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# Simplification of gold nanoparticle synthesis with low cytotoxicity using a greener approach: opening up new possibilities

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Gold nanoparticles (AuNPs) have diverse applications in the diagnosis and treatment of ailments. This study describes an extremely simplified synthesis of AuNPs using antioxidant-rich pollen extract as a local natural source. Ultraviolet-visible (UV-vis) spectroscopy, X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM) were used to characterize the synthesized AuNPs; strong UV-vis absorption at 534 nm confirmed their formation, the XRD pattern showed the presence of a crystalline structure, and TEM images showed them to be spherical nanoparticles with an average size of  $9.3 \pm 2.9$  nm. As synthesized AuNPs remained stable for up to two months under laboratory conditions without any sedimentation or change in the absorption value, presumably due to the protection afforded by the capping agents from pollen. AuNPs revealed low toxicity effects on MCF-7 and HUVECs cell lines (with an  $IC_{50}$  value of  $\sim 400 \mu\text{g mL}^{-1}$  for both the cell lines). The proposed method did not use any hazardous materials or high-energy consuming devices; thus this efficient protocol may be adapted for large-scale production using local resources.

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## 1. Introduction

Metallic nanoparticles (NPs), including gold, iron, nickel, copper and silver, are the subject of many investigations due to their wide range of applications in various scientific disciplines such as medicine, physics and chemistry.<sup>1–4</sup> Gold nanoparticles (AuNPs) have been used in biological sciences, pharmaceuticals, catalysis, immunization and cancer therapy.<sup>5,6</sup> There are different physical,<sup>7</sup> chemical<sup>8</sup> and biological<sup>9</sup> methods for the synthesis of NPs. NP synthesis by physical and chemical methods has some disadvantages namely multi-stage synthesis processes,<sup>10</sup> dependence on specialized equipment,<sup>11</sup> energy dependence,<sup>12</sup> and use hazardous chemicals<sup>13</sup> that pollute the environment.<sup>3,14,15</sup> Therefore, direct or indirect biological synthesis using living organisms such as bacteria, fungi and

plants has been considered.<sup>16,17</sup> On the other hand, due to the potential pathogenicity of some microbial agents and difficulty of cultivation of actinomycetes, bacteria and fungi, and the need for complex and expensive media, nowadays biological synthesis of NPs, especially gold, iron, and silver using different herbs has been considered. Among others, synthesis of gold nanostructures has been reported using a variety of plants such as *Aloe vera*,<sup>18</sup> alfalfa,<sup>19</sup> *Citrus aurantium* L. blossoms and *Rose damascena* oils,<sup>20</sup> plant surfactants and biological approaches.

The deployment of biological methods for NPs synthesis is protective to the environment, as these protocols, in the most extent, entails low-energy consumption, and avoids the use of solvents and toxic chemicals. The aim of this study has been to develop an eco-friendly synthesis of AuNPs using pine pollen (from male pinecones of the pine tree), as a low-cost procedure that exploits the safe bio-resource for the greener synthesis of AuNPs at room temperature without using environmentally harmful substances. Pollen extracts are known to be rich source of antioxidants and anti-inflammatory agents<sup>21</sup> and hence can be ideal source for reduction of salts. The synthesized AuNPs are characterized and their toxicity evaluation revealed them to be safe.

## 2. Materials and methods

### 2.1. Materials and cells

Hydrogen tetrachloroaurate(III) ( $\text{HAuCl}_4$ ) tetrahydrate was purchased from Merck (Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-

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diphenyltetrazolium bromide (MTT) was procured from Sigma-Aldrich (USA), and dimethyl sulfoxide (DMSO) was obtained from Sequoia Research Products. A human breast cancer cell line of MCF-7 and a human umbilical vein endothelial cell (HUVEC) line were secured from cell bank of Stem Cell Research Center, Kerman University of Medical Sciences. The cells were cultured in RPMI 1640 medium at 37 °C in a 5% CO<sub>2</sub> atmosphere with a 95% humidity. Deionized water was used throughout the study.

## 2.2. Synthesis of AuNPs

Pollen containing male pine cones were picked up from a pine tree in the spring. Male pine cones (2 g) were washed with water for 10 s to remove the surface dust, and then air dries at room temperature. Dried cones (1 g) was added to an Erlenmeyer flask containing 50 mL water, kept at room temperature for 12 h, and then filtered using a Whatman filter paper (no. 40). The filtered extract had pH = 5.6. 2 mL extract was added into 10 mL HAuCl<sub>4</sub> of 0.1 mM dissolved in water and manually mixed to complete the reaction within 2 h and the ensuing mixture was used for further evaluations.

## 2.3. Characterisation of nanoparticles

In order to investigate the physicochemical properties of AuNPs, the reaction mixture was evaluated by the following methods: UV-vis spectroscopy was performed by a spectrophotometer of Scan Drop from Analytic Jena (Germany) in the wavelength range of 400–700 nm. X-ray diffraction (XRD) analysis was performed by an X-ray diffractometer of X PERTPRO, PAN-alitical (the Netherlands) at a radiation wavelength of 1.54 Å and 2θ range of 20°–85°. For XRD sample preparation, 90 mL of the AuNPs suspension was centrifuged at 15 000 rpm for 10 min and was washed with water three times. The final sediment was dried at 60 °C for 24 h and the resulting powder was used. Fourier-transform infrared spectroscopy (FTIR) was done using a Tensor 27 spectrometer (Germany). For FTIR sample preparation, 10 mL of the extract alone was centrifuged at 6000 rpm for 6 min, and the supernatant solution was dried at 60 °C for 48 h. The resultant precipitate was analyzed by FTIR. Separately, 10 mL of the AuNPs suspension was centrifuged at 6000 rpm for 10 min, and washed with water three times. The final sediment was dried at 60 °C for 24 h and the resulting powder was analyzed by FTIR analysis of AuNPs. The shape, size and size distribution of AuNPs were studied using transmission electron microscopy (TEM) under a Carl ZEISS microscope (Germany) at an accelerating voltage of 80 kV. For TEM sample preparation, 5 mL of the AuNPs suspension was sonicated for 5 min, a droplet of the suspension was placed on a copper grid, and let to dry at room temperature.

## 2.4. Cytotoxicity assessment of AuNPs

MTT assay was used for evaluation of cytotoxicity of AuNPs toward MCF-7 cell (as a cancerous) and HUVEC (as a normal) lines. The cell lines were exposed to serial concentrations (0.78–800 μg mL<sup>-1</sup>) of AuNPs for 24 h. After incubation, 10 μL MTT was added into each well of ELISA plates and incubated for 4 h at 37 °C. Finally, DMSO was added and optical density was measured at 590 nm.

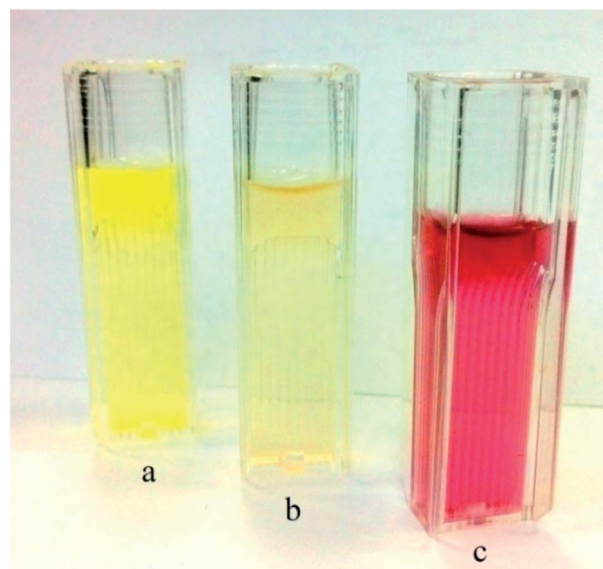


Fig. 1 Colors of HAuCl<sub>4</sub> solution (a), pine pollen extract (b), and bio-synthesized AuNPs (c).

## 3. Results

During the synthesis of AuNPs, the reaction progress could be monitored with the naked eye upon reduction of gold ions to the metallic state; color change of the extract from light yellow to red after addition of gold ions was the first sign of greener synthesis of AuNPs, (Fig. 1). The color change progression, depicting the reaction, occurred in less than an hour with no change in the color of the control extract (without addition of HAuCl<sub>4</sub>).

Absorption spectra of the pine pollen extract and the AuNPs dispersion are presented in Fig. 2. While the pine pollen extract had no noticeable absorption, the AuNPs dispersion showed a strong absorption peak at 534 nm indicating that the synthesis of AuNPs was successfully accomplished using the extract. The as-synthesized AuNPs remained stable at least for 2 months at 27 °C in dark without sedimentation or aggregation.

Fig. 3 depicts an XRD pattern of AuNPs. The diffractogram showed strong peaks at 38°, 44°, 64°, 77° and 82° which are

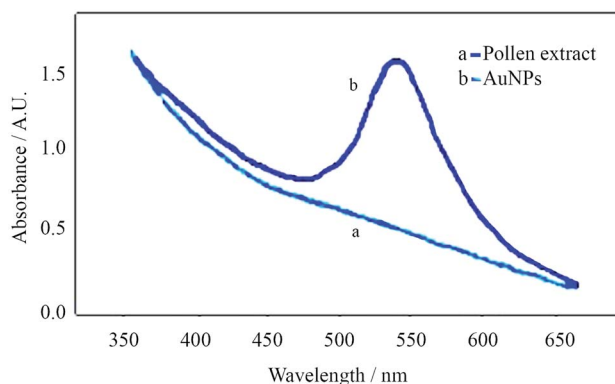


Fig. 2 Absorption spectra of the pine pollen extract (a) and the AuNPs dispersion (b).

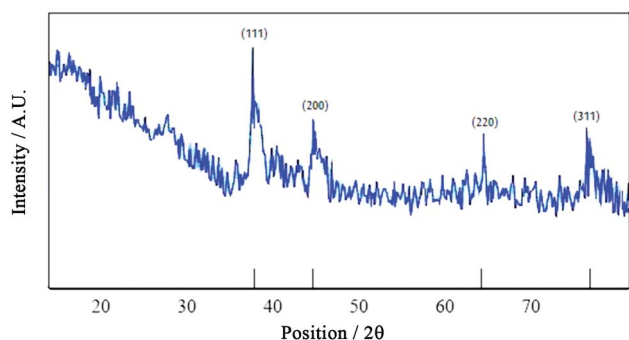


Fig. 3 XRD pattern of AuNPs.

related to crystalline planes of (1 1 1), (2 0 0), (2 2 0), (3 1 1) and (2 2 2), respectively. The XRD pattern also showed the presence of AuNPs crystals in the cubic structure.

Fig. 4 shows the FTIR spectra of the dried pine pollen extract and AuNPs. Spectrum of the extract indicated the presence of different functional groups, such as phenol, amide and carboxylic (Lee *et al.*, 2009), as part of the antioxidants. In the spectrum of AuNPs, bands located at 1628 and 1383  $\text{cm}^{-1}$  could be ascribed to carboxylic (C=O) group and C–O bond, respectively. The strong peak at 1073  $\text{cm}^{-1}$  also corresponded to an amide (N–H) group. Although the exact composition of the extract is unknown and is complex (Lee *et al.*, 2009), it appears that the aforementioned groups would play the major role in the reduction of gold ions, while the constituents bearing amide and carboxylic groups would play the major role of stabilization of AuNPs.

A TEM image from AuNPs and the corresponding size distribution histogram are shown in Fig. 5. The image showed spherical nanoparticles with an average size of  $9.3 \pm 2.9$  nm.

Cytotoxicity of the ensuing AuNPs was evaluated *in vitro*, and the results, as dependency of the cell viability on the AuNPs

concentration, are presented in Fig. 6; an  $\text{IC}_{50}$  value of  $\sim 400 \mu\text{g mL}^{-1}$  was attained for both the cell lines. Therefore, cytotoxicity of AuNPs toward both the cells was negligible, compared to anticancer drugs such as doxorubicin with an  $\text{IC}_{50}$  value of  $\sim 10 \mu\text{g mL}^{-1}$ .<sup>22</sup>

## 4. Discussion

In the present study, a greener strategy is developed for the synthesis of AuNPs without use of any chemical solvents; pine pollen extract showed displayed potential for the formation and stabilization of AuNPs. Therefore, this low-cost method, exploiting the local resources, has a potential to be used as a biological source for generating water-soluble AuNPs on a larger scale production. UV-vis absorption spectra of AuNPs is similar to those reported for chemically synthesized ones.<sup>23</sup> Change in the color of the synthesis mixture and the peak at 534 nm in UV-vis spectrum could be attributed to the surface plasmon resonance of AuNPs arising from the massive oscillations of free electrons upon interaction with light. The FTIR results confirmed stabilization of AuNPs by constituents bearing amide and acid functional groups. Up to now, proteins,<sup>24</sup> carbohydrates,<sup>25</sup> flavonoids,<sup>26</sup> *Citrus aurantium* L. blossoms and *Rose damascena* oils<sup>20</sup> have been reported as natural compounds for the synthesis of AuNPs. XRD results were quite in line with those reported for chemically synthesized AuNPs.<sup>27</sup> TEM image confirmed that the AuNPs size was smaller than those reported previously;<sup>28</sup> AuNPs in a size range of 30–60 nm have been produced using *Pycnoporus sanguineus*.<sup>29</sup> In order to inspect the repeatability and reproducibility of the synthesis method, AuNPs were synthesized under different conditions, UV-vis spectra were recorded, and relative standard deviation (RSD) of the absorbance values were obtained. Three independent syntheses were performed over one day (intra-day

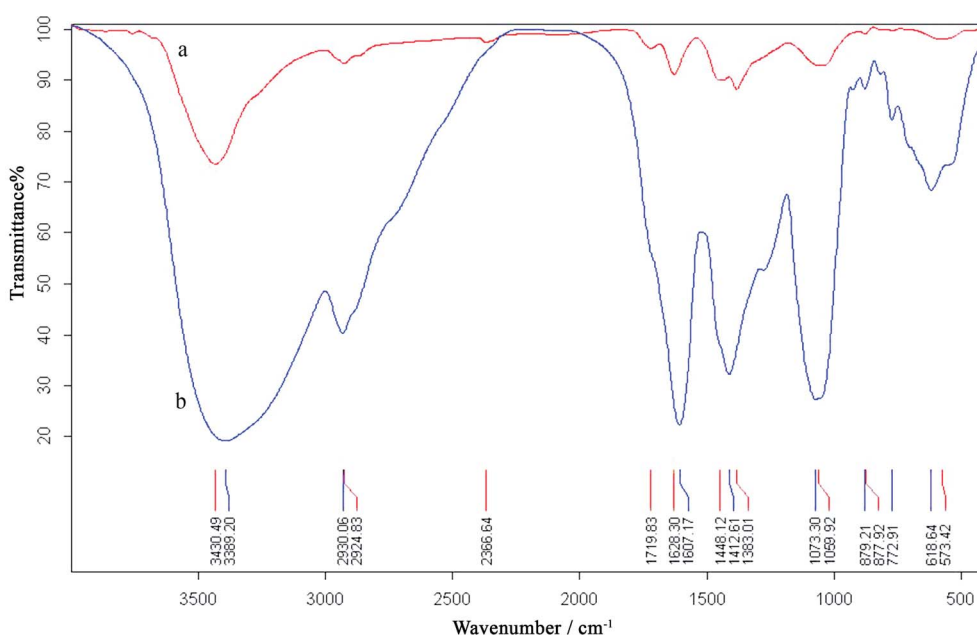
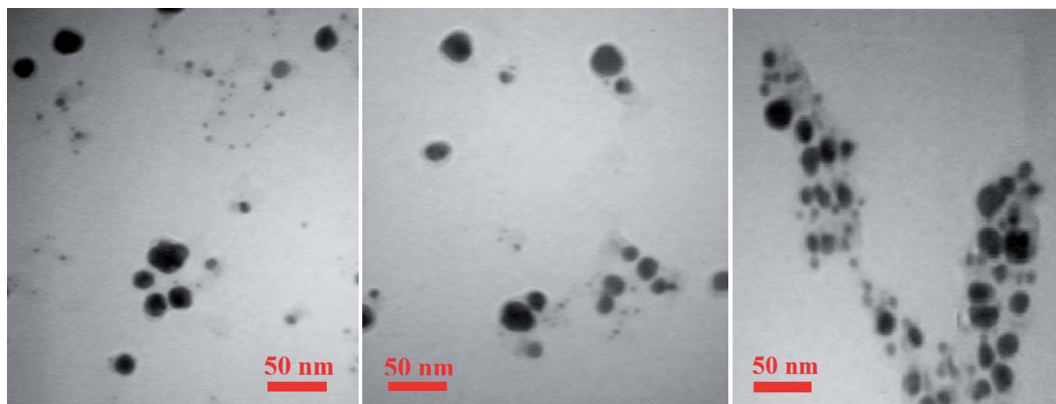
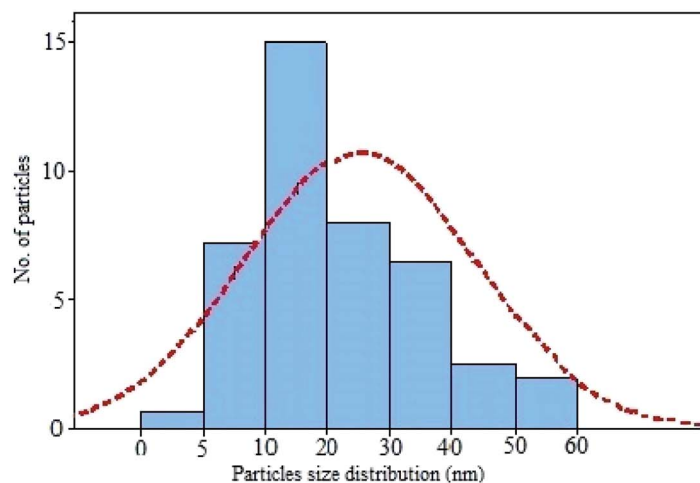


Fig. 4 FTIR spectra of the dried pine pollen extract (a) and AuNPs (b).





(A)



(B)

Fig. 5 A TEM image from AuNPs (A) and a size distribution histogram (B).

synthesis) and over three days (inter-day synthesis) using the same precursors ( $\text{HAuCl}_4$  and the extract); RSD values of 1.5% and 2.0% were obtained for these assays. In addition, three

independent extractions were conducted from pine pollen procured from the same tree, and AuNPs were synthesized; a RSD value of 5.7% was attained for the absorbance values.

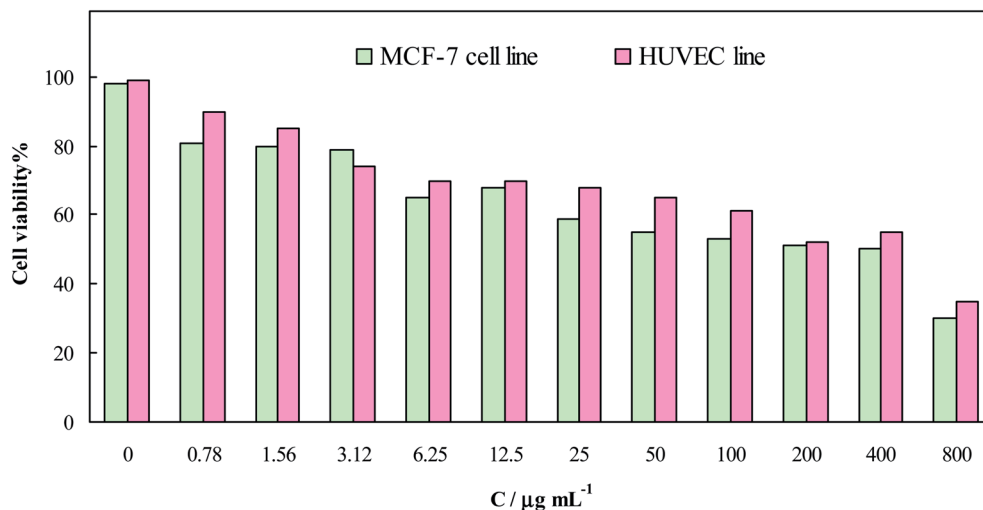


Fig. 6 Dependency of viability of MCF-7 cell and HUVEC lines on the AuNPs concentration.

**Table 1** Provides comparison with the earlier reported methods of plant-mediated AuNPs synthesis in terms of size, organs of the plant, stability, cytotoxic features

Plant	Organs of the plant	Size (nm)	Stability (days)	Cytotoxic features	Ref.
<i>Aegle marmelos</i> and <i>Eugenia jambolana</i>	Fruit	An average 18–28	More than 150	IC <sub>50</sub> ~ 163 µg mL <sup>-1</sup>	30
<i>Nerium oleander</i>	Stem bark	10–100	Several months	IC <sub>50</sub> ~ 74 µg mL <sup>-1</sup>	31
<i>Platycodon grandiflorum</i>	Leaves	3 to 80 with an average 15	NM	NM	32
<i>Pongamia pinnata</i>	Leaves	5–20	NM	NM	33
<i>Dracocephalum kotschy</i>	Leaves	7–22	150	IC <sub>50</sub> ~ 152 µg mL <sup>-1</sup> K 562 and 196 µg mL <sup>-1</sup> against HeLa cell line	34
<i>Taxus baccata</i>	Needles	Less than 20	More than 180	The maximum cell mortality of 22.15 and 26.5% were obtained after 72 h	35
<i>Moringa oleifera</i>	Flower	3 to 5 with triangular shapes	NM	An effect was not observed in the cancerous A549 cells	36
<i>Cryptolepis buchanani</i>	The whole plant	An average 11	NM	NM	37
<i>Terminalia catappa</i>	Leaves	10 to 35 with average of 21	120	NM	38
<i>Carica papaya</i> and <i>Catharanthus roseus</i>	Leaves	Less than 20	NM	Cell death was about 98% was found at 250 µg mL <sup>-1</sup> AuNPs: used against MCF7 and HepG2 cell lines.	39
<i>Jasminum auriculatum</i>	Leaves	8–37	In PBS was 2	IC <sub>50</sub> value of 104 µg mL <sup>-1</sup> against the human cervical cancer cell line	40
<b>Pine tree</b>	<b>Pine pollen</b>	<b>An average 9</b>	<b>Up to 60</b>	<b>IC<sub>50</sub> value of 400 µg mL<sup>-1</sup></b>	<b>This study</b>

<sup>a</sup> NM: not mentioned.

While three independent extractions were performed from pine pollen taken from different trees located at the same geographical region, RSD values for the synthesized AuNPs was

8.1%. Thus, the repeatability and reproducibility of the synthesis method was quite good and satisfactory (Table 1).

Although not all the reported methods for synthesis of AuNPs generally require environmentally detrimental

**Table 2** A comparison between the reported methods of AuNPs synthesis in terms of use of laboratory devices and energy consumption<sup>a</sup>

Bioresource	Water bath	Centrifuge	Mixer	Heater	Culture medium	Laminar air flow	Incubator	Ref.
<i>Streptomyces microflavus</i>	—	+	—	+	+	+	+	41
<i>Sumac</i>	—	+	+	+	—	—	+	42
<i>Garcinia mangostana</i>	—	—	+	+	—	—	+	43
<i>Black cardamom</i>	—	+	+	—	—	—	—	44
<i>Salvia officinalis</i>	—	+	+	+	—	—	+	45
<i>Lippia citriodora</i>	—	+	+	+	—	—	+	45
<i>Peanut skin</i>	—	—	+	+	—	+	+	46
<i>Deinococcus radiodurans</i>	+	+	—	+	+	+	+	47
<i>Dendropanax moribifera</i>	—	+	—	+	—	—	+	48
<i>leuropterus multiflorus</i>	—	+	+	+	—	—	+	49
<i>Ocimum sanctum</i>	—	+	+	+	—	—	+	50
<i>Aspergillus niger</i>	—	+	+	+	+	+	+	51
<i>Aspergillus terreus</i>	—	+	+	+	+	+	+	52
<i>Aegle marmelos</i>	+	—	—	+	—	—	+	53
<i>Botrytis cinerea</i>	+	+	+	+	+	+	+	54
<i>Olive</i>	—	—	+	+	—	—	+	55
<i>Melissa officinalis</i>	—	—	+	+	—	—	+	56
<i>Mentha piperita</i>	—	—	+	+	—	—	+	56
<i>Salvia officinalis</i>	—	—	+	+	—	—	+	56
<i>Marine algae</i>	—	+	+	+	—	—	+	57
<i>Actinomycetes</i>	+	+	+	+	+	+	+	58
<i>Cucurbita pepo</i>	+	—	+	+	—	—	+	59
<i>Coffea arabica</i>	+	—	+	+	—	—	+	60
<b>Pine pollen</b>	—	—	—	—	—	—	—	<b>This study</b>

<sup>a</sup> — No need, + need.





chemicals, synthesis using plants is more appropriate than those used fungal or microbial entities due to the need for additional culture and/or separation steps. Furthermore, our method described herein does not need any sophisticated laboratory devices and deploy local resources; Table 2 provides a comparison with the earlier reported methods for AuNPs synthesis from point of view of special devices with energy consumption.

In other words, the fact that no mechanical stirring was needed in this study is simply because the volumes used needed no mechanical stirring.

## 5. Conclusion

In brief, a greener synthesis of AuNPs is described method that occurs under ambient temperature and pressure without using chemical solvents. The ensuing AuNPs exhibited XRD pattern revealing the crystalline structure of AuNPs while the TEM image confirm the formation of spherical NPs with an average size of  $9.3 \pm 2.9$  nm. As synthesized AuNPs remained stable for up to two months under laboratory conditions, without any sedimentation or aggregation they displayed low toxicity indicating the possible applications in different areas of biological research. Due to simple procedure, employing a low-cost extract and high yield of AuNPs preparation, the proposed method can be scaled up for large-scale production of AuNPs and may stimulate further investigations.

## Conflicts of interest

There are no conflicts to declare.

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