https://doi.org/10.31925/farmacia.2022.5.22 **ORIGINAL ARTICLE**

ANTIBACTERIAL ACTIVITY OF *PEGANUM HARMALA* EXTRACTS AND THEIR GREEN SILVER NANOPARTICLES AGAINST ACNE ASSOCIATED BACTERIA

MUHANNAD I. MASSADEH [1]*, MONA AL-MASRI [1], ABU-QATOUSEH L.F. [2]

[1]*Department of Biology and Biotechnology, Faculty of Science, 13133, The Hashemite University, Zarqa, Jordan*
[2]*Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan*

**corresponding author: massadeh@hu.edu.jo*

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Abstract

Acne vulgaris is a chronic inflammatory skin disease associated with abnormal *Propionibacterium acnes* proliferation and presence of acne associated bacteria. The aim of this study was to examine the antibacterial effect of *Peganum harmala* extracts and their silver nanoparticles on acne associated bacteria. The synthesis of silver nanoparticles (AgNPs) using *P. harmala* extract was characterized by UV-Vis Spectroscopy, ICP-AE Spectroscopy, FT-IR Spectroscopy and scanning transmission electron microscope (STEM). The susceptibility of *P. acnes* and other acne associated bacteria to *P. harmala* extracts and AgNPs was tested by using agar well diffusion and broth microdilution methods. The MIC values for AgNPs against *P. acnes* and *S. aureus* were the same (12 µg/mL) while for *S. pyogenes* it showed less MIC and MBC value (6 µg/mL). The methanolic extract displayed a higher antibacterial activity compared with the aqueous extract against *P. acnes, S. pyogenes, S. aureus* and *K. pneumoniae* (26.5 ± 3.5, 19.5 ± 0.5, 35± 1 and 19±1 mm, respectively) and their MIC values against *S. pyogenes* and *K. pneumoniae* were the same (1 mg/mL) while for *P. acnes*, the maximum MIC value was 2 mg/mL. The results revealed no significant effect of metallic nanoparticles and methanolic *P. harmala* extract on *P. acnes* and *S. pyogenes* co-culture. The antibacterial activity of *P. harmala* extracts and their silver nanoparticles on *P. acnes* and other acne associated bacteria is recommended for further studies such as *in vivo* and anti-inflammatory studies as it might be considered as a treatment option for acne cases..

Rezumat

Acneea vulgară este o boală inflamatorie cronică a pielii asociată cu proliferarea anormală a bacteriei *Propionibacterium acnes* și prezența bacteriilor asociate acestei boli. Scopul acestui studiu a fost de a examina efectul antibacterian al extractelor de *Peganum harmala* și al nanoparticulelor lor de argint asupra bacteriilor asociate acneei. Sinteza nanoparticulelor de argint (AgNPs) folosind extractul de *P. harmala* a fost caracterizată prin spectroscopie UV-Vis, spectroscopie ICP-AE, spectroscopie FT-IR și microscop electronic cu transmisie de scanare (STEM). Susceptibilitatea *P. acnes* și a altor bacterii asociate acneei la extractele de *P. harmala* și AgNP-uri a fost testată prin utilizarea metodelor de microdiluție și difuzie. Valorile CMI pentru AgNP-uri împotriva *P. acnes* și *S. aureus* au fost aceleași (12 pg/mL), în timp ce pentru *S. pyogenes* au arătat mai puține valori CMI și MBC (6 pg/mL). Extractul metanolic a prezentat o activitate antibacteriană mai mare în comparație cu extractul apos împotriva *P. acnes, S. pyogenes, S. aureus* și *K. pneumoniae* (26,5 ± 3,5, 19,5 ± 0,5, 35 ± 1 și respectiv 19 ± 1 mm) și valorile CMI față de *S. pyogenes* și *K. pneumoniae* au fost aceleași (1 mg/mL), în timp ce pentru *P. acnes*, valoarea maximă a CMI a fost de 2 mg/mL. Rezultatele nu au evidențiat nici un efect semnificativ al nanoparticulelor metalice și al extractului metanolic de *P. harmala* asupra culturii de *P. acnes* și *S. pyogenes*. Activitatea antibacteriană a extractelor de *P. harmala* și a nanoparticulelor lor de argint asupra *P. acnes* și a altor bacterii asociate acneei este recomandată pentru studii ulterioare, cum ar fi studii *in vivo* și antiinflamatorii, deoarece ar putea fi considerată o opțiune de tratament pentru acnee.

Keywords: Acne-associated pathogens, AgNPs, Antimicrobial activity, *P. acnes*, *P. harmala*

Introduction

The human skin consists of a different microbial flora related to skin health. This microbial flora includes *Staphylococcus sp., Propionibacterium sp., Streptococcus sp., Corynebacterium sp.* and *Malassezia sp.* [8]. In acne vulgaris lesion, which is considered the most frequent skin disease, *Propionibacterium acnes* plays an important role in the development of inflammatory acne vulgaris [16]. *P. acnes* is a Gram-positive, aerotolerant, immotile bacterium that colonizes hair follicles and uses sebum as a nutrient source [26]. In addition to *P. acnes, Streptococcus pyogenes* or group A streptococci, a Gram-positive bacterium which colonizes the throat or skin and are responsible for several infections. Group A streptococcus is recognized for streptococcal toxic shock syndrome and necrotizing fasciitis which involves destruction of the skin and soft tissues in severe cases. Another

skin microbiome, *S. aureus* that is considered the most notable member of skin microbiota, it plays a role as a pathogen in many skin infections such as impetigo, folliculitis, and their co-existence with other microorganisms in acne lesions. The prevalence and resistance patterns of *S. aureus* and *S. pyogenes* in the oropharynx of patients with acne are more than those without acne [17], which indicates that both bacteria are associated with acne, but the exact mechanism is still not known.

Many medicinal plants are used to inhibit *P. acnes* growth due to their antimicrobial and antioxidant properties. In addition to their safety, these alternative treatments for acne vulgaris can be used instead of antibiotics to reduce the spread of bacterial resistance to antibiotics [30]. One of these medicinal plants is harmal *(Peganum harmala*), a wild plant that belongs to the *Zygophyllaceae* family. This plant contains phyto-alkaloids such as harmaline, harmine and peganine that are considered as antimicrobial substances [31]. It was tested on many Gram-positive and Gram-negative bacteria [13]. This value of *P. harmala* could be considered as a substitute for antibiotics especially in the case of *P. acnes* taking note that it was not tested against this pathogen.

Nowadays, to enhance the activity of plant extracts against pathogens, the extracts are subjected to further treatment using metals to synthesize nanoparticles of improved efficiency. The major advantage of using plant extracts for silver nanoparticle synthesis in particular is that they are non-toxic, safe and easily available [15]. In fact, the phytochemicals are involved directly in the reduction of the silver ions and the formation of silver nanoparticles [23]. Synthesis of silver nanoparticles using *P. harmala* extract was reported and their antibacterial activity was studied against some Gram-positive and Gram-negative bacteria [5, 6]. To the best of our knowledge, *P. harmala* extracts and their silver nanoparticles have never been involved in any study against acne associated pathogens and/or treatment and hence, this study aimed to examine the antibacterial effect of *P. harmala* extracts and their silver nanoparticles on acne associated bacteria.

Materials and Methods

Microorganisms

A control strain of *P. acnes* (NCTC 747) provided by Dr. Luay Abu-Qatouseh (Petra University), a clinical isolate of *S. pyogenes* provided by the Medical Laboratory of Ministry of Health (Central Laboratories directorate), *S. aureus* ATCC 6538 and *K. pneumoniae* ATCC 13883 provided by the Faculty of Medicine (The Hashemite University, Jordan) were all used as test pathogens throughout this study. *P. acnes* and *S. pyogenes* were maintained on Tryptic Soy Agar (TSA) at 37°C under anaerobic conditions using OXOID anaerobic jar and AnaeroGen atmosphere generation systems, while *S. aureus* and *K. pneumoniae* were cultured on Muller Hinton (MH) Agar/Broth at 37°C under aerobic conditions.

Aqueous P. harmala seeds extract preparation

Whole *P. harmala* plant was collected in July 2020 from Al-Hallabat area of *Zarqa* Governorate. The plant was identified and authenticated taxonomically by Dr. Salim Abderrahman (Medicinal plants experts, Dept. of Biology, Hashemite University, Jordan). The seeds were collected and stored in clean container for further use. According to [6], the seeds were washed by double distilled water (Sigma-Aldrich HPLC Plus Water) and completely dried at room temperature. After drying, the seeds were freshly grinded into crude powder using electric grinder. Thereafter, a 5 g from this powder was mixed with 50 mL double distilled water in a water path running at 70°C for 15 min, and then filtered twice by Whatman filter paper #1. Micro filtration was applied using sterile syringe membrane filters (0.22 mm) to obtain clear aqueous extract.

Qualitative phytochemicals screening

The aqueous seed extract of *P. harmala* was studied for the presence of alkaloids, flavonoids, glycosides, phenols, saponins, steroids and tannins using standard protocols [23].

Methanolic P. harmala seeds extract preparation

The seeds of *P. harmala* were washed, completely air dried and grinded into crude powder. A 5 g of the crude powder were macerated for 48 hr with stirring in 50 mL of 80% methanol (80:20) at a ratio of 1:10 (w/v). The extract was filtered twice by Whatman filter paper #1. For extract defatting, a 50 mL petroleum ether were used to remove the organic layer and this step was repeated three times. Methanol removal was achieved by using rotary evaporator (Heidolph/Germany) running at 40°C and low pressure. The solid plant extract was obtained after drying in an oven at 40°C until dryness. Thereafter, the weight of the solid plant extract was recorded and it was stored at 4°C for further experiments [2, 9, 24].

Thin layer chromatography of P. harmala extracts

The plant extract alkaloidal fraction was separated using TLC plates [14]. A freshly prepared aqueous extract was used and the dried methanolic extract was dissolved in a solvent containing DMSO and methanol at a ratio of 1:10 to obtain a concentrated solution. The samples were loaded as narrow spots onto the silica plates with capillary micropipettes many times (over spots) and the plates were dipped in a jar containing the mobile phase (ethyl acetate-methanol-ammonia; 8.5:0.1:0.05) that was freshly

prepare. After separation was completed, the TLC plate was completely dried in a fume hood. The bands were visualized under short UV light (254 nm) and long UV light (365 nm).

Silver nanoparticles synthesis

According to [6] with some modifications, 50 mL of 3mM $AgNO_3$ solution was heated to 80°C in foiled 100 mL Erlenmeyer flask with continuous stirring speed of 1100 rpm. A 4 mL of aqueous *P. harmala* seed extract was added dropwise with at a rate of 34 µL/min. The change in the solution colour from yellow to red brown was the first indication for AgNPs synthesis. The remaining Ag^+ ions in the solution were removed by centrifugation using a refrigerated centrifuge (NOVA, Turkey) running at 10.000 g and a temperature of 10°C. This step was repeated in triplicate using double distilled water. After centrifugation, the clear AgNPs were stored at refrigerator for further analysis and use.

Characterisation of silver nanoparticles

The formation of the nanoparticles was confirmed qualitatively by the gradual colour change from pale yellow into red brown colour. The bio-reduction of $AgNO_3$ salt solution to biogenic AgNPs mediated by *P. harmala* seed aqueous extract was tracked by UV–Visible spectroscopy (UV-1900, SHIMADZU, Japan). All spectrophotometric analyses were carried out in quartz cuvettes. The optical properties of AgNPs were determined using a UV-Visible spectrophotometer at a wavelength range 800-300 nm to measure the surface plasmon resonance (SPR) of the silver nanoparticles as an indicator for nanoparticles synthesis [25].

The yield of silver nanoparticles in the colloidal solution was measured by using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES; GBC E1475, USA) in Hamdi Mango Centre for Academic Research (University of Jordan/Amman) by calculating the difference in Ag^+ ions concentration between the AgNPs solution and the standard $AgNO_3$ stock solution. The conversion of Ag^+ ions to Ag^0 atoms was expressed as the amount of AgNPs in the solution [1].

The morphology and size characteristics for the synthesized AgNPs were tested using Scanning Transmission Electron Microscope (STEM; Versa 3D, FEI, Netherlands) in Cell Therapy Centre/ University of Jordan/Amman [10]. Fourier-Transform Infrared Spectroscopy (FT-IR) analysis for the AgNPs and *P. harmala* seeds extract were tested in the Chemistry department of The Hashemite University, Jordan to evaluate the interaction between functional groups of the *P. harmala* extract and the synthesized AgNPs at a wavelength range 3600-400/cm [32].

Antiacne effect of P. harmala extracts and synthesized AgNPs

Agar well diffusion method

The anti-acne activity of AgNPs, aqueous and methanolic *P. harmala* seeds extracts was tested against the test pathogens mentioned employing the agar well diffusion method [3]. For this purpose, the AgNPs were used at a concentration of 0.099 mg/mL and aqueous seeds extract was freshly prepared and used as a stock extract whereas, the methanolic extract was used at a concentration of 100 mg/mL DMSO. Initially, the standardized culture (1.5×10^8 CFU/mL) for each pathogen was prepared under optimum conditions and the culture turbidity was adjusted to 0.5 McFarland standard using spectrophotometer (OD_{600} value was in range 0.08 - 0.1).Thereafter, TSA/MHA agar plates were inoculated with a 100 µL of the previous standardized culture (1.5×10^8 CFU/mL) of each pathogen and spread with sterile cotton swabs. Wells of 6 mm in diameter were punched in the agar plates containing the bacterial lawn. A 40 µL volume from the synthesized AgNPs colloidal stock solution, aqueous and methanolic *P. harmala* seeds extracts were poured into the wells. The plates were left at room temperature for 10 min to allow the diffusion of the AgNPs solution/extracts into the agar bacterial lawn. Neomycin antibiotic discs were used as a positive control and double distilled water and DMSO were used as a negative control. The TSA plates were incubated under the optimum incubation conditions for *P. acnes* and *S. pyogenes* for 48 hr and 24 hr, respectively under anaerobic conditions at 37°C. While the optimum conditions for *S. aureus* and *K. pneumoniae* grown on MHA plates for 24 hr under aerobic conditions at 37°C. After incubation, the zone of inhibition was measured in millimetres.

MIC and MBC determination by broth micro-dilution method

According to the CLSI protocol, the standardized culture (1.5×10^8 CFU/mL) of each pathogen was diluted with sterile TSB/MHB to a ratio of 1:20 to achieve standard inoculum size (10^5 CFU/mL) and it was used within 15 min after adjustment. Thereafter, a 200 µL of sterile TSB/MHB were added to a 96 wells plates. A 200 µL of AgNPs stock solution (99 µg/mL) or methanolic seeds extract (16 mg/mL) were added to the first well. A two-fold serial dilution with up and down mixing was performed to reach well number 9. A 20 µL from previous standard cultures were added into the first well to reach well number 11 except wells 10 and 12. These wells were remained for media sterility control and AgNPs or methanolic extract sterility control. The *P. acnes* and *S. pyogenes* 96 wells plates were incubated anaerobically for 24 hr at 37°C while *S. aureus* and *K. pneumoniae* 96

wells plates were incubated aerobically for 24 hr at 37°C. The OD_{600} values were recorded for all plates before and after incubation using ELISA microplate reader. Moreover, for MBC determination, a 100 µL were subcultured from previous 96 well plates and spread using a sterile cotton swap to TSA/MHA plates. The experiments were repeated three times.

Effect of AgNPs and methanolic extract on P. acnes and S. pyogenes co-culture

According to [18, 29] with some modifications, in order to study the effect of methanolic extract and AgNPs on *P. acnes* and *S. pyogenes* co-cultures, a single colony from each *P. acnes* and *S. pyogenes* was incubated separately under anaerobic conditions overnight in 25 mL TSB at 37℃. After incubation, a 0.5 mL from each culture were transferred into 25 mL of fresh TSB and incubated anaerobically for 20 hr in case of *P. acnes* and 4 hr in case of *S. pyogenes* to make sure that the pathogens entered the logarithmic phase. Thereafter, the OD_{600} values for both bacterial cultures were recorded. A 1 mL from each culture was transferred to a fresh 160 mL sterile rich media consisting of 10 g/L yeast extract, 3 g/L TSB, 1.5 g/L KH_2PO_4 and 20 g/L sucrose as a selective carbon source and the pH was adjusted to 7 by using sterile 1 M NaOH. After inoculation, a 1 mL of AgNPs stock solution or methanolic seeds extract of a concentration of 100 mg/mL were added into *S. pyogenes, P. acnes* and Co-culture bottles at zero time and/or after 24 hr of incubation to the study the effect of time for the AgNPs /extract addition. To provide anaerobic conditions for all bottles, nitrogen gas was sparged aseptically in order to remove dissolved oxygen from the cultures. The bottles were closed with rubber cork stopper and incubated at 37℃ in a shaking incubator (HUMAN Lab, Korea) with shaking at 200 rpm for 72 hr. After incubation, the OD_{600} and pH were recorded to monitor the growth and acids production.

Statistical analysis

One-way Analysis Of Variance (ANOVA) was used to test for significant differences between mean values of $P \leq 0.05$ were considered as statistically significant. Statistical analysis was carried out using 2016 Microsoft Excel. The results were expressed as mean ± standard deviation (SD).

Results and Discussion

P. harmala seeds extract and Qualitative screening for Phytochemicals

A 400 mL of methanolic *P. harmala* seeds extract were prepared and filtered as previously mentioned. This liquid extract was evaporated and completely dried to obtain 7 g of dried, gummy methanolic extract. On the other hand, the aqueous *P. harmala* seeds extract was freshly prepared in subsequent for each experiment.

The aqueous seed extract of *P. harmala* was used for nanoparticles synthesis in order to find out the type of active phytochemicals involved in the reduction process. Table I shows the phytochemicals present in the seed extract of *P. harmala* and the results represent the presence of alkaloids, phenols, saponins, steroids and tannins.

Table I

Phytochemical screening of the aqueous *P. harmala* seeds extract

Sample	Aqueous seed extract	+/-
1	Alkaloids	+
2	Flavonoids	-
3	Glycosides	-
4	Phenols	+
5	Saponins	+
6	Steroids	+
7	Tannins	+

+/- = present or absent

Silver nanoparticles characterization

In order to check the green synthesis of AgNPs, the results demonstrates that the reaction mixture was turned from yellow into brownish colour due to the excitation of surface plasmon resonance in silver nanoparticles, which indicates the reduction of $AgNO_3$. The UV–Vis spectrum shows the absorption broad peak of around 461 nm. The nano-colloidal solution was analysed for AgNPs yield using ICP-AES. The initial concentration of Ag^+ in $AgNO_3$ stock solution was approximately 250 ppm while the final concentration of Ag^+ in AgNPs solution dropped to 150 ppm, thus the percentage of Ag^+ ions conversion into Ag^0 atoms was 40% with an AgNPs concentration in colloidal solution of about 0.099 mg/mL.

FT-IR analysis was carried out to identify the possible interactions between silver atoms and bioactive molecules, which may be responsible for the reduction and stabilization of silver nanoparticles. The FT-IR spectra of the *P. harmala* extract and the synthesized AgNPs present similar peaks with a small shift in both spectra. The colours indicate the detection of molecules of different functional groups. There was a shifting in the peaks between the two figures representing the AgNPs and extract spectra *i.e.* the peaks of the red colour that was detected at 3352.69 cm^{-1} for the *P. harmala* extract spectrum shifted to 3395.69 cm^{-1} for the AgNPs-extract spectrum; the yellow colour of the peak at 2924.33 cm^{-1} for the *P. harmala* extract spectrum shifted to 2890.74 cm^{-1} for the AgNPs-extract spectrum; the grey colour represent the peak at 1626.50 cm^{-1} for the *P. harmala* extract spectrum shifted to 1629.49 cm^{-1} for the AgNPs-

extract spectrum; the blue colour represent the peak at 1556.12 cm^{-1} for the *P. harmala* extract spectrum shifted to 1400.00 cm^{-1} for the AgNPs-extract spectrum. Figure 1 presents the STEM images of AgNPs synthesised using *P. harmala* seed extract. In Figure 1A, the dark filed image indicates that the AgNPs were present on average particle sizes of about 11.74 nm. Figure 1B represent the bright field image for AgNPs which shows that the synthesized particles were uniform, spherical, unaggregated in shape.

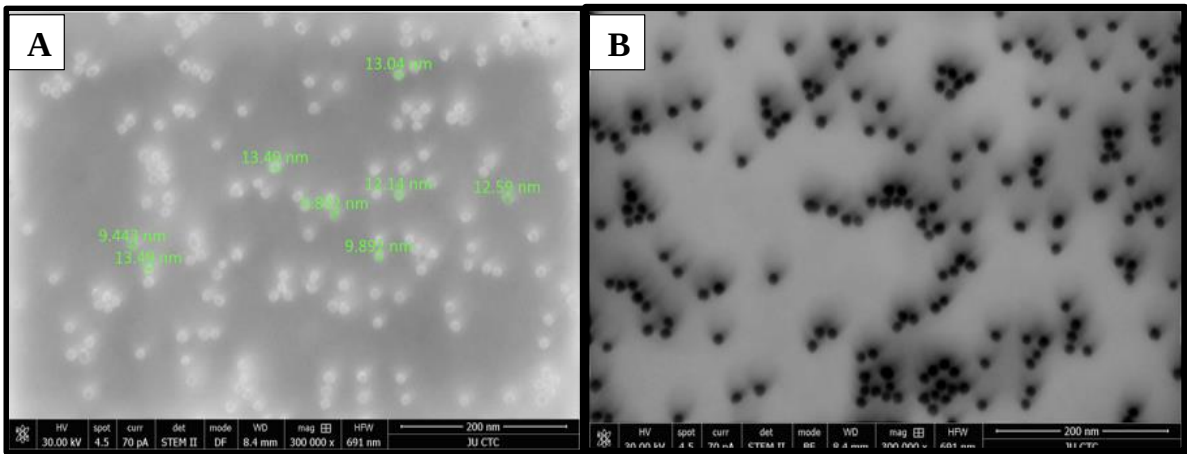

Figure 1.
The STEM image for the synthesized AgNPs (300.000X)

Thin Layer Chromatography for P. harmala seeds extracts

The alkaloidal fraction of both methanolic and aqueous extracts were separated using ethyl acetate: methanol: ammonia (8.5: 0.1: 0.05) as the mobile phase. According to the TLC results, the methanolic extract showed more intensive spot than aqueous extract and both extracts showed the same R_f (0. 29) for both spots revealed (Figure 2).

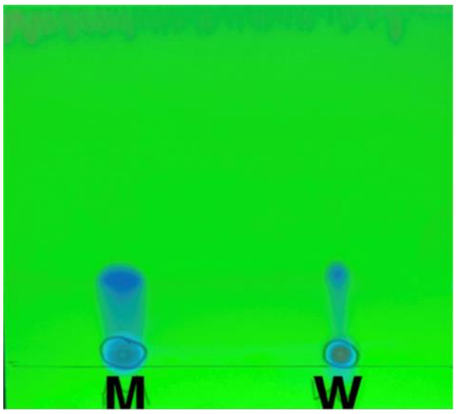

Figure 2.
TLC for methanolic and aqueous *P. harmala* **(M)** Methanolic and **(W)** Aqueous *P. harmala* extracts

Antibacterial activity of P. harmala extracts and the AgNPs

The activity of AgNPs, aqueous and methanolic seeds extracts against acne associated bacterial strains was studied using the agar well diffusion test. As shown in Table II, the AgNPs displayed the highest antibacterial activity against *S. aureus* (20 ± 1). *P. acnes* and *S. pyogenes* were sensitive to AgNPs as the activity was almost the same as the activity of the positive control used. On the other hand, *K. pneumoniea* exhibited a slight resistance to AgNPs as compared to the control. The methanolic extract displayed the highest antibacterial activity against all test pathogens. *P. acnes, S. pyogenes* and *K. pneumoniea* were sensitive to the aqueous extract (19 ± 1, 17 ± 2 and 17± 1 respectively) while *S. aureus* displayed resistance as compared to the positive control.

Table II
Antibacterial activity of the synthesized AgNPs, methanolic and aqueous plant extract

Pathogen	Zone of inhibition (mm)				
	Methanolic extract	**Aqueous extract**	**AgNPs**	**Positive control**	**Negative control**
P. acnes	26.5 ± 3.5	19 ± 1	14 ± 1	12	0.0
S. pyogenes	19.5 ± 0.5	17 ± 2	15 ± 1	15	0.0
S. aureus	35 ± 1	20 ± 1	20 ± 1	24	0.0
K. pneumoniae	19 ± 1	17 ± 1	10 ± 1	15	0.0

***** Each value represents the Mean ± standard deviation. Positive control: Neomycin. Negative control: DMSO/Distilled water

MIC and MBC for the methanolic P. harmala extract and the synthesized AgNPs

The MIC and MBC values for the methanolic extract and the AgNPs are presented in Table III. Accordingly, the MIC and MBC values for the methanolic extract were the same in the case of *S. pyogenes* and *K. pneumoniae* (1 mg/mL) while against *P. acnes* the values were higher by 2 folds (2 mg/mL). On the other hand, the MIC and MBC values against *S. aureus* were lower (0.125 mg/mL). On the other hand, the results revealed that the effect of AgNPs against *P. acnes* and *S. aureus* were the same (12 µg/mL) while in the case of *S. pyogenes* it was more sensitive (6 µg/mL) and *K. pneumoniae* was the most sensitive pathogen to AgNPs.

Table III

MIC and MBC for the methanolic plant seed extract and AgNPs

	MIC		**MBC**	
Pathogen	**Methanolic extract (mg/mL)**	**AgNPs (µg/mL)**	**Methanolic extract (mg/mL)**	**AgNPs (µg/mL)**
P. acnes	2	12	2	12
S. pyogenes	1	6	1	6
S. aureus	0.125	12	0.125	12
K. pneumoniae	1	3	1	3

The effect of methanolic P. harmala seeds extract and AgNPs on P. acnes and S. pyogenes co-culture

The effect of *P. harmala* methanolic extract/AgNPs on *P. acnes* showed that the growth of *P. acnes* growth in liquid culture was reduced as represented by the OD_{600} values after the addition of 1 mL of *P. harmala* methanolic extract/AgNPs by 24 hr while *P. acnes* showed the maximum OD_{600} value at the absence of AgNPs/extract (Table IV and V). Moreover, in the case of *S. pyogenes* growth the OD_{600} value was reduced after the addition of AgNPs /extract at zero time. The OD_{600} value was increased for *S. pyogenes* culture at addition of extract/AgNPs by 24 hr. On the other hand, the OD_{600} values for *P. acnes* and *S. pyogenes* co-cultures recorded slightly the same values.

To monitor the effect of AgNPs/extract addition on the acids production in cultures, the pH values before incubating all cultures were adjusted at pH = 7. After 72 hr of incubation, the pH values were recorded for all cultures as represented in Table IV and V. The maximum pH values recorded for *P. acnes* cultures were in the range of 6.8 with no significant difference from the initial pH value. On the other hand, the pH values for *S. pyogenes*, *P. acnes* co-cultures and *S. pyogenes* cultures were dropped to the range of 5.2 with no significant variation in pH values at the same type of the culture.

Table IV

Effect of methanolic plant seed extract on *P. acnes*, *S. pyogenes* and their co-culture after 72 hrs of incubation

	OD_{600} & pH			
	Zero time		***72 hr**	
Cultures		**- extract**	**0 hr + extract**	**24 hr + extract**
P. acnes	0.009	0.697 ± 0.04	0.586 ± 0.07	0.313 ± 0.09
	7.00	6.93 ± 0.03	6.87 ± 0.02	6.95 ± 0.04
S. pyogenes	0.009	0.719 ± 0.15	0.544 ± 0.05	0.927 ± 0.08
	7.00	5.32 ± 0.2	5.43 ± 0.6	5.24 ± 0.5
Co-culture	0.004	1.159 ± 0.12	1.136 ± 0.08	1.142 ± 0.04
	7.00	5.66 ± 0.5	5.9 ± 0.3	5.85 ± 0.3

*Each value represents the Mean ± SD. - extract: No extract addition. 0 hr + extract: extract was added at zero time. 24 hr + extract: extract was added after 24 hr of incubation. The highlights represent the culture pH values.

Table V

Effect of the synthesized AgNPs on *P. acnes*, *S. pyogenes* and their co-cultures after 72 hrs of incubation

	OD_{600} & pH			
	Zero time		***72 hr**	
Cultures		**- AgNPs**	**0 hr +AgNPs**	**24 hr + AgNPs**
P. acnes	0.003	0.806 ± 0.03	0.723 ± 0.05	0.546 ± 0.04
	7.00	6.85 ± 0.05	6.73 ± 0.05	6.88 ± 0.04
S. pyogenes	0.005	1.02 ± 0.02	0.803 ± 0.22	0.944 ± 0.07
	7.00	5.02 ± 0.5	5.18 ± 0.2	5.06 ± 0.2
Co-culture	0.006	1.104 ± 0.06	1.098 ± 0.02	1.102 ± 0.05
	7.00	5.03 ± 0.5	5.07 ± 0.3	5.04 ± 0.5

*Each value represents the Mean±SD. -AgNPs: No AgNPs addition. 0 hr + AgNPs: AgNPs were added at zero time. 24 hr + AgNPs: AgNPs were added after 24 hr of incubation. The highlights represent the culture pH values.

There are numerous pathogens associated with acne lesions such as *P. acnes, S. pyogenes, S. aureus* and *K. pneumoniae* whose pathogenic mechanisms and genes associated with virulence factors are believed to play a significant role in the development of acne [16]. Although antibiotics have been included in the therapeutic regimes of acne, the number of reports on resistance by acne-causing bacterial species is escalating over recent years [21]. The adverse effects associated with benzoyl peroxide, retinoids, isotretinoids, azelaic acid, and salicylic acid and other widely used antiacne agents could not be neglected [22]. This necessitates the development of novel therapeutic agents with high efficacy and low side effect profiles.

In this study, two *P. harmala* seeds extracts and synthesized silver nanoparticles have been tested against acne associated bacterial strains. With respect to the zone of inhibition and the MIC and MBC concentrations, methanolic extract of *P. harmala* seeds extract was more effective than aqueous extract against *P. acnes, S. pyogenes, S. aureus* and *K. pneumoniae* (26.5 ± 3.5, 19.5 ± 0.5, 35 ± 1 and 19 ± 1 mm respectively). The reason for the difference between the antibacterial effects of these two extracts against acne associated bacteria was justified from the TLC results as the intensity of bands appeared more intense for *P. harmala* methanolic extract due to the difference in the number of active phytochemicals existing in these extracts (Figure 2**)**. These results are in agreement with [11] who studied the antibacterial activity of different parts of *P. harmala* against multi-drug resistant bacteria. They found that 100 mg/mL of methanolic seeds extract showed antibacterial activity against *S. pyogenes, S. aureus* and *K. pneumoniae.* In general, many medicinal plants were tested for their antibacterial effect on *P. acnes i.e. Eucalyptus globulus, Mentha rotundifolia, Inula viscosa, Utrica dioica, Malva sylvestris, Quercus calliprinos, Arum palaestinum* and *Achillea odorata* [2]. They found that only *E. globulus, A. palaestinum, U. dioica, I. viscosa and M. rotundifolia* have potential anti-*P. acnes* activity.

The green synthesis approach was applied for silver nanoparticles (AgNPs) by using *P. harmala* aqueous seeds extract. Before AgNPs synthesis, the qualitative analysis of phytochemicals that was responsible for nanoparticles reduction was tested (Table I). The results showed that alkaloids may be responsible for the nanoparticles synthesis due to the presence of hydroxyl and carbonyl groups of alkaloids that act as a reducing agent for the reduction of silver ions to AgNPs and capping agent to prevent agglomeration [6]. Mariselvam *et al.* claimed that alkaloids, carbohydrates, terpenoids, tannins, saponins and phenolic compounds found in the extract of *Cocos nucifera* are all responsible for nanoparticles synthesis [19]. In this manner, Sathishkumar *et al*. found that flavonoids presented in the fractionated leaf extract of *Coriandrum sativum* induced AgNPs synthesis and as well as acted as a capping agent to prevent agglomeration [25]. Recently, Fahmy *et al.* green synthesised platinum and palladium nanoparticles using *P. harmala* L. seeds alkaloids fraction [12]. Accordingly, these previous literatures indicated that alkaloids and a few more phytochemicals present in plant extract are involved in the AgNPs synthesis.

The AgNPs synthesized from *P. harmala* aqueous seeds extract were characterized using UV-Vis spectroscopy, FT-IR, STEM and ICP-AES. The ICP-AES was used to test the Ag^+ ions conversion to Ag^0 atoms and the result of this test was slightly in agreement with [1] who reported a 48% conversion of Ag^+ ions to Ag^0 atoms using rosemary leaf extract. The UV-visible spectrum (Figure 1) for the synthesized AgNPs showed a broad peak near 461 nm due to the presence of different size ranges of nanoparticles. In other words, the broadening of the spectrum was due to the change in the distribution of particles, coalescence and particle shape in the colloidal solutions [20]. The UV-Visible and FT-IR spectra (Figure 2) results are in agreement with the results of Azizi *et al.* who synthesized AgNPs using *P. harmala* extract and the maximum absorption wavelength was determined at 447 nm [6]. The FT-IR spectra of *P. harmala* seeds extract and synthesized AgNPs revealed that the synthesized AgNPs contained natural compounds from the *P. harmala* extract on the shell of the nanoparticles that are responsible for the stability. The peaks at 3352.69 cm^{-1} (for the *P. harmala* extract spectrum) and at 3395.69 cm^{-1} (for the AgNPs-extract spectrum) were assigned to OH-stretching of hydroxyl groups or the NH group of the amines or amides. The absorption band at 2924.33 cm^{-1} (for the *P. harmala* extract spectrum) and the band at 2890.74 cm^{-1} (for the AgNPs-extract spectrum) are attributed to CH stretching. The bands assigned to the C=C group are at 1556.12 cm^{-1} (for the *P. harmala* extract spectrum) and 1400.00 cm^{-1} (for the AgNPs-extract spectrum). The bands at 1626.50 cm^{-1} (for the *P. harmala* extract spectrum) and 1629.49 cm^{-1} (for the AgNPs-extract spectrum) are assigned for carbonyl groups that have a high affinity for silver atoms that are responsible for reduction and stabilization of them. The CH and NH groups act as stabilizing agents that corresponds to proteins. Additionally, the peak at 1626.50 cm^{-1} and 1629.49 cm^{-1} (attributed to the carbonyl group) was significantly diminished in the FT-IR spectrum of AgNPs, which may suggest

conjugation of nanoparticles with the *P. harmala* seed extract *via* carbonyl groups [6].

There are many factors that affect the antimicrobial activity of AgNPs including shape and size of AgNPs. Therefore, the STEM images (Figure 1) of the synthesized AgNPs showed a uniform spherical shape with an average particles size of about 11.74 nm. Alomar *et al.* found that the AgNPs of *P. harmala* extract showed various particles sizes and the presence of aggregation in AgNPs colloidal solution [4]. Silver nanoparticles have been reported for the antibacterial activity against various pathogens; however, there are very few reports on anti- acne associated bacterial activity. The agar well diffusion method for acne associated bacteria showed apparent inhibition as shown in Table II. The antibacterial activity result demonstrates that the inhibition zone of AgNPs against *P. acnes, S. pyogenes, S. aureus* and *K. pneumoniae* were found to be 14 ± 1, 15 ± 1, 20 ± 1 and 15 ± 1 mm by well diffusion method respectively. The highest activity was reported for the methanolic extract which could be due to the difference in concentration of active compounds between methanolic extract (100 mg/mL) and AgNPs colloidal solution (0.9 mg/mL). Furthermore, for more quantitative examination and in order to know the antibacterial efficacy of AgNPs, MIC assay was performed. The MIC results indicated that the MIC of AgNPs was found to be 12, 6, 12 and 3 µg/mL against *P. acnes, S. pyogenes, S. aureus* and *K. pneumoniae* respectively (Table III).

The mechanism of the bactericidal effect of silver colloid particles against bacteria and the antibacterial property of AgNPs may be due to the adsorption of Ag atoms to cell walls, deactivation of membranous enzymes and interaction of ions with proteins on the cell wall leading to protein denaturation and porous structures formation [28]. Moreover, the AgNPs can interact with the bacterial cell wall by the thiol group which lead to cell death by causing seizure of the respiratory chain reaction. Sathishkumar *et al*. studied the anti-acne effect of green synthesized silver nanoparticles using *Coriandrum sativum* leaf extract and they found that the green synthesized nanoparticles size was found to be in the range of 37 nm and the minimal inhibitory concentration (MIC) of AgNPs for acne causative agent *P. acnes* was found to be at 3.1 µg/mL [25].

The *P. acnes* and *S. pyogenes* co-cultures were performed in order to study the synergistic effect of the two types of acne associated bacteria by the addition of *P. harmala* methanolic extract and synthesized AgNPs in the presence of sucrose as a selective carbon source. The effect of AgNPs/methanolic *P. harmala* extract on *P. acnes* and *S. pyogenes* cultures exhibited a decrease in OD_{600} value after the addition of AgNPs/methanolic extract by 24 hr for *P. acnes* and at zero time for *S. pyogenes* which could be due to the rapid multiplication of *S. pyogenes* compared to *P. acnes* that lead to entering the exponential phase more quickly thus the effect of AgNPs/methanolic extract appeared early. Unnikrishnan *et al*. reported that *S. pyogenes* enters the exponential phase after 3 hr, mid log phase after 4 h, late log phase after 8 hr, and stationary phase after 9 hr [27]. On the other hand, Balgir *et al*. found that *P. acnes* enters to the exponential growth phase after 24 hr, stationary growth phase after 48 h, 72 hr and late-stationary growth phase after 144 hr [7]. Nevertheless, *P. acnes* and *S. pyogenes* co-cultures recorded slightly the same OD_{600} values which may be due to the presence of both types of bacteria that help each other to exhibit resistance to AgNPs/methanolic extract. Moreover, these results are confirmed by the pH reads (Tables IV and V) where the pH values for *P. acnes* cultures showed a stable pH for the culture after the addition of the AgNPs/methanolic extract at 24 hr due to the reduction of bacterial growth. Whereas the culture of *S. pyogenes* showed a drop in pH values from the initial pH (7) with no significant variation in the pH values in the same type of cultures while the co-culture of *S. pyogenes* and *P. acnes* showed slightly the same reads due to the synergic positive effect of both bacterial growths. Another explanation might be correlated to the presence of sucrose as a selective carbon source for *S. pyogenes* which leads to inhibition of acids production by *P. acnes* [29].

It is worth mentioning that *S. pyogenes* is a sucrose utiliser that can produce secondary metabolites such as acids (lactic acid) that lowers the pH value. However, for re-balance the skin normal flora in the acne lesion should enhance the growth of the normal flora more than *P. acnes* which might be achieved by using a selective carbon source as a solution to the imbalance of the skin normal flora. Consequently, our results are in agreement with previous studies claiming that the prevalence and resistance patterns of *S. aureus* and *S. pyogenes* in the oropharynx of patients with acne are more than those without acne [16, 17], which indicates that both bacteria are associated with acne lesion, but the exact mechanism is still not known.

Conclusions

The present study indicates that the AgNPs were successfully green, eco-friendly synthesized by *P. harmala* seeds aqueous extract with an average size of 11.74 nm. The main constituents of the plant extract include alkaloids, phenols, saponins, steroids and tannins. The green synthesis of AgNPs

was confirmed by microscopic and spectroscopic analyses such as UV-Vis, ICP-AES, FT-IR and STEM. These AgNPs exhibited antibacterial activity against *P. acnes* and other acne associated bacteria. The AgNPs have displayed the highest inhibition zone against *S. aureus* (20 ± 1 mm). The MIC values for AgNPs against *P. acnes* and *S. aureus* were the same (12 µg/mL) while for *S. pyogenes* it showed less MIC and MBC value (6 µg/mL). The methanolic extract displayed a higher antibacterial activity compared with the aqueous extract against *P. acnes, S. pyogenes, S. aureus* and *K. pneumoniea* (26.5 ± 3.5, 19.5 ± 0.5, 35 ± 1 and 19 ± 1 mm, respectively) and their MIC values against *S. pyogenes* and *K. pneumoniae* were the same (1 mg/mL) while for *P. acnes* the maximum MIC value was 2 mg/mL. No significant effect of *P. harmala* methanolic extract/AgNPs on *P. acnes* and *S. pyogenes* co-culture.

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Conflict of interest

The authors declare no conflict of interest.

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