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Green synthesis of gold nanoparticles using Damiana induce antiseptic activity against gram-negative bacteria

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Abstract

Interest in green synthesis methods for nanoparticle production with potential use in biology, pharmacy, and medicine is a forefront field in research. Among the routes described in the literature, the use of plant extracts as reducing and capping agents is one of the most promising. *Turnera diffusa* commonly called Damiana is a wild shrub traditionally used as aphrodisiac, diuretic, and for cancer and diabetes treatments. Its extracts are rich in flavonoid, phenolic, terpenoid, and other active compounds that possess antiallergic, antiviral, and antifungal activities. Besides, flavonoids have been reported to have a redox potential

sufficient for metal ion reduction. Thus, this study reports a novel and facile method for green gold nanoparticle (AuNPs) synthesis using *T. diffusa* extracts. The samples were fully characterized by scanning electron microscopy, acrylonitrile butadiene styrene ultraviolet-visible, dynamic light scattering, X-ray photoelectron, and inductively coupled plasma optical emission spectroscopies. The results showed high efficiency of *T. diffusa* extracts to reduce and stabilize the AuNPs which showed spherical shape, 24 nm diameter, negative surface charge, and high colloidal stability at biological pH and alkaline media. Additionally, its antimicrobial activity was evaluated showing high antiseptic capacity against gram-negative *Salmonella enteritis* and *Escherichia coli* ATCC 8739 bacteria.

Keywords: Green Synthesis, AuNPs, *Turnera Diffusa* Extracts, Antimicrobial NPs

Introduction

The first reports on colloidal gold synthesis for applications in biology and medicine date back before 1583, when David de Planis-Campy prepared colloidal gold in water as a "longevity elixir" [1]. Since that day, the application of gold nanoparticles (AuNPs) in the biomedical field has been extremely wide. Its uses involve biolabeling, diagnosis, plasmon biosensing, vaccination, photothermal cancer therapy, immunoassay, as a vehicle for transporting drugs, antigens, DNA, and some other applications [2–10].

AuNPs are commonly synthesized by colloidal chemistry methods. The most common oxidized compound is $HAuCl_4$, which is reduced by agents such as sodium citrate, sodium borohydride, hydrazine, ascorbic acid, and more. These methods can produce particles with controlled geometrical and optical properties, narrow size distribution, and can functionalize the nanoparticle surface [11,12]. However, it is important to note that some of these methods

also produce several toxic by-products, so the development of ecologically friendly routes remains a research challenge [13].

Currently, the use of green synthesis methods to produce colloidal nanoparticles has been a forefront field in research. Green synthesis is ecologically friendly, cost-effective, does not use high temperatures or pressures, and reduces the generation of toxic chemical by-products [14]. In the production of AuNPs, a wide variety of derived compounds from algae, bacteria, fungi, viruses, and plant extracts have been used [14–18]. However, among all these compounds, the use of plant extracts -as reducing and stabilizing agents- is of particular interest since most plants are generally inexpensive, available, and non-toxic. Several extracts of *Salvia officinalis* [19], *Lippia citriodora* [14], *Sterculia acuminata* [20], *Pelargonium graveolens* [21], or *Punica granatum* [22] have been used as reducing and capping agents in AuNP green synthesis, suggesting that some water-soluble organic compounds including flavonoids can reduce and stabilize Au (III) [16]. Moreover, the results have shown that AuNPs coated with organic layers from plant extracts show biocompatibility, low toxicity, high stability, and synergy in their response as catalyzers and immunostimulant systems [8,23].

On the other hand, *Turnera diffusa* -commonly referred to as damiana- is a wild shrub that grows in tropical and subtropical regions of America. Its traditional use dates back to ancient Mayan and Mexican indigenous groups that prepared medicinal beverage for giddiness treatment and as an aphrodisiac [24,25]. Currently, several damiana products have been commercially available for their use as aphrodisiac, diuretic, and cancer and diabetes treatments [26,27]. *T. difussa* extracts contain more than 47 organic compounds comprised of flavonoids, terpenoids, saccharides, phenolics, and cyanogenic derivatives [28]. Recently, our group has reported that *T. difussa* extracts show antimicrobial activity against Gram-

negative bacterium [29]. This property -together with the possibility that *T. difussa* extracts can reduce and stabilize AuNPs- makes the AuNPs@Damiana systems a novel compound with potential uses in biotechnology and biomedicine.

In this sense, this study reports a novel and facile method for AuNP green synthesis using *T. diffusa* extracts as reducing and stabilizing agents. The AuNPs were fully characterized by scanning electron microscopy (SEM), ultraviolet-visible spectroscopy (ABS UV-VIS), dynamic light scattering spectroscopy (DLS), X-ray photoelectron spectroscopy (XPS), and inductively coupled plasma optical emission spectroscopy (ICP-OES). The samples were evaluated as antimicrobial agents against *Salmonella enteritis*, *Escherichia coli* ATCC 8739, and *Listeria monocytogenes*, showing enhanced microbial inhibition on Gram-negative bacteria compared to the control treatments.

Results and discussion

The most important reducing and capping agents in plant extracts are proteins, polysaccharides, flavonoids, and terpenoids [23]. According to our previous report *T. diffusa* extracts include at least 47 chemical compounds comprising flavonoids, terpenoids, saccharides, phenolics, and cyanogenic derivatives [29].

The complexity in the chemical structures of each compound -together with their natural intermolecular interactions- makes it difficult to assign the first steps of the process for Au (III) complexation, reduction, and stabilization. However, the reduction of gold ions to Au^0 and their agglomeration -through oligomeric clusters that form and stabilize AuNPs- can be due mainly to the functional groups CH_3, CH_2, OH, COOH or CHO exposed on flavonoids. These functional groups have demonstrated to have sufficient redox energy to reduce noble metal ions and form metallic nanoparticles [14]. This process is one of the most accepted

mechanisms in oxide and metallic nanoparticle production by using plant extracts rich in phenolic compounds [13,30]. This fact was confirmed in the SEM micrograph (Fig 1a), which showed spherical AuNPs@Damiana particles with a size distribution of 24 ± 3 nm in diameter free of agglomerations (Fig. 1b). Furthermore, Fig 1c shows the evolution on time of Localized Surface Plasmon Resonance (LSPR) during AuNP synthesis. Interestingly, these spectra showed a blueshift in their maximum peaks concerning time, which denoted a decrease in particle size. This behavior is unusual during AuNP synthesis because crystal size grows after nucleation is expected [8]. The maximum intensity in this spectrum was located at 540 nm -usually assigned to AuNPs of ~40 nm in diameter- while 15 min later its maximum absorption shifted to 530 nm that corresponded to AuNPs of ~27 nm in diameter. Likewise, a higher absorption intensity was interesting to note after 15 min of reaction, denoting a greater amount of formed AuNPs. Finally, the blueshift spectra can be assigned to the decrease in the dielectric field surrounding the AuNP surface induced by the active compounds of *T. diffusa* extracts.

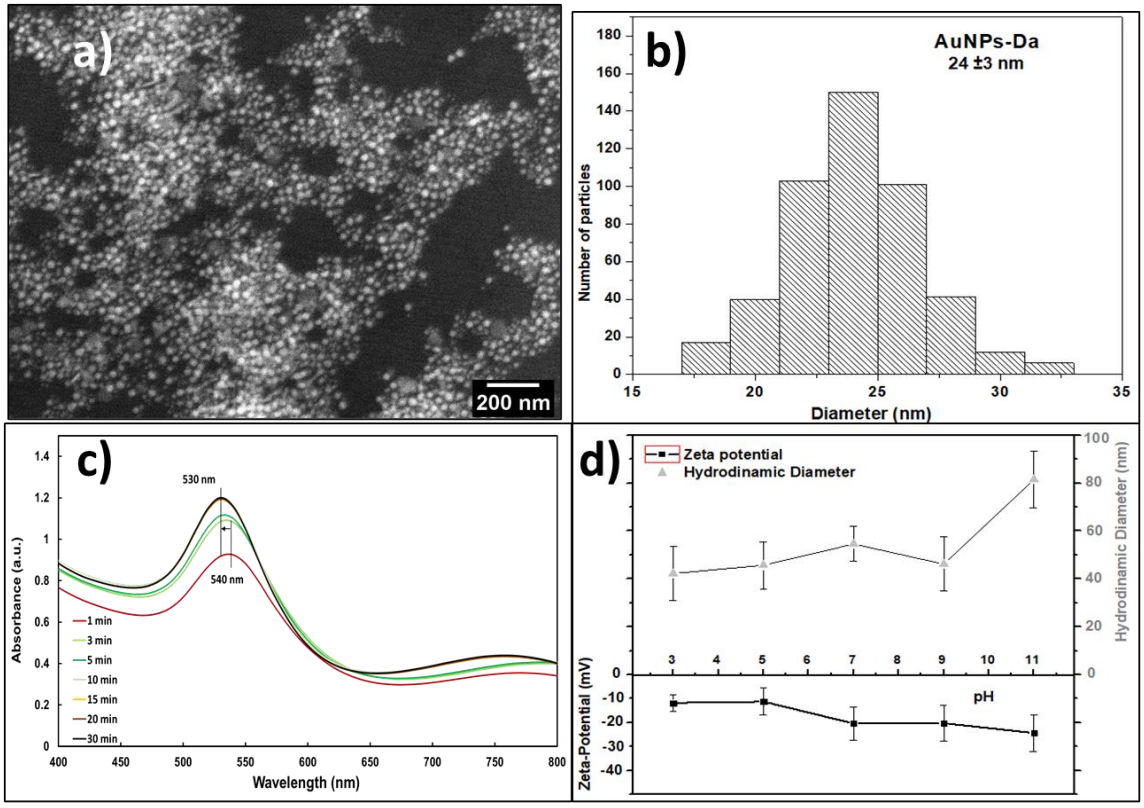

Figure 1. **(**a) Scanning electron microscopy (SEM) micrograph of AuNPs@Damiana synthesized by using *Turnera diffusa* extracts and their (b) size distribution; (c) Evolution on time of local surface plasmon resonance (LSPR) of AuNPs@Damiana; d) Zeta Potential and Hydrodynamic Diameter of AuNPs@Damiana

The DLS analysis was carried out at a pH ranging from 3 to 11. Each pH was adjusted using two aqueous solutions: (1) sodium hydroxide (0.1 M), and (2) hydrochloric acid (1% v/v). The AuNPs@Damiana analysis through SEM (Fig. 1a) and DLS (Fig. 1d) revealed different diameter scales. From pH 3 to 9, the hydrodynamic diameter (HD) maintained ~50 nm; however, in a very alkaline medium the HD increased up to ~80 nm. It is important to note, that a size result by DLS involved HD of the nanoparticles in suspension. The different

values observed for the same AuNPs@Damiana samples were a consequence of the cloud formed by molecules coming from the *T. difussa* extracts on the nanoparticle surface, which resulted in larger HD [31]. On the other hand, ZP did not show any isoelectric point, which meant the AuNPs@Damiana surface charge was always negative from pH 3 to 11 due to anion adsorption on the particle surface in all ranges. In acidic media, the ZP was ~-10 mV, while surface charge increased to ~-30 mV in alkaline medium, nanoparticles remained stable up to 3 months as observed in the experiments. The Au concentration in the AuNPs@Damiana sample was 0.0145 mg/mL determined by ICP-OES, which allowed to estimate the Au amount used in the biological assays [32].

Figure 2 depicts the XPS survey spectrum indicating that the principal elements were Au, C, and O, which confirmed the presence of organic species adsorbed on AuNPs@Damiana surface (Fig 2a). According to the deconvoluted high-resolution spectra, Fig. 2b shows two characteristic signals related to Au $4f_{7/2}$ and Au $4f_{5/2}$, where the most intense peaks at 84.0 eV and 87.7 eV were attributed to the presence of metallic gold (Au^0) and those at 84.9 eV and 88.6 eV corresponded to Au^{1+}. The C 1s spectra depicted in Fig. 2c revealed the existence of chemical states of C–C, C–H, and C=C (284.8 eV) as well as C–O, and C=O groups (287.2 eV) [33]. The presence of oxygenated organic groups was confirmed by peaks at 531.9 eV and 532.9 eV detected in the O 1s spectra (Fig. 2d), which were typically assigned to C–O/C=O and C–OH, respectively. Therefore, these oxygenated organic groups were functionalizing AuNPs@Damiana, probably interacting with Au^{1+} species.

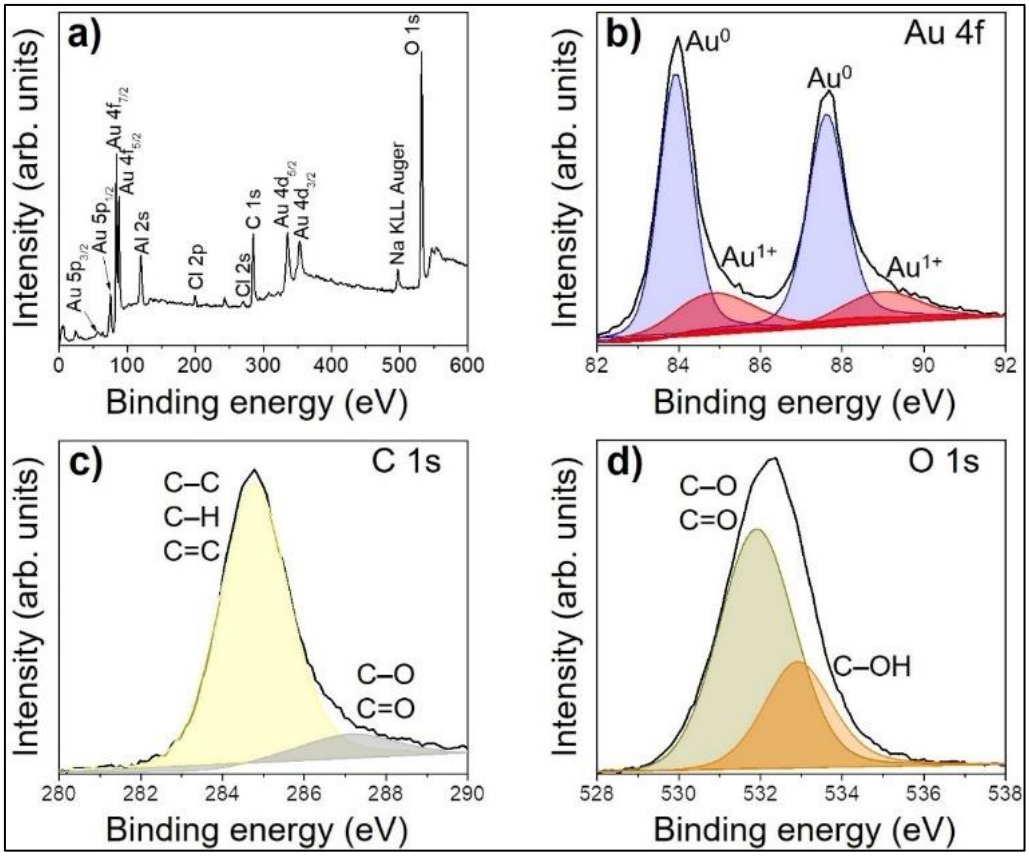

Figure 2. X-ray photoelectron (XPS) analysis of AuNPs@Damiana: (a) survey spectrum, and high-resolution spectra for (b) Au 4f, (c) C 1s and (d) O 1s. The Al 2s signal detected in (a) corresponds to the holder.

The results demonstrated that AuNPs@Damiana had growth inhibitory activity ($P < 0.05$) against Gram-negative (*S. enteritidis* and *E. coli* ATCC 8739) but not against Gram-positive (*Listeria monocytogenes*) bacterial pathogens when compared to the control group (untreated bacteria) (Figure 3). Differences on bacterial growth were not detected among the tested AuNPs@Damiana concentrations. Gram-positive bacteria have a thick cell wall composed of peptidoglycan whereas Gram-negative bacteria have a very thin cell wall with an outer

lipopolysacharide (LPS) cell membrane, allowing them more receptivity to AuNPs@Damiana effects. On this regard, several green synthetized AuNPs using plant extracts have demonstrated antibacterial activity against Gram-negative bacteria [34,35]. However, Aljabali et al. [36] did not find antibacterial activity using AuNPs synthetized with *Ziziphus zizyphus* extract. Probably, Gram-negative bacteria could be more susceptible to metallic NPs because their positive charges interacted with the negatively charged LPS, resulting in membrane holes and cell death [37]. Additionally, antibacterial activity of Damiana extracts has been demonstrated, and AuNPs adsorbed bioactive compounds could contribute to this effect [29].

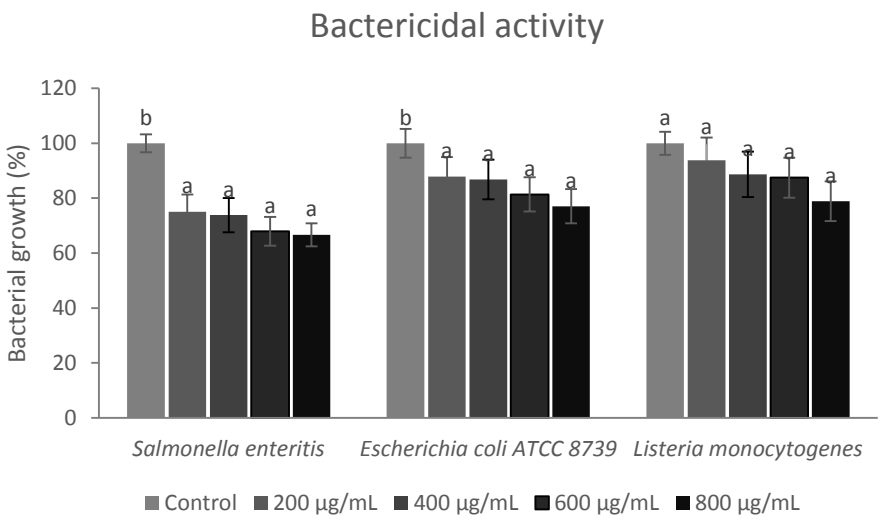

Figure 3. Green gold nanoparticle (AuNPs@Damiana) antimicrobial activity on *Salmonella enteritis*, *Escherichia coli* ATCC 8739 and *Listeria monocytogenes.*

Conclusions

Overall, these results highlighted a novel route for AuNPs green synthesis using *T. diffusa* extracts. AuNPs@Damiana showed a spherical shape with a negative surface charge induced by the organic cloud of *T. difussa* extracts adsorbed on the nanoparticle surface. Additionally, its antimicrobial activity was evaluated showing high antiseptic capacity against *Salmonella enteritis* and *Escherichia coli* ATCC 8739. The results in this study suggest that AuNPs@Damiana might be a promising system with potential use in nanomedicine and biotechnological applications.

Experimental

Extract preparation and AuNPs@Damiana synthesis

Turnera diffusa leaves were collected from a wild shrub located at Todos Santos, Baja California Sur (BCS), Mexico. Leaves were washed with cold water, dehydrated by convection, crushed and sieved to obtain small fragments (<500 µm), and boiled at 100 °C in a round-bottom flask of distilled H_2O, in which 10 g of processed leaves were added. The mixture was kept under magnetic stirring for 10 min and later left to cool down naturally. The supernatant was separated by centrifugation (3000 rpm/5 min) and lyophilized to finally obtain a dehydrated extract.

The AuNP synthesis using *T. diffusa* extracts was performed as follows. In a round-bottom flask, 7.2 mL of distilled H_2O were mixed with 1.8 mL of $HAuCl_4$ (5 mM) aqueous solution. The mixture was stirred vigorously and heated at 60 °C for 5 min to later add 1 mL of *T. diffusa* extract (10 mg/mL) with a volumetric rate of 1 mL/30 sec until color solution turned to violet. The reaction was stopped after 15 min and finally left to cool down naturally. The sample was labeled as AuNPs@Damiana.

Characterization and antimicrobial assays

Particle size and shape were examined by SEM using JEOL JSM-7600F (JEOL, Tokyo, Japan) equipment. Optical absorption was analyzed with a UV–visible spectrometer (Lambda 25, PerkinElmer, Waltham, USA). Hydrodynamic Diameter (HD) and Zeta potential (ZP) were analyzed by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS 3600 (Malvern, UK). Au quantification in samples was determined by ICP-OES (VARIAN model 730-ES, CA, USA). The surface chemical analysis of NPs was performed by XPS with a K-Alpha Thermo Scientific spectrometer (XPS, Waltham, USA) using Al Kα X-ray monochromatic source (1486.6 eV) at 12 kV and 40W.

Briefly, bacterial pathogens (*Salmonella enteritis*, *Escherichia coli* ATCC 8739 and *Listeria monocytogenes*) were cultured in Luria Broth agar (LB; BD, Franklin Lakes, USA). The microplate assay method was used for determining AuNPs@Damiana antibacterial activity [29]. Each microplate well was filled with 100 μL of LB and 20 μL of AuNPs@Damiana at concentrations of 0, 200, 400, 600 and 800 μg/mL. Then, 10 μL of bacterial suspension (1 x 10^6 cells/mL) were inoculated and incubated at 28 °C for 24 h. Bacterial growth was determined at 600 nm (iMark™, BioRad, Hercules, USA) and expressed as a percentage of the control bacteria without AuNPs@Damiana.

Declaration of Competing Interest

The authors declare that they do not have a conflict of interest.

Acknowledgments

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References

1 Antonii, F. Bibliopolio Frobeniano Hambg. **1618**, 205

2 Jeong, H.-H.; Choi, E.; Ellis, E.; Lee, T.-C. *J. Mater. Chem. B* **2019**, *7* (*22*), 3480–3496. doi:10.1039/C9TB00557A

3 Tello-Olea, M.; Rosales-Mendoza, S.; Campa-Córdova, A. I.; Palestino, G.; Luna-González, A.; Reyes-Becerril, M.; Velazquez, E.; Hernandez-Adame, L.; Angulo, C. *Fish Shellfish Immunol.* **2019**, *84*, 756–767. doi:10.1016/j.fsi.2018.10.056

4 Dykman, L.; Khlebtsov, N. *Chem. Soc. Rev.* **2012**, *41* (*6*), 2256–2282. doi:10.1039/C1CS15166E

5 Lee, N.; Lee, D.-W.; Lee, S.-M. *Biomacromolecules* **2018**, *19* (*12*), 4534–4541. doi:10.1021/acs.biomac.8b01221

6 Wang, C.; Zhu, W.; Luo, Y.; Wang, B.-Z. *Nanomedicine Nanotechnol. Biol. Med.* **2018**, *14* (*4*), 1349–1360. doi:10.1016/j.nano.2018.03.007

7 Pokharkar, V.; Bhumkar, D.; Suresh, K.; Shinde, Y.; Gairola, S.; Jadhav, S. S. *J. Biomed. Nanotechnol.* **2011**, *7* (*1*), 57–59. doi:10.1166/jbn.2011.1200

8 Hernandez-Adame, L.; Angulo, C.; Delgado, K.; Schiavone, M.; Castex, M.; Palestino, G.; Betancourt-Mendiola, L.; Reyes-Becerril, M. *Int. J. Biol. Macromol.* **2019**, *134*, 379–389. doi:10.1016/j.ijbiomac.2019.05.065

9 Ali, M. R. K.; Wu, Y.; El-Sayed, M. A. *J. Phys. Chem. C* **2019**, *123* (*25*), 15375–15393. doi:10.1021/acs.jpcc.9b01961

10 Salazar-González, J. A.; González-Ortega, O.; Rosales-Mendoza, S. *Expert Rev. Vaccines* **2015**, *14* (*9*), 1197–1211. doi:10.1586/14760584.2015.1064772

11 Sengani, M.; Grumezescu, A. M.; Rajeswari, V. D. *OpenNano* **2017**, *2*, 37–46. doi:10.1016/j.onano.2017.07.001

12 Liu, S.; Lämmerhofer, M. *Electrophoresis* **2019**, *40* (*18–19*), 2438–2461. doi:10.1002/elps.201900111

13 Ishak, N. A. I. M.; Kamarudin, S. K.; Timmiati, S. N. *Mater. Res. Express* **2019**, *6* (*11*), 112004. doi:10.1088/2053-1591/ab4458

14 Elia, P.; Zach, R.; Hazan, S.; Kolusheva, S.; Porat, Z.; Zeiri, Y. *Int. J. Nanomedicine* **2014**, *9*, 4007–4021. doi:10.2147/IJN.S57343

15 Colin, J. A.; Pech-Pech, I. E.; Oviedo, M.; Águila, S. A.; Romo-Herrera, J. M.; Contreras, O. E. *Chem. Phys. Lett.* **2018**, *708*, 210–215. doi:10.1016/j.cplett.2018.08.022

16 Camas, M.; Celik, F.; Camas, A. S.; Ozalp, H. B. *Part. Sci. Technol.* **2019**, *37* (*1*), 31–38. doi:10.1080/02726351.2017.1287794

17 Molnár, Z.; Bódai, V.; Szakacs, G.; Erdélyi, B.; Fogarassy, Z.; Sáfrán, G.; Varga, T.; Kónya, Z.; Tóth-Szeles, E.; Szűcs, R.; Lagzi, I. *Sci. Rep.* **2018**, *8* (*1*), 3943. doi:10.1038/s41598-018-22112-3

18 Slocik, J. M.; Naik, R. R.; Stone, M. O.; Wright, D. W. *J. Mater. Chem.* **2005**, *15* (*7*), 749–753. doi:10.1039/B413074J

19 Oueslati, M. H.; Tahar, L. B.; Harrath, A. H. Green *Chem. Lett. Rev.* **2020**, *13* (*1*), 18–26. doi:10.1080/17518253.2019.1711202

20 Bogireddy, N. K. R.; Hoskote Anand, K. K.; Mandal, B. K. *J. Mol. Liq.* **2015**, *211*, 868–875. doi:10.1016/j.molliq.2015.07.027

21 Shankar, S. S.; Ahmad, A.; Pasricha, R.; Sastry, M. *J. Mater. Chem.* **2003**, *13* (*7*), 1822–1826. doi:10.1039/B303808B

(22 Dash, S. S.; Bag, B. G. *Appl. Nanosci.* **2014**, *4* (*1*), 55–59. doi:10.1007/s13204-012-0179-4

23 Singh, P.; Kim, Y. J.; Zhang, D.; Yang, D. *Trends Biotechnol*. **2016**, *34* (*7*), 588–599. doi:10.1016/j.tibtech.2016.02.006

24 Kumar, S.; Sharma, A. *Pharm. Biol.* **2006**, *44* (*2*), 84–90. doi:10.1080/13880200600591758

25 Tousson, E.; Hafez, E.; Zaki, S.; Gad, A.; Elgharabawy, R. M. *Biomed. Pharmacother.* **2020**, *132*, 110819. doi:10.1016/j.biopha.2020.110819

26 Avelino-Flores, M. del C.; Cruz-López, M. del C.; Jiménez-Montejo, F. E.; Reyes-Leyva, J. *J. Med. Food* **2014**, *18* (*3*), 299–305. doi:10.1089/jmf.2013.0055

27 Esquivel- Gutiérrez, E. R.; Saavedra- Molina, A.; Alcaraz- Meléndez, L. *Faseb J.* **2016**, *30*, 1100.8-1100.8. doi:10.1096/fasebj.30.1_supplement.1100.8

28 Zhao, J.; Pawar, R. S.; Ali, Z.; Khan, I. A. *J. Nat. Prod.* **2007**, *70*, 289–292. doi:10.1021/np060253r

29 Reyes-Becerril, M.; Ginera, P.; Silva-Jara, J.; Macias, A.; Velazquez-Carriles, C.; Alcaraz-Meléndez, L.; Angulo, C. *Fish Shellfish Immunol.* **2020**, *100*, 418–426. doi:10.1016/j.fsi.2020.03.045

30 Panche, A. N.; Diwan, A. D.; Chandra, S. R. *J. Nutr. Sci.* **2016**, 5. doi:10.1017/jns.2016.41

31 Ortega-Berlanga, B.; Hernández-Adame, L.; del Angel-Olarte, C.; Aguilar, F.; Rosales-Mendoza, S.; Palestino, G. *Chem. Eng. J.* **2020**, *385*, 123414. doi:10.1016/j.cej.2019.123414

32 Jia, H.; Gao, X.; Chen, Z.; Liu, G.; Zhang, X.; Yan, H.; Zhou, H.; Zheng, L. *CrystEngComm* **2012**, *14*, 7600–7606. doi:10.1039/C2CE25840D

33 Dinya, Z.; Komáromi, I.; Lévai, A. *J. Electron Spectrosc. Relat. Phenom.* **1992**, *59*, 315–325. doi:10.1016/0368-2048(92)87011-A

34 Lokina, S.; Suresh, R.; Giribabu, K.; Stephen, A.; Lakshmi Sundaram, R.; Narayanan, V. Spectrochim. Acta. A. *Mol. Biomol. Spectrosc.* **2014**, *129*, 484–490. doi:10.1016/j.saa.2014.03.100

35 Markus, J.; Wang, D.; Kim, Y.-J.; Ahn, S.; Mathiyalagan, R.; Wang, C.; Yang, D. C. *Nanoscale Res. Lett.* **2017**, *12*, 46. doi:10.1186/s11671-017-1833-2

36 Aljabali, A. A. A.; Akkam, Y.; Al Zoubi, M. S.; Al-Batayneh, K. M.; Al-Trad, B.; Abo Alrob, O.; Alkilany, A. M.; Benamara, M.; Evans, D. *J. Nanomater. Basel Switz.* **2018**, *8* (*3*). doi:10.3390/nano8030174

37 Otunola, G. A.; Afolayan, A. *J. Biotechnol. Biotechnol. Equip.* **2018**, *32*, 724–733. doi:10.1080/13102818.2018.1448301