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Role of *Punica granatum*-derived nanosilver in helminth protection

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Abstract

Pomegranate is a long-known edible fruit with medicinal properties. Helminth infections are among the most common human illnesses worldwide, due to a lack of suitable sanitary systems and safe drinking water sources. Although these illnesses are seldom deadly, they cause development limitations in children, which damage their school performance and make them susceptible to other illnesses. The present study has been conducted to investigate the anthelmintic activity of silver nanoparticles (AgNPs) synthesized from Pomegranate peel extract (PPE-AgNPs). Also, the cytotoxicity of PPE-AgNPs was tested in liver (Huh7), colon (HCT116) and breast (MDA-MB 231) cancer lines. Three doses were used (5, 2.5 and 1.25 mg/mL) to study the anthelmintic activity of PPE-AgNPs. *Eisenia fetida* was used as a model worm. Also, Mebendazole was used as a reference drug. Phytochemical analysis using FT-IR showed the presence of 13 compounds. In all tests, a dose-dependent effectiveness was shown. The most efficient dose, 5 mg/mL showed the time to paralysis and death were 8.118 ± 0.26 and 9.338 ± 0.14 min, respectively. In all treated worms, histological examination revealed significant malformation of surface architecture of worms. We conclude that PPE-AgNPs has strong anthelmintic properties and low cytotoxicity, stimulating its use in the biomedical field.

Keywords: Pomegranate; anthelmintic activity; cytotoxicity; silver nanoparticle.

Practical Application: *Punica granatum*-derived nanosilver against helminthiasis.

1 Introduction

Although most studies focused on using pomegranate peel in the food industry due to the presence of natural antioxidant and phenolic compounds (Kaderides et al., 2015), other investigators used pomegranate as a treatment for a variety of diseases (Kaderides et al., 2015).

In many countries, parasitic infections brought on by protozoa and helminths result in significant death and economic loss (Mehlhorn, 2001). Helminthiasis has remained a major health risk for the majority of individuals living in developing countries (El-Badry et al., 2019). The main complaints of worm infection are anemia and weakness caused by malnutrition (Jones & Berkley, 2014). Anthelmintic medicines, as they are now used, cause some problems in the human body, particularly in the liver and kidney (Tripathi, 2008; Hong, 2018). Furthermore, the high expense of medications has sparked interest in medicinal plants as a potential source of anthelmintic medications (Dkhil et al., 2019).

According to several research, the pomegranate fruit's (*Punica granatum*) belongs to Punicaceae with various parts, particularly the peel, may function as possible antimicrobial agents. As a result, a safe natural alternative to synthetic antimicrobial agents may be suggested. The high tannin content, particularly punicalagin, present in pomegranate extracts has been identified as the primary component responsible for such antibacterial action (Rosas-Burgos et al., 2017).

Pomegranate peel, which accounts for approximately 50% of the fruit weight, is distinguished by the presence of high molecular weight phenolics, ellagitannins, proanthocyanidins, complex polysaccharides, flavonoids, and significant amounts of microelements, all of which have anti-mutagenic, antioxidant, antimicrobial, and apoptotic properties (Dikmen et al., 2011; Li et al., 2006; Prakash et al., 2013; Ricci et al., 2006; Tezcan et al., 2009).

Nanoparticles are gaining traction due to their attractive characteristics. The use of harmful substances limits the uses of physical and chemical processes, despite the fact that they are more often used to create nanoparticles. Due to this issue, safe eco-friendly green processes are essential for creating nanoparticles (Rajathi et al., 2012). It is generally known that silver nanoparticles have a wide range of biological uses both in vitro and in vivo due to their extraordinary characteristics. When compared to traditional antihelmintic agents, silver nanoparticles demonstrated strong antihelmintic action (Raji et al., 2012).

In this study, we evaluated the anthelmintic effect of green synthesized silver nanoparticles using Pomegranate peel extract.

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2 Materials and methods

2.1 Collection of materials and extracts preparation

Fresh Pomegranate (*Punica granatum*) were collected from the local market of Cairo, Egypt. Then the plant's material for the study was identified and authenticated by the taxonomists at Botany Department in Helwan University.

Dried and ground peel (about 100 g) of Pomegranate were cut into small pieces then submitted to extraction with 300 mL methanol (70%) for 24 h. After extraction, the solvent was filtered and then evaporated by Rotavapor. The obtained garlic extract was stored at −20 °C until being used (Raju et al., 2008).

2.2 Synthesis of AgNPs

In accordance with Murugan et al. (2016), 5 mL of Pomegranate peel extract was combined with silver nitrate ($AgNO_3$, 8×10^{-3} M, ~ 67.93 mg) in 45×10^3 µL of methanol to produce nanosilver. The size and shape of nanosilver are then determined by transmission electron microscopy using a JEOL JEM-2100 (JEOL Ltd., Tokyo, Japan) (Murugan et al., 2016).

2.3 Infrared spectroscopy

For Pomegranate peel extract analysis, we used a Nicolet 6700 Fourier-transform infrared spectroscopy (FT-IR) optical spectrometer from ThermoScientifific (Waltham, MA, United States). We mixed the powder of the extract (10 mg) with 100 mg of potassium bromide powder (1:99 wt%) to obtain a translucent sample disk that we then loaded into an FTIR spectroscope with a scan range of 400-4000 cm−1. The chemical bonds in a molecule can be determined by interpreting the infrared absorption spectra (Pakkirisamy et al., 2017).

2.4 MTT cytotoxicity assay

According to Satyavani et al. (2012), the cytotoxic activity of PPE-AgNPs was tested in liver (Huh7), colon (HCT116) and breast (MDA-MB 231) cancer lines by using the 3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. To assess the half maximal cytotoxic concentration (IC50), different concentrations of PPE-AgNPs were prepared in 10% DMSO in ddH_2O. Cell viability was evaluated by the MTT colorimetric technique where the absorbance of formazan solutions was measured at λmax 540 nm with 620 nm as a reference wavelength using a multi-well plate reader (BMGLABTECH®FLUOstar Omega, Germany) (Satyavani et al., 2011).

2.5 Experimental worms

Adult earthworms belonging to species of *Eisenia fetida* were used in this study as its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings (Thorn et al., 1977). In addition, because of easy availability, earthworms have been used widely for the initial evaluation of anthelmintic compounds in vitro (Ajaiyeoba et al., 2001). Worms in distilled water were used as a control. In this experiment, the time to reach paralysis and death state was expressed in minutes (Dkhil, 2013). Three doses were used (5, 2.5 and 1.25 mg/mL) to study the anthelmintic activity of PPE-AgNPs. We used a reference drug, Mebendazole (Saudi Pharmaceutical Industries, Riyadh, Saudi Arabia) with a concentration of 10 mg/mL (Murugamani et al., 2012).

2.6 DPPH radical scavenging method for antioxidant activity

Using 2,2-diphenyl-1-picrylhydrazyl, the free radical scavenging activity of PPE-AgNPs was determined (DPPH). In a summary, 80 mL of a methanolic solution of DPPH (100 mM) was combined with 20 mL of PPE-AgNPs (1 mg/mL), and the mixture was then incubated for 30 min at 25 °C in the dark. At 517 nm, the absorbance was measured, and the radical scavenging activity was estimated (Ghosh et al., 2013).

2.7 Histological changes

Immediately after paralysis and death experiment, the treated and control worms were prepared for histological study following the method of Drury & Wallington (1973). Briefly, specimens were fixed in 10% formalin for 24 h, then dehydrated by graded ethanol series and embedded in paraffin. Tissues were then cut into thin sections using a microtome, stained with hematoxylin and eosin (H & E), and examined and photography using an Olympus B×61 microscope (Tokyo, Japan) (Drury & Wallington, 1967).

2.8 Statistical analysis

All values are expressed as the means and standard deviations. Significance was evaluated using t-test at $p \leq 0.05$ using a statistical package program (SPSS version 17.0).

3 Results

As illustrated in the figure, the biosynthesized silver nanoparticles are spherical in appearance and range in size from 10 to 30 nm (Figure 1).

IR analysis showed that the extract of Pomegranate peel extract exhibited strong bands at 3293.63 Cm-1, 1716.87 Cm-1, 1614.74 Cm-1, 1224.69 Cm-1, 1340.12 Cm-1, 919.19 Cm-1, 867.06 Cm-1, 816.30, 775.19 Cm-1, 554.89 Cm-1 and 516.05 Cm-1 and medium bands at 2935.66 Cm-1 and 1027.84 Cm-1 (Figure 2, Table 1). These bands confirm the presence of O-H stretching for alcohol, C-H stretching and C=C stretching for alkene, S=O stretching for sulfoxide, and C-CI stretching for halo compounds.

Results of DPPH radical scavenging method showed that PPE-AgNPs possessed 67.7 ± 0.7% antioxidant activity.

3.1 MTT cytotoxicity assay

The cell viability of several cancer lines of liver (Huh7), colon (HCT116) and breast (MDA-MB 231) cancer lines was determined after 24 h stimulation. The cytotoxic effect of PPE-AgNPs on the Huh7 (Figure 3), HCT116 (Figure 4) and MDA-MB 231 (Figure 5) cell lines was tested using an MTT assay. The viability of cells has a direct dose-dependent manner, meaning cell viability decreased progressively with higher

Table 1. Infrared (IR) spectrum of pomegranate peel extract by frequency range.

Absorption (cm^{-1})	Appearance	Transmittance (%)	Group	Compound Class
3293.63	Strong, broad	18.71	O-H stretching	Carboxylic acid
2935.66	medium	16.67	C-H stretching	alkane
1716.87	strong	9.75	C=O stretching	α,β-unsaturated ester
1614.74	strong	9.17	C=C stretching	α,β-unsaturated ketone
1224.69	strong	6.95	C-O stretching	alkyl aryl ether
1340.12	strong	7.61	S=O stretching	sulfone
1027.84	medium	5.83	C-N stretching	amine
919.19	strong	5.22	C=C bending	alkene monosubstituted
867.06	strong	4.92	C-H bending	1,2,4-trisubstituted
816.30	strong	4.63	C-H bending	1,3-disubstituted or 1,2,3,4-tetrasubstituted
775.19	Strong	4.40	C-H bending	1,2,3- trisubstituted
554.89	strong	3.15	C-Cl stretching	halo compound
516.05	strong	2.93	C-Br stretching	halo compound

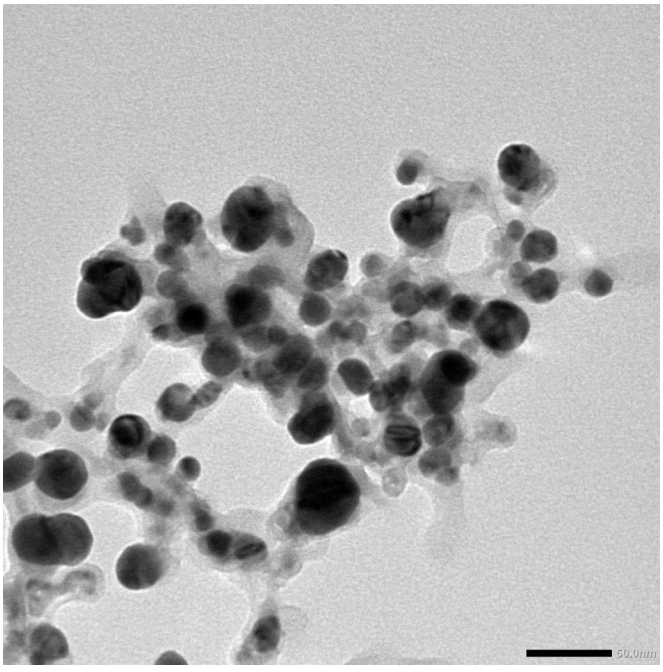

Figure 1. Transmission electron microscopy image of AgNPs biosynthesized by using pomegranate peel extract.

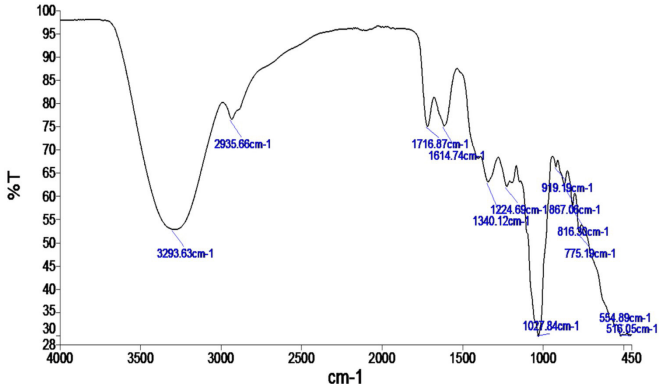

Figure 2. FT-IR spectrum of Pomegranate (*Punica granatum, punicaceae*) Peel Extract (PPE).

concentration of nanoparticles. The IC50 was 222.06 ± 3 for Huh7 cells, 41.5 ± 2 for HCT116 and 92.5 ± 9 µg/mL.

3.2 Anthelmintic activity of PPE-AgNps

The biosynthesized nanoparticles from pomegranate peel extract produced a relatively comparable anthelmintic activity with the conventional anthelmintic agent (mebendazole) against live adult *E. fetida* worms. The most efficient dose, 5 mg/mL showed the time to paralysis and death were 8.118 ± 0.26 and 9.338 ± 0.14 min, respectively. However, the reference drug mebendazole (10 mg/mL) Showed almost similar effect (6.622 ± 0.42 and 9.398 ± 0.77 for paralysis and death time, respectively) compared to the 5 mg/mL PPE- AgNps (Table 2).

3.3 Microscopic examinations

Light microscopic examination revealed uniform normal body architecture for control worms, without any alterations to the surface of worms (Figures 6). On the other hand, all worms exposed to PPE-AgNPs had alterations in the overall topography, including a decrease in size along the length and homogenous body wall shrinkage accompanied with cuticular thickness (Figures 7). All worms treated with the reference medication mebendazole showed the same types of disruption.

4 Discussion

Consumers are becoming more aware of the importance of diet to their health and, as a result, are willing to purchase foods high in bioactive compounds. Consumer acceptance of healthy products, on the other hand, is influenced by quality and sensory properties. Many investigations have found that various herbal extracts have anthelmintic properties (Mehlhorn et al., 2011;

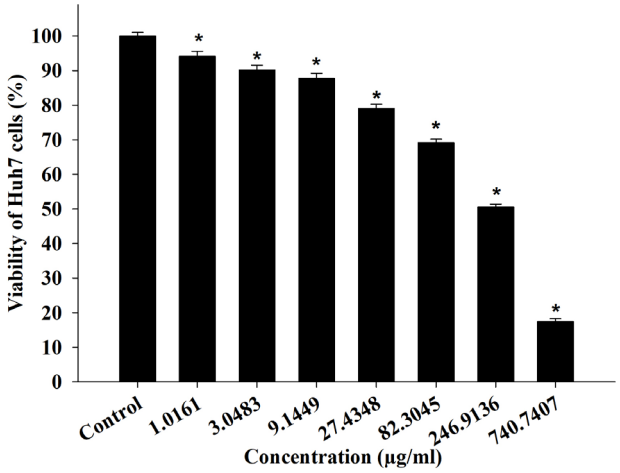

Figure 3. Cytocompatibility evaluation of PPE-AgNPs using liver (Huh7), cancer line. *Significance change with respect to control group (values are mean ± SD).

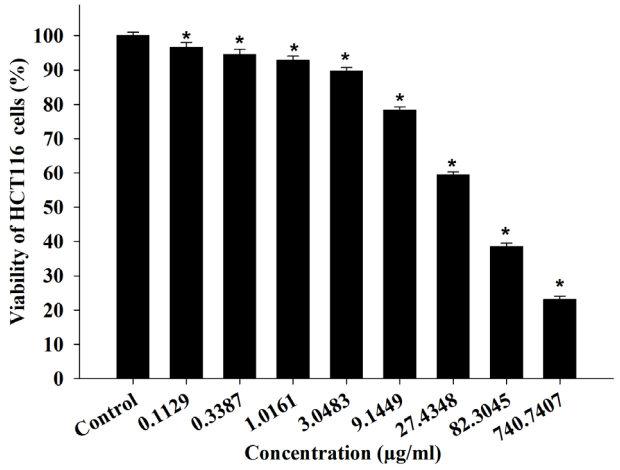

Figure 4. Cytocompatibility evaluation of PPE-AgNPs using colon (HCT116), cancer line. *Significance change with respect to control group (values are mean ± SD).

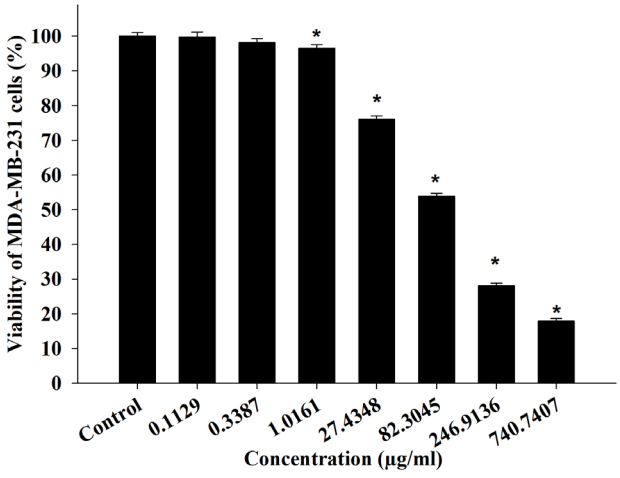

Figure 5. Cytocompatibility evaluation of Bio-AgNPs using breast (MDA-MB 231), cancer line. *Significance change with respect to control group (values are mean ± SD).

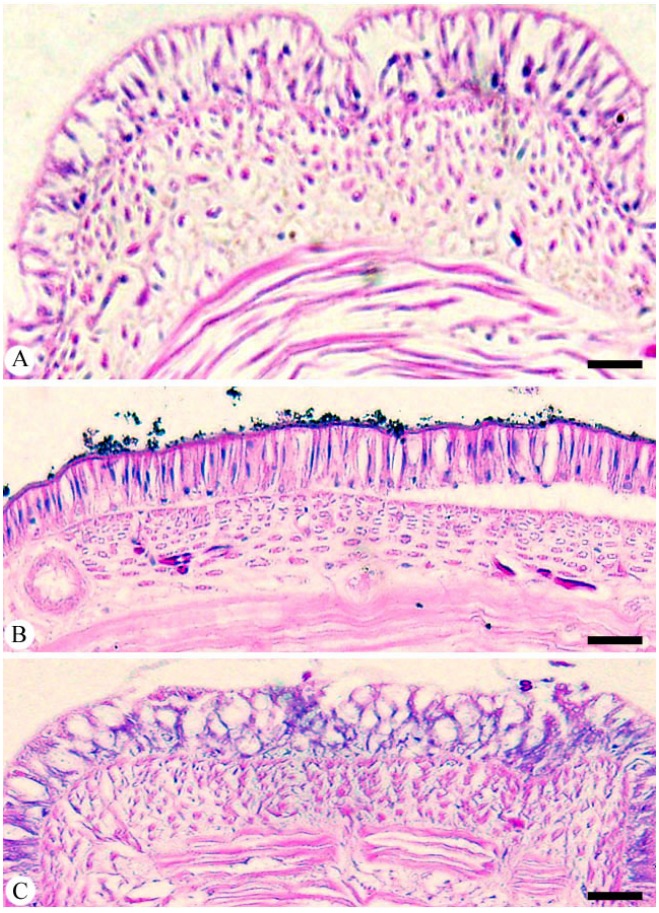

Figure 6. Effect of PPE-AgNPs on the histology of *E. fetida*. (A) Worms in distilled water (control). (B) Worms in 5 mg/mL (PPE-AgNps). (C) Worms in the reference drug (Mebendazole). Sections stained with hematoxylin and eosin, scale bar = 25 µm.

Table 2. Anthelmintic activity of AgNps synthesized from Pomegranate Peel Extract (PPE) (Bio-AgNps).

Group	Time Taken for paralysis (min)	Time Taken For death (min)
Bio-AgNps (5 mg/mL)	8.118 ± 0.26	9.338 ± 0.14
Bio-AgNps (2.5 mg/mL)	12.77 ± 2.42	14.444 ± 2.50
Bio-AgNps (1.25 mg/mL)	26.85 ± 4.78	28.995 ± 4.99
Mebendazole (10 mg/mL)	6.622 ± 0.42	9.398 ± 0.77

Dkhil et al., 2019). Many studies used earth worms as the model for the anthelmintic activity evaluation due to the physiological similarities between the *E. fetida* worms and some intestinal round worms that infect people (Cáceres et al., 2017; Hawsah et al., 2023). Pomegranate peel nanoparticles can kill *E. fetida* entirely in a short period of time, comparable to Mebendazole, probably due to the presence of active phytochemical components in the peel extract.

In this work, silver nanoparticles extracted from pomegranate peel were evaluated for their anthelmintic properties. The peel of the pomegranate is rich in polyphenols such gallic acid, ellagic acid,

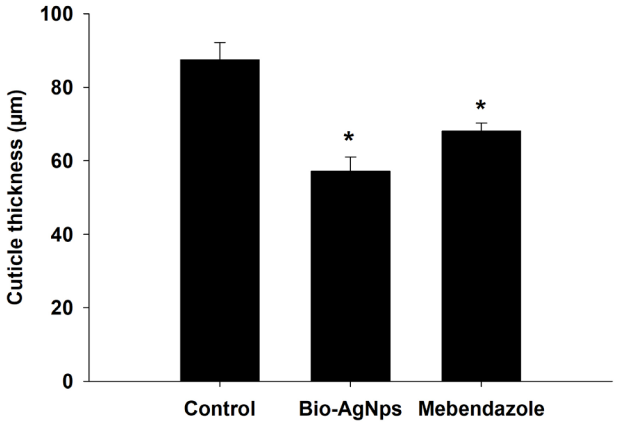

Figure 7. Cuticle thickness of *E. fetida* with various treatments. *Significance change with respect to control group.

and ellagic tannins. It has been used to make tinctures, cosmetics, medicinal formulas, and recipes for cuisine (Nasr et al., 1996). The high tannin content of pomegranate extracts, particularly punicalagin, has been identified as the primary component responsible for such antimicrobial action (Rosas-Burgos et al., 2017). Flavonoids make up almost 0.2-1.0% of the weight of the entire pomegranate fruit and are concentrated in the peel in amounts of around 30% of the total fruit anthocyanidins (Fischer et al., 2011; Zhao et al., 2013).

Histopathology has validated the in vitro investigation and examined the topographical effects of PPE-AgNps on the worms to assess the anthelmintic activity. The cuticle is an important feature in annelids and Nematodes worms because it protects and covers the worm's body while also supporting internal organs (Meyer et al., 2021).

The cuticular surface of the worms treated with nanoparticles in the current study showed extraordinary modifications, including significant shrinking. The cuticle of parasites has been linked to one of the several target areas via which anthelmintic agents interact (Kundu et al., 2015). This is in agreement with (Hawsah et al., 2023) who described how anthelmintic treatments caused modifications to the worms' body surfaces. As a result, any damage to the worm's body surface induced by medication or extract therapy may result in paralysis and death of the worm.

According to Govarthanan et al. (2016), the cytotoxicity test is crucial for toxicology inquiry since it may explain the cellular response to harmful substance and offer details on cell death and survival (Govarthanan et al., 2016). In this work, the MTT test was used to estimate the cell survival rate. Therefore, the Huh-7, HCT116 and MDA-MB 231 cancer cell line's in vitro cytotoxicity of was assessed at various doses, and the findings showed that cell viability is directly dose-dependent. This study agreed with (Fernandes et al., 2018) stated that Pomegranate peel extract produced stable AgNP with antimicrobial and anthelmintic action and low cytotoxicity, stimulating its use in the biomedical field.

5 Conclusion

According to the findings, AgNps synthesized from Pomegranate Peel Extract exhibits has strong anthelmintic properties and could be used as a food additive to farm animals for protection against helminthiasis. In vivo studies are needed in the future to understand the mechanism of PPE-AgNps action on both the parasite and the host.

Ethical approval

The experiments were approved by Helwan University's Research Ethics Committee for Laboratory Animal Care (approval no.: HU-IACUC/Z/MA0901-22).

Conflict of interest

The author(s) declare that they have no conflict of interest regarding the content of this article.

Availability of data and material

The data used to support the findings of this study are included within the article.

Author contributions

FAT, RA and MAD contributed to study design. AMM, MIA and MAM contributed to data acquisition. MAD, RA, AMM and FAT organized the database, performed the statistical analysis. All authors revised, improved, read, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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