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EFFECT OF ELEVATION ON BIOLOGICAL ACTIVITY OF *JUNIPERUS PROCERA* HOCHST. EX ENDLICHER POPULATIONS GROWN IN AL SODA MOUNTAINS, SAUDI ARABIA

ASHWAG HASSAN[1], SULAIMAN ALRUMMAN[1] AND MAHMOUD MOUSTAFA[1,2*]

[1]Department of Biology, Faculty of Science, University of King Khalid University, Saudi Arabia
[2]Department of Botany and Microbiology, Faculty of Science, South Valley University, Qena, Egypt
[*]Corresponding author's email: mfmostfa@kku.edu.sa

Abstract

In the present study, leaves and berries of healthy and diseased (dieback) of *Juniperus procera* plants have been collected from four different elevations site in Al Soda Mountains, Saudi Arabia to study their antimicrobial activity against *Staphylococcus aureus*, *Klebsiella oxytoca* and *Candida albicans*. Also, the synthesized silver nanoparticles from healthy and diseased leaves and berries of *J. procera* have been tested against the same microbes using well agar diffusion methods. All extracts showed effective inhibiting properties against tested microbes at different rates depending on the type of microbe itself, type of solvent and plant location. *C. albicans* recorded the highest inhibition zone gained from ethyl alcohol extract of diseased leaves (3.16 ± 0.11cm) compared to other extracts and microbes, followed by water extract of healthy berries with a zone of inhibition (3.13 ± 0.15cm) against *K. oxytoca*. Chloroform extract obtained from diseased berries recorded antibacterial activity with an inhibition zone of (2.74 ± 0.11cm) against *S. aureus*. The synthesized silver nanoparticles showed potent inhibition activity against all microbes in the range between (3.52 ± 0.05cm) against *S. aureus*, (3.46 ± 0.05cm) against *K. oxytoca* and (3.42 ± 0.11cm) against *C. albicans*. Our findings proved that leaves and berries of *J. procera* from all locations whether healthy or diseased had antimicrobial activity against tested human pathogenic microbes which it may be used as a safe, economical, and powerful natural medicine better than synthetic chemicals.

Key words: *Juniperus procera*; Elevations; Antimicrobial activity; Solvent extracts; Silver nanoparticles.

Introduction

The west south of Saudi Arabia has a unique environmental condition especially Sarwat Mountains therefore many endemics weed plants growing there. One of the most important plants for Saudi Arabia's ecosystem is *Juniperus procera* making a continuous forest in many areas of Sarwat Mountains. *J. procera* forests are growing in Al Soda Mountains, KSA in height ranging from 2700 to 3000 meters above sea level covering about 95% of Sarwat Mountains. It is a perennial evergreen plant that grows in cold regions and one of the most important trees at the global level on which humans have depended on it in many of the requirements of life for thousands of years. *J. procera* (locally named Arar) have an attractive scene in its growing places and refreshing air because it contains a large number of volatile oils and has aroma smell beautiful if burned (Al-Attar *et al*., 2016).

Juniperus procera Hochst ex Endl originally identified by (Hochstetler) and reported by (Endlicher) in 1847 in Ethiopia and is the only one that exists naturally in the north and south hemispheres, all other species of *Juniperus* are found in the northern hemisphere. These forests are work to modify the climate, improve soil by increasing fertility, combat air pollution, break wind and reduce noise. *Juniperus* plant is considered as perennial trees whereas it lives for hundreds of years. All *Juniperus* spp are dioecious where the male and female have reproductive organs in separate individuals (cones) (Adams, 2011).

The scientific name of *Juniperus procera* indicates the characteristics of the tree where the (*Juniperus*) means the ruggedness and the (*procera)* means higher which mean the ruggedness is higher where the plant shows its tolerance to be in difficult environmental conditions like drought, low temperature and in erosive soil of some areas of the world (Maharia *et al*., 2013).

These amazing trees have a lot of benefits and use. For example, it has been using by local people as a remedy for treating cold, gastritis, cough, wound healing, rheumatism, gout and for treating the urinary tract inflammations, kidney and gallbladder stones. Its wood could be used for building purpose, making fire and cooking. Around the world, the medical uses of *Juniperus* are also well known for many as for Bosnian, Lebanese, and Turkish people. They are often using the berries of *Juniperus* trees to treat skin diseases like skin rash and eczema. Industrially, at the local and global level tar oil that is extracted from *Juniper* tree is being used for many purposes, for example, in southern Saudi Arabia tar oil called "Safwa" is prepared and used in coating doors, windows, and Potsherds (Samaha *et al*., 2017).

The Dieback known also as (apical death) is the disease that affects woody plants in which a tree begins to die from the tip of its leaves or roots backward starting at the tips. The causes of dieback in *Juniperus procera* have not been determined yet. Some researchers reported that the *Junipers* dieback due to many causes such as drought, pollution, fire, insects, and pathogens. *Juniper* tree globally faces the danger of fading and extinction for many reasons like urban expansion, construction of roads, some incorrect practices of some visitors to forest areas (El-Juhany, 2015). In this study, therefore, antimicrobial effects of healthy and diseased leaves and berries of various solvents extracts gained from *J. procera* plants which growing in various elevations in Al Soda Mountains, KSA against some human pathogenic microbes had been examined.

As the antimicrobial activity of synthesized silver nanoparticles from medicinal plants such as *Boswellia ovalifoliolata* and *Shorea tumbuggaia* proved a potent inhibiting effect against pathogenic microorganisms (Savithramma *et al*., 2011). This study aslo is aimed to synthesis silver nanoparticles using leaves and berries extracts obtained from healthy and diseased *J. procera* plants as natural antibiotic agents had been examined.

Materials and Methods

Materilas collection: *J. procera* leaves and berries from both plants either healthy or diseased have been collected in February 2018 from four different sites in Al Soda Mountains, Kingdom of Saudi Arabia. The elevations of collection sites lied between at the first appearance of *J. procera* in the mountain at height (2737 meters) and at (3000 meters) above sea level (Fig. 1).

Extract preparation: Each sample was dried totally in shade for three weeks, then all samples ground into a fine powder using mortar and pestle. To get *J. procera* active principles from the dry materials, 10 ml from each solvent (ethyl alcohol, chloroform, petroleum ether, methanol, and water) had been added to 3.7 gm from the dried powdered samples. All sample were placed into a rotary shaker apparatus at 140 RPM at 30ºC for 7 days to complete obtaining the extracts from plants. After that extract from each sample had been dried using drying ovens adjusted at 50ºC until the solvent was evaporated totally. All extracts were dissolved in (10 ml) sterile dimethyl sulfoxide (DMSO) (Zeraib *et al*., 2014).

Pathogenic microorganisms' preparation: Three microbial strains; one Gram-positive bacteria (*Staphylococcus aureus*), one Gram-negative bacteria (*Klebsiella oxytoca*) and one candida (*Candida albicans*) had been used. All clinical isolates were obtained from the Microbiology Laboratory, Biology Dep., Faculty of Science and Microbiology Laboratory, Faculty of Medicine, King Khalid University, Saudi Arabia. The microorganism strains were sub cultured in nutrient broth then incubated at 30ºC for 24 h for bacteria and 48 h for candida.

***In vitro* antimicrobial activity:** The effectiveness of antimicrobial activity of each extract was done by applying the agar well diffusion method according to (Moufid *et al*., 2012; Bakht *et al*., 2013). *J. procera* obtained from leaves and berries extracts had been examined for their antimicrobial activity. 20 ml from sterilized nutrient agar media were poured into sterile Petri dishes, and then left for about one hour at room temperature for media solidification. Thereafter, holes (0.6 ml) had been made in the agar media by a sterile cork-borer tool, and then 1ml from sub cultured m strains distributed over solid agar. After that, each hole was filled with 0.1ml of plant extracts and left the plates for about one hour at room temperature for well diffusion of extracts into the agar, then incubated at 30ºC for 48 h. All experiment was carried out in triplicate and the mean diameter of the inhibition zone was registered.

Silver nanoparticles

Plant extracts preparation for silver nanoparticles: Plant extracts of leaves and berries from both healthy and diseased *J. procera* obtained from different elevations were prepared using 3 gm from dried powdered samples and dissolved in (50 ml) of deionized water (DIW).

Silver nanoparticles synthesized using *J. procera* extracts: In order to synthesize silver nanoparticles utilizing *J. procera* extracts, (0.2gm) of $AgNo_3$ were dissolved in 100 ml of deionized water. From the stock, 25 ml of $AgNo_3$ solution had been placed on a heater at 70ºC, with the addition of 2ml of plant extracts. The color change was observed over time from light yellow to yellow then to dark brown and the synthesized nanoparticles had been used for antimicrobial test (Puišo *et al*., 2013).

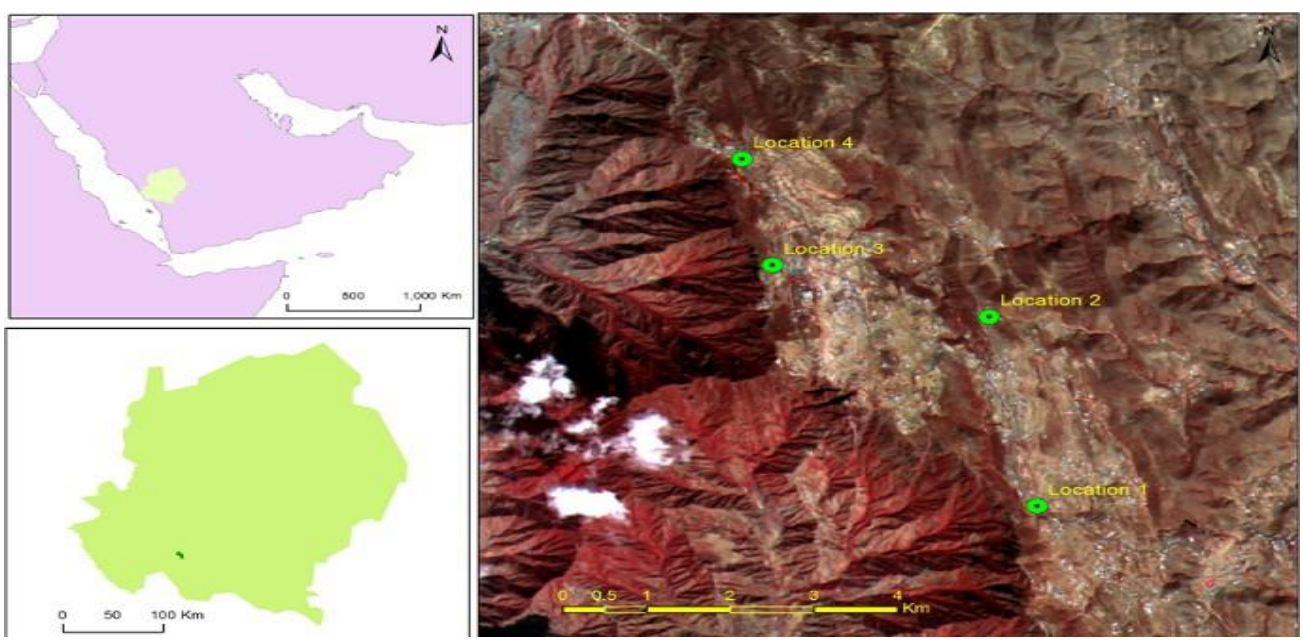

Fig. 1. Locations site of *J. procera* plants in Al Soda Mountains, Kingdom of Saudi Arabia.

Negative and positive controls: Dimethyl sulfoxide (DMSO) had been used as a negative control and synthetic antibiotic (Clariva-250mg) was dissolved in (10 ml) dimethyl sulfoxide (DMSO) and used as a positive control.

UV-Vis spectroscopy: The optical density of solutions was measured using a Shimadzu spectrophotometer. Spectroscopy quickly detects the formation of silver nanoparticles as the colored solution of nanoparticles showed a peak of ~450 nm.

Energy dispersive x-ray spectroscopy (EDX): This technique is used to determine the elemental composition of a sample. In this study, it was used to confirm that the nanoparticle suspension contains silver.

Scanning electron microscope: Scanning electron microscope (SEM) (JOEL, Japan, Model- 6360) was used to sketch construal picture of silver nanoparticles produced by extract of *J. procera*. The specifics voltage applied, the magnification used and the scale of the image material are inserted on the images.

The statistical analysis: The Graph Pad Prism program (version 7) was used to do statistical analysis. One-way analysis of variance (ANOVA) was used to define statistically significant variations between *J. procera* various extracts. A *p*-value of less than 0.05 was considered statistically significant.

Results and Discussion

Anti-candidal properties of *J. procera* leaves and berries extracts: The antimicrobial analysis results of extracts obtained from healthy and diseased leaves and berries of *J. procera* demonstrated that all extracts showed an anticandidal effect against *C. albicans* with inhibition zones ranging from (3.16 ± 0.11cm) to (1.13 ± 0.15cm) (Figs. 2A and B). The strongest inhibitory zones were recorded from ethyl alcohol extract of diseased leaves at (2846m) with zone of inhibition (3.16 ± 0.11cm), water extract from diseased leaves at (2737m) (3.14 ± 0.11cm) followed by chloroform extract from diseased berries at (3000m) (3.12 ± 0.17cm). Moreover, the petroleum ether and chloroform extracts from healthy and diseased berries at 2737m and 3000m showed slight lower activity with zones of inhibition (3.09 ± 0.17cm) and (2.93 ± 0.11cm) respectively. On the other hand, the methanol extract from diseased berries at (3000m) represented the lowest activity with an inhibition zone of (1.13 ± 0.15cm) against *C. albicans*. This could explain the results obtained by Baydar *et al*., (2004) and Kali, (2015) proofed that *J. procera* is full of phenolic compounds which have antimicrobial inhibitory effect. *J. procera* found to have high contents of phenolic, flavonoid and totarol which cause strong antibacterial and anticandidal activity (Smith *et al*., 2007). In addition, the antimicrobial activity of the plants can be affected by various factors like storage conditions, time of collection and the location of plant species (Matu *et al*., 2003). Arya *et al*., (2015), investigated about 10 plants growing in different regions in India namely Indus, Nubra, and Suru. They observed that the total phenolic and total antioxidant capacity had been influenced by several factors like the solvent type, area environmental conditions and genetic variations among the plants. Therefore, the current study sheds light on the application of *J. procera* extract to be used in pharmaceutical medicine against *C. albicans*.

Antibacterial properties of *J. procera* leaves and berries extracts against *K. oxytoca*: The results of antimicrobial screening unveiled that all these extracts have an antibacterial effect against *K. oxytoca* (Figs. 2C and D). The highest antibacterial potentials were seen from the water extract of healthy berries with a zone of inhibition (3.13 ± 0.15cm) at (3000m). Chloroform and petroleum ether extracts from healthy berries and diseased leaves at (2737m) showed somewhat less activity with zones of inhibition ranging from (3.09 ± 0.17cm) to (3.06 ± 0.11cm). While the methanol and ethyl alcohol extracts of healthy leaves and diseased berries at 2737m and 2940m exhibited the minimum activity compared with other solvents with inhibition zones (1.5 ± 0.01cm) and (1.4 ± 0.11cm). The results revealed a significant variation between the inhibitions zones caused by various extract of *J. procera* plants that may be due to variations in the sensitivity of the examined microorganisms (Ali *et al*., 2015). Antimicrobial activities recorded in our study showed to be depend on the solvents types applied and plant elevations. In agreement with other study by Mala *et al*., (2015), examined 77 extracts from 24 therapeutic plants versus 12 infection microorganisms and found that the hexane and methanol extracts showed a different level of antimicrobial growth. Hammami *et al*., (2011), showed that inhibition zones depended also on the type of tree either male or female, where the female trees exhibited higher inhibition zones because of their distinctive chemical composition. Our results showed that the smallest inhibition zones resulted from methanol extract from diseased berries of *J. procera* followed by the ethyl alcohol of diseased leaves and berries, this again support that each plant growing in a specific location had its own chemical compounds that affect specific microbes by various degrees.

Antibacterial properties of *J. procera* leaves and berries extracts against *S. aureus*: *In vitro*, all solvents extract from four elevations displayed varying level of antibacterial activities against *S. aureus* (Figs. 2E and F). Among various solvents tested, the chloroform of diseased berries extracts at 3000m showed the highest antibacterial activity with a zone of inhibition (2.79± 0.11cm). While the ethyl alcohol and petroleum ether of leaves and berries extracts from various elevations had somewhat lower antibacterial activity compared with methanol extract with inhibition zones ranging from (2.62 ± 0.12cm) to (2.51 ± 0.10cm). Likewise, the ethyl alcohol, chloroform and petroleum ether of healthy berries and diseased leaves and berries extracts at 2737m, 2846 m and 2940 m had also lower antibacterial activity with a zone of inhibition (2.56 ± 0.11cm) to (2.46 ± 0.15cm). On the other hand, the petroleum ether extracts of healthy berries at 2846m and water extract of diseased berries at 2940m had the lowest inhibitory activity (1.16± 0.15cm). These results disclose that the *J. procera* either healthy or diseased at various elevations could inhibit the growth of tested microorganisms. These results also support the previous study by Zeraib *et al*., (2014), who proved that the essential oils of *J. thurifera* prevent the growth of fourteen bacterial strains with a zone of inhibition ranging from 0.6 cm to 1.7cm.

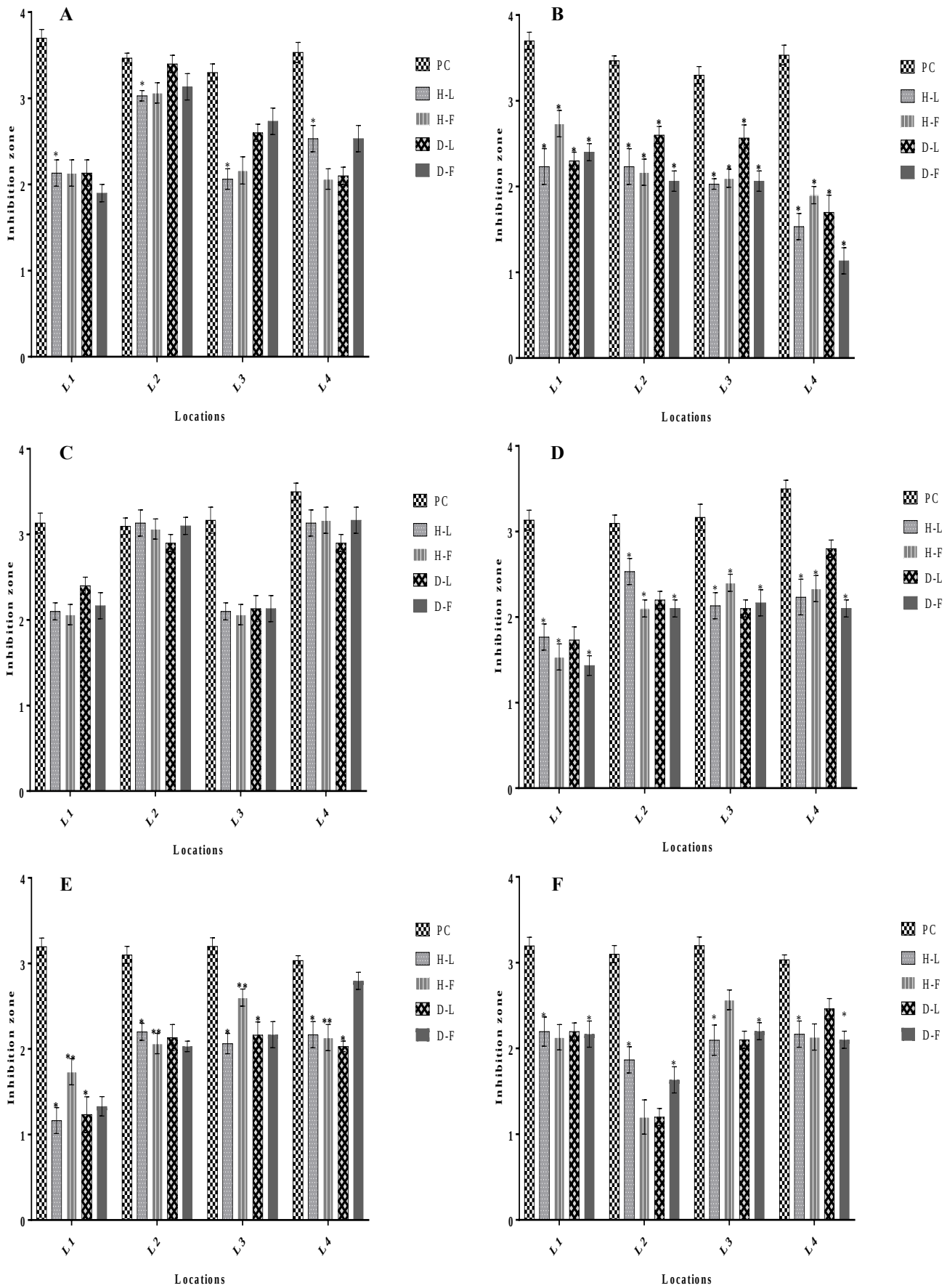

Fig. 2. Anti-candidal activity of ethyl alcohol extracts (A) and methanol extracts (B) against *C. albicans*; antibacterial activity of water extract (C) against *K. oxytoca*; ethyl alcohol extract (D) against *K. oxytoca*; chloroform extract (E) against *S. aureus*; petroleum ether extract (F) against *S. aureus* obtained healthy and diseased leaves and berries of *J. procera* plants. L1 (site1); L2 (Site 2); L3 (Site3); L4 (Site 4). Inhibition zone in centimeters and statistical difference at ***P*<0.05 and ****P*<0.01.

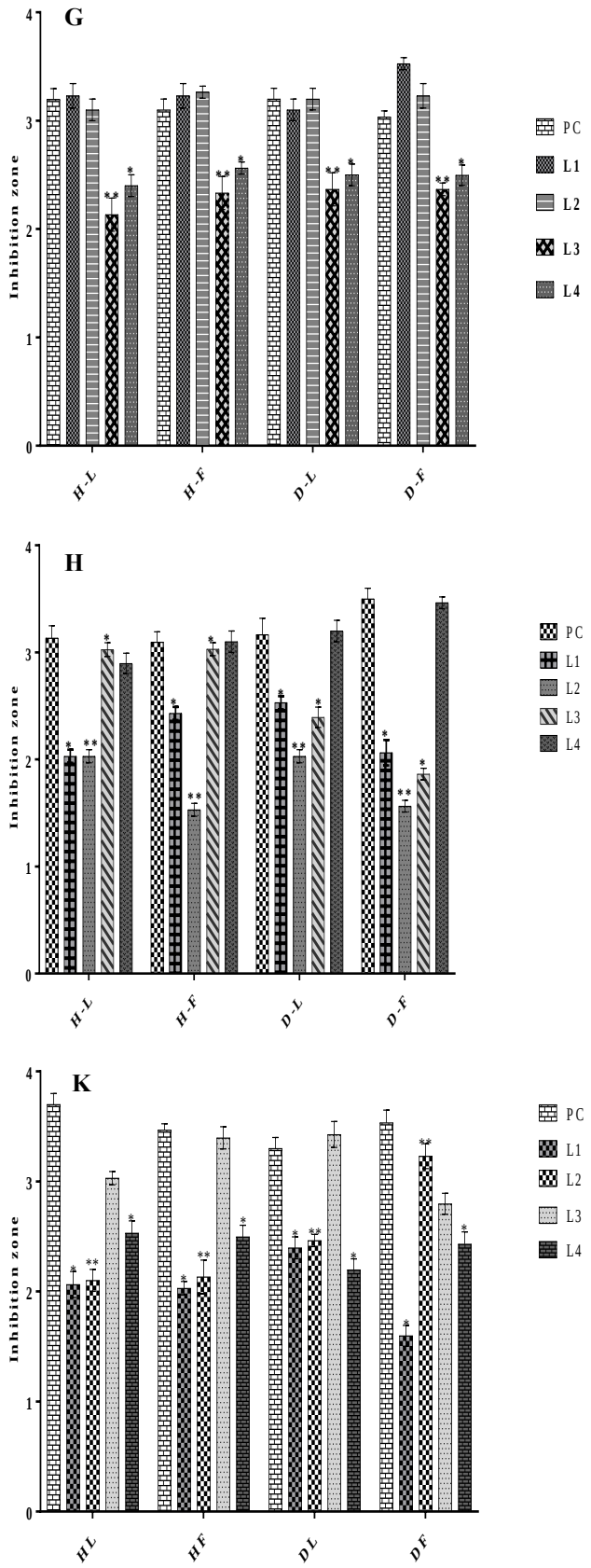

Fig. 3. Antibacterial activity of silver nanoparticles from healthy and diseased leaves and berries of *J. procera* plants against *S. aureus* (G); against *K. oxytoca* (H) and against *C. albicans* (K). L1 (site1); L2 (site 2); L3 (site3); L4 (site 4). HL (healthy leaves); HF (healthy fruits); DL (diseased leaves); DF (diseased fruits). Inhibition zone in centimeters and statistical difference at ***P*<0.05 and ****P*<0.01.

Impact of silver nanoparticles from *J. procera* against human pathogenic microbes: Synthesized silver nanoparticles gained from leaves and berries of both healthy and diseased *J. procera* plants proved potent inhibitory activites which ranged between (3.52 ± 0.05cm) against *S. aureus*, (3.46 ± 0.05cm) against *K. oxytoca* and (3.42cm ± 0.1cm) against *C. albicans*. Results showed that the highest antimicrobial activity from AgNPs found to be against *S. aureus* from diseased berries at (2737m), diseased berries extract at (3000m) against *K. oxytoca* and from diseased berries extract at (2940m) against *C. albicans.* However, results found that the lowest antimicrobial activity obtained from AgNPs of diseased berries extract of *J. procera* at (2846m) against *K. oxytoca* (Figs. 3G, H and K). Previous studies showed that silver nanoparticles had a potent inhibiting effect against gram-negative bacteria than gram-positive organisms (Shrivastava *et al*., 2007). Petica *et al*., (2008) demonstrated that silver nanoparticles had a profound effect against both Gram-negative and Gram-positive bacteria. Our result found that both Gram-negative and Gram-positive bacteria found to be also sensitive to the influence of synthesized silver nanoparticles either from leaves or berries extracts of both healthy and diseased *J. procera* plants.

Therefore, either the biosynthesized silver nanoparticles or crude extract of *J. procera* could be used as safe medicines against various human pathogenic microbes. Confirmation the biosynthesis of silver nanoparticles from leaves and berries extract of *J. procera* collected from four different elevations, administered from solution color change as the extract changed from light yellow to yellow then to dark brown. UV–vis spectrophotometer analysis had been applied to observe the formation of the silver nanoparticles (Figs. 4A, B, C and D). The absorption peaks of leaves and berries extract for healthy and diseased *J. procera* utilizing silver nanoparticles showed a wavelength ranged in between 440 nm to 470 nm. Femi-Adepoju *et al*., (2019) informed that the absorbance at about 460 nm for silver is a feature of these famous metal particles and formation the silver nanoparticles from the plant extracts. The synthesized nanoparticles also ascertained by using Scanning Electron Microscope (SEM) and Energy Dispersive X-ray analysis (EDX) having nanoparticles scale and Ag only as shown in (Fig. 4F). As these microorganisms found to cause a lot of diseases to human, for example, the bloodstream infection in hospitals caused mainly by *S. aureus* and *C. albicans* (Lin *et al*., 2009). It was found that *S. aureus* is in charge of various illnesses such as central nervous system (CNS) infections, skin problems, endocarditis, soft tissue bacteremia and pneumonia (Liu *et al*., 2011).Therefore, it is worth to find natural antibiotics form plant origin against *S. aureus, K. oxytoca* and *C. albicans*.

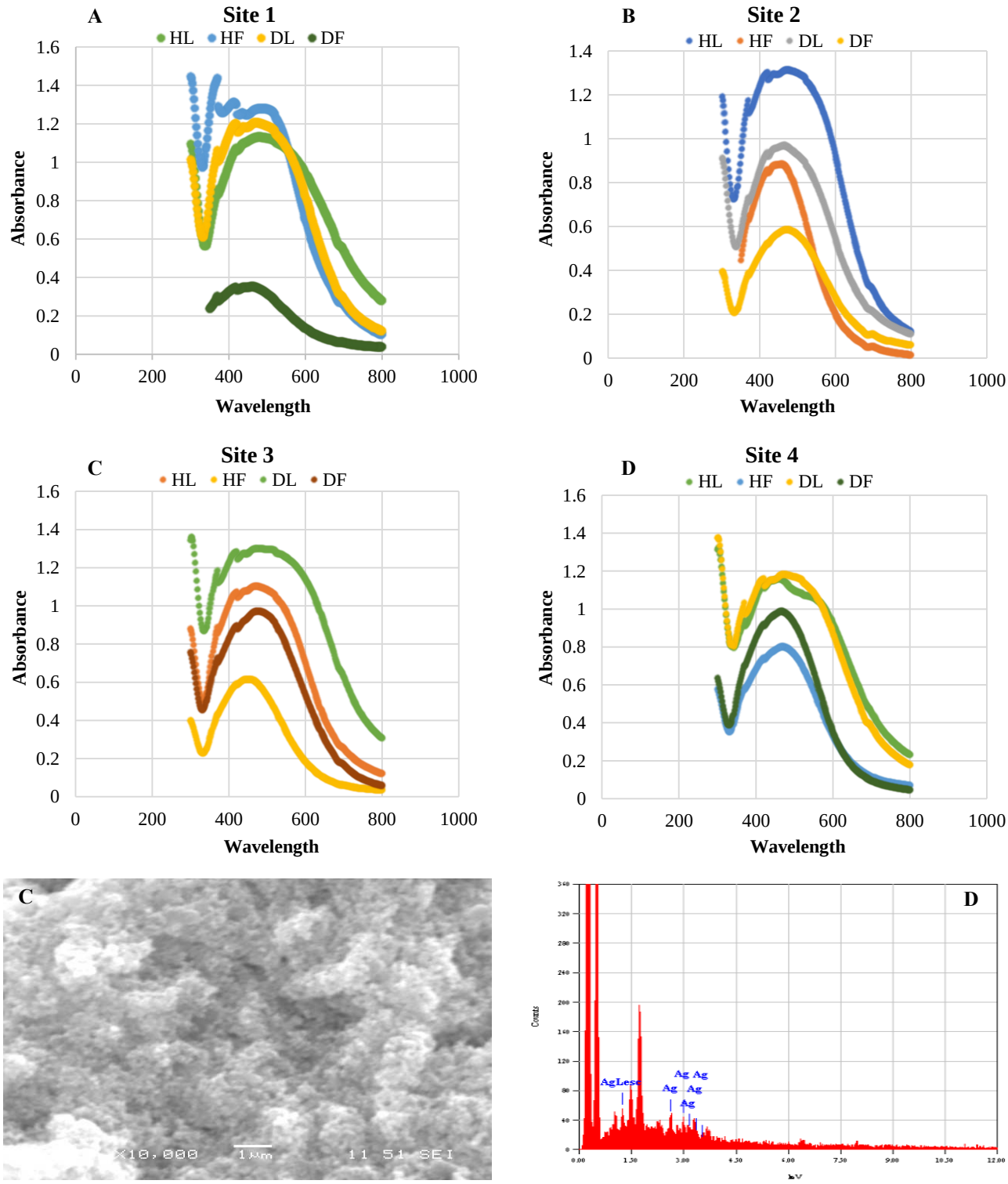

Fig. 4. UV–vis spectrophotometer of silver nanoparticles synthesized from leaves and berries extracts of healthy and diseased *J. procera* for various sites (A, site 1; B, site 2; C, site 3; D, site 4). HL (healthy leaves); HF (healthy fruits); DL (diseased leaves); DF (diseased fruits). (E), SEM images of the synthesized silver nanoparticles; (F), EDX image.

Conclusions

Crude solvent extract from healthy and diseased *J. procera* obtained from of leaves and berries exhibited significant potent antimicrobial activity against all pathogenic microbes. Hence, this study recommends using *J. procera* extracts as an alternative drug better than synthetic chemicals, supporting the traditional use of *J. procera* plants. This study also proposed that the dieback disease not from microbial infection, it may be physiological phenomena as the plant extract itself had a potent antimicrobial activity. This study also highlighted the possible application of silver nanoparticles from leaves and berries of *J. procera* against various pathogenic strains as having strong antimicrobial activity.

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