.8/\lambda \quad (1)$$

Where (Eg) = band gap energy (eV), and wavelength (λ) = absorption peak value.

The ZnO was observed under a scanning electron microscope (SEM) (Tescan, MIRA3 LMU, London, UK). The samples were sputter-coated with gold before observation under SEM. Both low and high-magnification images were obtained to confirm the uniformity of the particle sizes and to determine the exact size of the particle, respectively. The high-magnification SEM images were interpreted by ImageJ software to determine the size of the particles (Razura-Carmona *et al.*, 2022a).

The EUSJ-impregnated treatments were observed in a SEM (SNE, 3200M, Viontec) to evaluate changes in the morphology of the nanomaterials.

Minimum inhibitory concentration (MIC)

L. monocytogenes, *S. aureus*, *Salmonella paratyphi*, *Streptococcus mutans,* and *Escherichia coli* strains were reactivated in nutrient broth at 35 ± 1 °C/ 18 h, once the culture was obtained it was adjusted to 0.5 optical density (DensiCHEK™ Plus Standards, USA). Two hundred µL of adjusted culture was added in a microplate, then eight microdilutions (120 - 20 µg/mL) of each treatment were made. They were incubated at 35 ± 1 °C/ 24 h and plate was rebated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) (Razura-Carmona *et a*l., 2022b).

Results and Discussion

Scanning electron microscopy

One of the most important parameters in the synthesis of materials is the particle size distribution, since being vehicles of substances provides a starting point to the behavior of its release, so that the more homogeneous its size will be in the same order the release of the charged molecules (Razura-Carmona *et al*., 2022c). Figure 2 shows the micrographs of uncharged ZnO, (a) shows an area where a cluster of the synthesized powder is identified (b) represents the same area but at higher magnification, (c) and (d) are representative samples of individual nanoparticles used to measure and evaluate morphology, this information is translated to Figure 2 (e).

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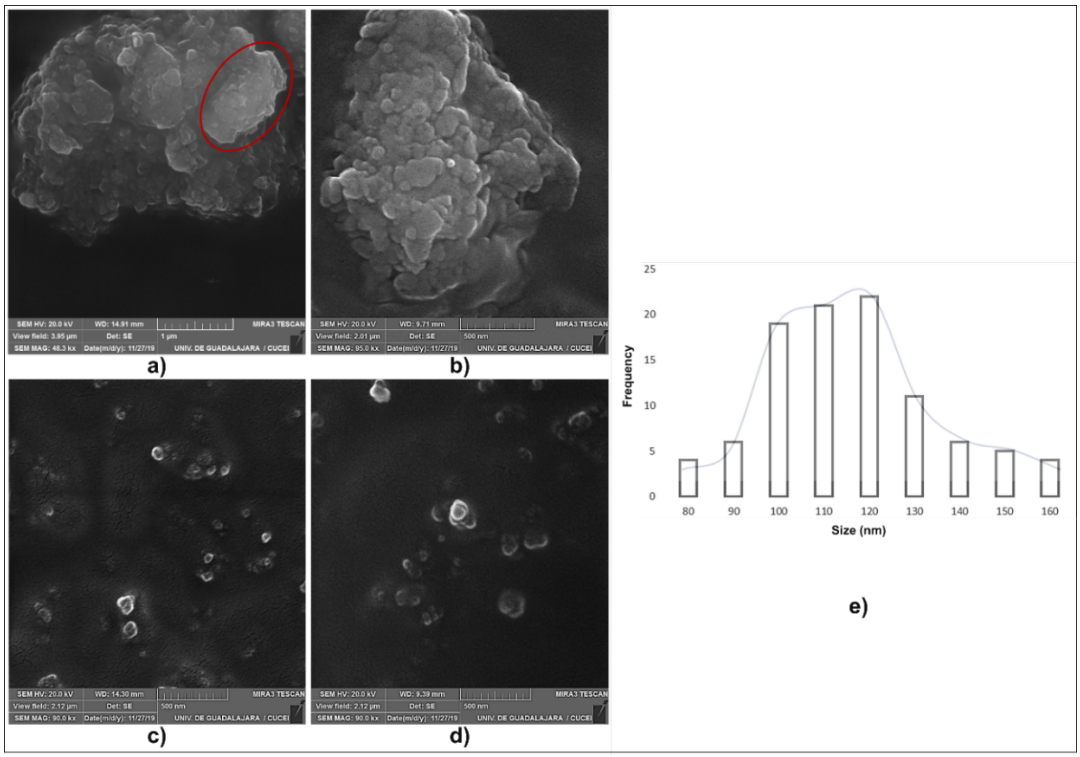

Figure 2. Micrographs of zinc oxide nanoparticles and particle size distribution; a) marks the area of the micrograph b), c) and d) random areas to measure individual particle size.

The monomodal distribution represents a homogeneous particle size, being the 120-100 nm dimensions the most abundant for these materials, Aquino *et al*. (2018) synthesized ZnO by the precipitation method and obtained average particle sizes of 90.4 ± 10.6 nm, Razura-Carmona *et al.* (2022b) mention that not only the material synthesis methodology can affect the material size, also the oxide precursor has impact on the shape and size of the particles. Therefore, the size obtained in this study is within the quantifiable parameters for ZnO nanoparticles.

One of the most widely used techniques in the ceramics industry is impregnation with metals since this contributes to the development of materials with fewer pores, which gives them greater strength; however, structurally, the original structure is also modified, so the analysis of the morphology after impregnation is important for the study of nanomaterials (Guerrero *et al.,* 2015). Figure 3 shows the micrographs of the different treatments impregnated with EUSJ, (a) T0.5 %, (b)T1.0 %, (c) and (d) T1.5 % at different magnification. In these figures there is no structural modification of the ZnO nanoparticles, the methodology used is not invasive for the material since

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we do not use pressure to impregnate. As ZnO is a mesoporous structure, the molecules present in the extract will be trapped inside the nanoparticle, however; others will be attached to the surface, this will depend on the size, polar surface area, charge, and other parameters to determine the number of molecules available for each particle (Bouzid *et al*., 2015; Suarez & Brito 2020; Razura-Carmona *et al*., 2022a).

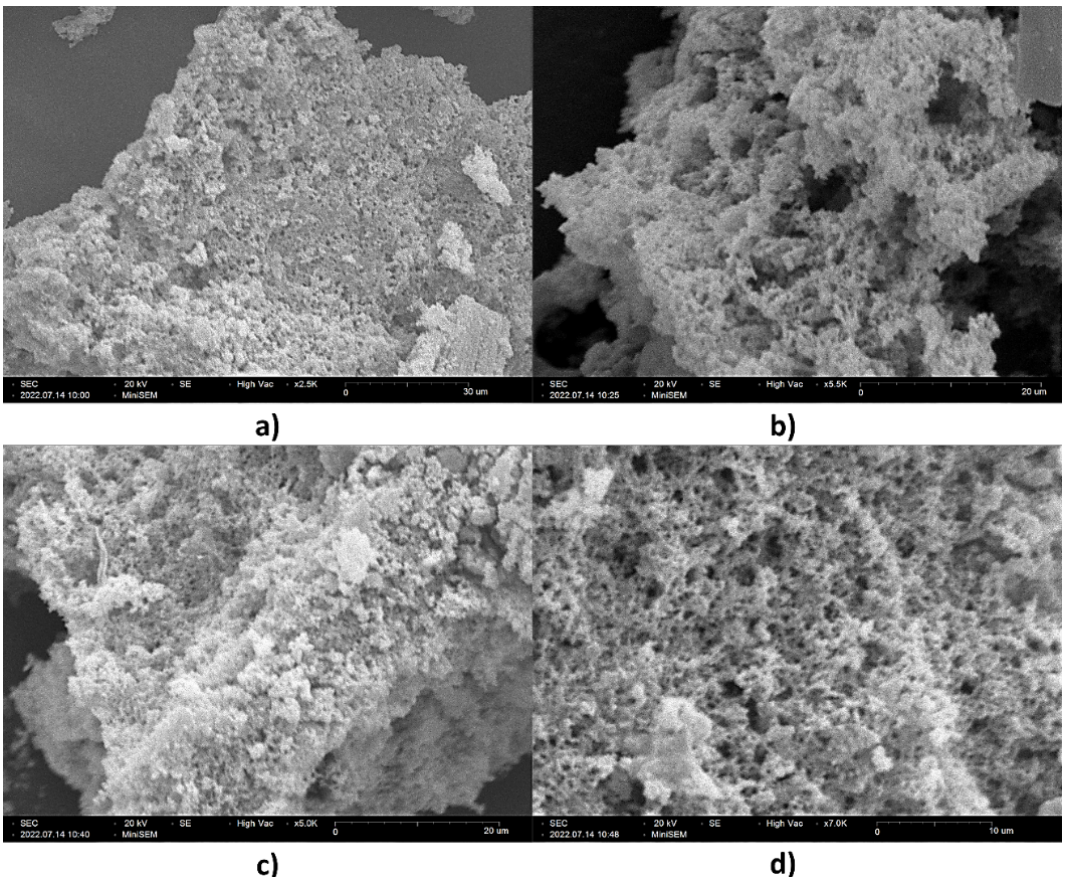

Figure 3. Micrographs of ZnO nanoparticles impregnated with ultrasound-assisted extract of by-products of *Hibiscus sabdariffa L.* ; a) T0.5 %, b) T1.0 %, c), and d) T1.5 %.

UV-vis

The size of nanoparticles plays an important role in modifying all the properties of materials. Therefore, the size evolution of semiconducting nanoparticles such as ZnO nanoparticles is

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essential to explore the properties of materials. UV-visible absorption spectroscopy is a widely used technique to examine the optical properties of nanoparticles. The absorption spectrum of ZnO powder is shown in Figure 4. In other studies, ZnO presents a strong absorption band at 355 nm, however; in Figure 4 a) and b) three excitonic absorption peaks are found at 220, 273, and 333 nm due to the presence of Jamaican extract as described by Navidad-Murrieta *et al*. (2020). Figure 4 c shows that the T0.5 %, T1.0 %, and EUSJ treatments do not present the signal at 273 nm; Razura-Carmona *et al*. (2022c) describe the specific signal for the material between 280 and 270 nm; therefore, the presence of EUSJ in higher concentration in the T1.5 % treatment decreases the ZnO signal. 5 % decreases the signal of ZnO, on the other hand, this is also attributed to the impregnation process because ZnO nanoparticles are mesoporous structures bioactive compounds are attached to the structure of the material, so the signal of the nanomaterial is well below the wavelength of the forbidden band of 358 nm (Eg = 3.46 eV) (Siddique *et al.,* 2018).

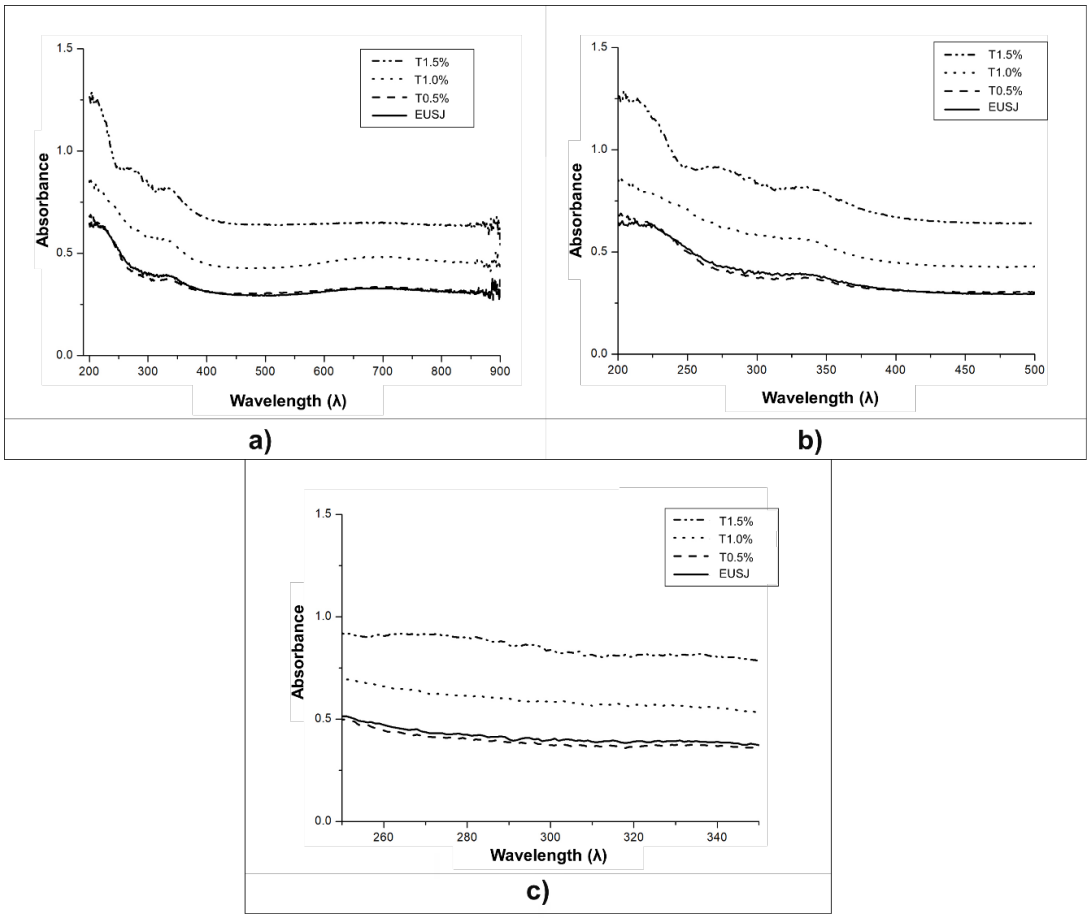

Figure 4. UV-vis spectrum of treatments and control (T0.05 %, T1.0 %, T1.5 %, EUSJ).

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Minimum inhibitory concentration (MIC)

The results obtained in Table 1 show that the three concentrations of nanoparticles impregnated with EUSJ show antimicrobial activity against *L. monocytogenes*, *S. aureus*, *S.* paratyphi, *S. mutans* and *E. coli* strains, with T0.5 % being the least effective treatment compared to T1.0 % and T1.5 %; however, there is no significant difference (α = 0.05) between the use of 1.0 % and 1.5% concentration of ZnO to the incorporation of EUSJ. *S. aureus* and *S. mutans* showed higher sensitivity to ZnO treatments but did not show the same behavior with EUSJ. Razura-Carmona *et al*. (2022b) describe the minimum inhibitory concentration (MIC) for unloaded ZnO nanoparticle treatments is 250µg/mL for *E. coli*, *L.monocytogenes* , and *S. aureus* strains. So, the result shown is a potentiated effect of EUSJ incorporated into the ZnO matrix, similar results are described by Razura-Carmona *et al*. (2022b) using an optimized extract of mangiferin for impregnation resulting in MIC = 180µg/mL.

Rajeshkumar *et al.* (2018) demonstrated that there is a retained portion inside the mesoporous structure of ZnO, which confers stability for the chemical structures allowing a gradual release of the phytochemicals. Fu *et al*. (2011) described that using the precursor Zinc sulfate they obtained a 60 % retention of mangiferin from an extract, occurring after the third hour of exposure in a liquid medium its first release.

Table 1. Minimum inhibitory concentration (MIC) of treatments on *L. monocytogenes, S. aureus*, *S. paratyphi*, *S. mutans*, and *E. coli*.

Treatments	MIC (µg/mL)				
	L. monocytogenes	***S. aureus***	***S. paratyphi***	***S. mutans***	***E. coli***
EUSJ	200^{e}	200^{e}	200^{e}	200^{e}	200^{e}
T0.5 %	160^{d}	140^{c}	160^{d}	140^{c}	160^{d}
T1.0 %	100^{b}	80^{a}	100^{b}	80^{a}	100^{b}
T1.5 %	100^{b}	80^{a}	100^{b}	80^{a}	100^{b}

EUSJ: ultrasonic assisted extract of *Hibiscus* by-product, T 0.5 %: extract added with 0.5 % zinc oxide, T1.0 %: extract added with 1.0 % zinc oxide, T1.5 %: extract added with 1.5 % zinc oxide, to the statistical group.

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Conclusions

ZnO nanoparticles synthesized with zinc nitrate precursor by the sol-gel method are shown to be a suitable vehicle to encapsulate bioactive molecules present in extracts, impregnation is an economical and non-invasive process to load such metabolites. The development of ZnO nanoparticles impregnated with ultrasonic extracts of by-products of *Hibiscus sabdariffa* L shows a potential antimicrobial effect, so the implementation of this type of materials could be suggested in future research in the development of sanitizers.

Authors' contribution

Experimental, F.F.R.-C., T. P. A.-M., and J.A.S.-B.; methodology S.G.S.-A, A.P.-L, P. U. B.-R. and A. Y. B.-D.; investigation, F.F.R.-C.; writing—original draft preparation, F.F.R.-C.; writing—review and editing, A.P.-L. and M. I. G.-P..; project administration, S.G.S.-A.; funding acquisition, Translate, J.A.S.-B. All authors have read and agreed to the published version of the manuscript.

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Ethical statements

Not applicable for studies not involving humans or animals.

Informed Consent Statement

Not applicable for studies not involving humans or animals.

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Conflicts of Interest

The authors declare no interest conflicts.

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