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ORIGINAL ARTICLE

Simultaneous effect of medicinal plants as natural photosensitizers and low-level laser on photodynamic inactivation

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Abstract

Photodynamic inactivation (PDI) technology is a promising alternative to antibiotics. This technology is defined as the inhibition of bacterial growth with photosensitizers while irradiated with low-level laser light in the wavelength of 532 ± 2.08 nm. A challenging area in this field is selecting photosensitizers with antibacterial potential. In this paper, to enhance the antibacterial efficiency, the photosensitizers (the selected plant extracts) with a high absorption peak at the selected laser frequency, 532 nm, were prepared. Low-concentration ethanolic plant extracts of *Hibiscus sabdariffa* and *Opuntia ficus-indica* were found to exhibit significant antibacterial activity against, *Acinetobacter baumannii ATCC 19606* and, *Staphylococcus aureus ATCC 33591* as two important human pathogenic bacteria. The effectiveness of these natural photosensitizers was measured by determining their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values and by performing a time-killing assay in the absence and the presence of laser irradiation. Our results showed that the combination of low-level laser irradiation and the selected photosensitizers had excellent potential for treating in vitro bacterial infections. Therefore, PDI technology has great potential as a viable alternative to traditional antibiotics for combating bacterial infections. This study presents a promising avenue for further exploration of PDI and the use of laser technology in medical science.

Keywords Photodynamic inactivation (PDI) · Medicinal plants · Antibiotic-resistant bacteria · Medicinal natural photosensitizer · Low-level laser

Introduction

Medicinal plants, rich in bioactive molecules, have intrigued researchers [1–3]. In the industry, cost-effective, environmentally sustainable antibacterial skin ointments have been developed [1, 3]. A pressing concern is the rise of antibiotic-resistant infections due to overusing antibiotics. Multidrug-resistant pathogens discredit antibiotics rapidly [4–6]. As antibiotics lose effectiveness, international research explores alternatives like photodynamic inactivation (PDI)/photodynamic therapy (PDT) [7–12]. The photodynamic action, the therapeutic effect of combining light with chemical matters, was accidentally discovered by German medical researchers Raab and his coworker. Photodynamic inactivation, also known as PDI, is a challenging method that is performed by using the precise cooperation of the key factors, light, oxygen, and photosensitizers that can be targeted the cells of pathogenic bacteria and inactivate or kill them. The most common phenomenon in applying light in the medical field

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is the absorption process, among other processes such as reflection, transmission, and scattering [13]. So, the other PDI key factors are photosensitive materials, and photosensitizers (PSs), which could optimize the inactivation process by absorption of light in a specific wavelength of light.

The primary mechanism in the PDI method includes the absorption of photons by the photosynthetic molecules, which yields the photophysical and photochemical processes and consequently a photopharmacological effect. The processes initiate when the photosynthetic molecules are excited by the absorption of laser light. The excited energy is dissipated as radiative or non-radiative decay; Radiation decay occurs through fluorescence and phosphorescence effects, and non-radiative decay, includes the inter-system crossing from the singlet excited state to the triplet excited state. The importance of the decay is the production of active pharmaceutical species. Especially type I and II of PDIs processes begin after the phosphorescence decay that led to forming the reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) and also pigment radicals. These species cause high oxidative damage to PSs, which is responsible for damage to bacterial cell membranes and destroying their enzymes and nucleic acids [14].

The selection of PSs is crucial due to their side effects, limiting their usage in the PDI method. Thus, choosing photosensitizers with no adverse health effects and antibacterial properties is highly valuable. Some PDI studies have proposed chemical photosensitizers like porphyrin, phthalocyanines, and chlorine [13, 15–19]. However, the limited availability of chemical photosensitizers without side effects restricts their use with any laser light wavelength. Plant PSs, such as B. orellana, C. longa, C. xanthorrhiza, G. blepharophylla, and H. sabdariffa, containing natural compounds like curcuminoids, alkaloids, and porphyrin, have been historically excellent sources. These plants offer effective therapeutic compounds and serve as alternatives to antibacterial agents for treating human infections. They are also used to inhibit the growth of gram-positive and gram-negative bacteria [20–23]. Consequently, the best choice for these photosensitizers is using plant pigments, which possess two positive attributes: no harm and antibacterial properties, owing to their bioactive compounds, including phenolic compounds [24–26].

Moreover, some studies of the PDI method have been confirmed on Gram-positive and Gram-negative bacteria by using photosensitizers with high-power lasers [27, 28]. However, usage of high-power lasers would be associated with serious damage for long periods, cost, and also side effects. Therefore, low-power lasers have attracted significant interest among researchers due to their cost-effectiveness and very few side effects [29, 30]. Kyungsu Kang and colleagues have proposed utilizing low-level laser and light with natural photosensitizers in the PDI method [14, 31, 32] and, other studies are mentioned [33, 34]. In the other studies, the PDI performance has been improved by selecting the maximum absorption peak of photosensitizers according to the laser wavelength [35].

In this paper, we attempted to improve the PDI efficiency and investigated the enhancement of the antibacterial properties of the selected photosensitizers on Gram-positive and Gram-negative bacteria. To this aim, the correct and accurate selection of natural photosensitizers was the priority of the research. Therefore, we purposefully started to survey the plant extracts that have excellent antibacterial properties in addition to a high absorption peak according to our low-power laser. Also, gas chromatography-mass spectrometry (GC/MS) was performed to study the chemical composition of the selected great natural photosensitizers including *H. sabdariffa* and *O. ficus-indica*. To evaluate the antibacterial properties of the natural pigments, well diffusion was performed to determine the zone of inhibition. Also, we have investigated the effectiveness of utilizing a 532 nm low-level laser in accompanying the selected pigments as good photosensitizers by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) and also the time-killing assay in the absence and the presence of the low power laser.

Materials and methods

Collection and extraction

This paper evaluated two bacterial groups, *Staphylococcus aureus ATCC 33591,* and *Acinetobacter baumannii ATCC 19606,* which are two important Gram-positive and Gram-negative human pathogenic bacteria, respectively.

Plant extracts were selected based on their previous demonstrated effectiveness at inhibiting bacterial growth against both Gram-positive and Gram-negative bacteria [36–39]. These studied groups were Leaf and branch of *Hibiscus sabdariffa*, the fruits of *Capsicum annuum group, Purple leaves of B.oleracea, Cornus mas,* and *Opuntia ficus-indica* as it is shown in Table 1. The choice of plant species and their potential impact on human health was carefully considered, as these plants have been examined by global organizations, such as the International Union for Conservation of Nature (IUCN) and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), to ensure they are not on the endangered red list and are not threatened with extinction. The plant species used in this study *H. sabdariffa*, purple leaves of *B. Olrtacea, Capsicum annuum group, Cornus mas, Opuntia,* were all collected in a responsible manner, identified and confirmed taxonomically by the herbarium of the Islamic Azad University, Central

Table 1 Collection of plant samples

Source	Common name	The used part	References	The used parts in this paper	City, Country
Hibiscus sabdariffa	*Hibiscus*	Leaf and branch	[40–42]	Leaf and branch	Mecca, Saudi Arabia
Purple leaves of B.oleracea	*Red cabbage*	Fruit	[43, 44]	Fruit	Shiraz, Iran
Capsicum annuum group	*Red bell pepper*	Fruit	[45, 46]	Fruit	Shiraz, Iran
Cornus mas	*Cornelian Berries*	Fruit	[47, 48]	Fruit	Ghazvin, Iran
Opuntia ficus-indica	*Fig opuntia*	Plants	[49, 50]	Fruit	Kelardasht, Iran

Tehran Branch of Iran, and are presented alongside their useful parts in Table 1. Of these plant species, *H. sabdariffa*, purple leaves of *B. Olrtacea,* and *Capsicum annuum group,* are safe for human consumption, while *Cornus mas* is classified as Least Concern (LC) and *Opuntia* is Data Deficient (DD). In subsequent sections, low concentrations of these extracts were studied for their effect on Gram-positive and -negative bacteria.

Plant samples collection and extract preparation

In this paper, we used five samples, namely, *H. sabdariffa*, purple leaves of *B. olrtacea, Capsicum annuum group, Cornus mas,* and *Opuntia*, were mainly purchased from a local market in Kelardasht, Iran. All the samples, except *H. sabdariffa*, were sourced from various regions in Iran, as indicated in Table 1. The species used were confirmed by Central-Tehran University herbarium.

To prepare the samples, the shade drying method [51] was used. The plant parts were washed and then dried in the shade at room temperature to prevent the reduction of effective ingredients [52]. After drying, all plants were ground, and fresh fruits were washed and sliced to increase the level of solvent contact with plant materials, which optimizes compound extraction [52].

Preparation of plant extracts by Soxhlet and maceration methods

This study employed two extraction techniques, maceration and Soxhlet, details of which can be found in Table 2.

Table 2 The solvents and methods of extraction

Plants	Solvents: Ethanol	
	Methods:	
	a. Soxhlet	b. Maceration
H. sabdariffa	✓	-
Purple leaves of B. olrtacea	✓	✓
Capsicum annuum group	✓	✓
Cornus mas	-	✓
Opuntia	✓	✓

a. Soxhlet extraction Method

The Soxhlet extraction method involved adding 5 g of dried plant material and 40 g of sliced fruit to 100 mL of 70% ethanol in D.W. (v/v) and extracting the mixture at a temperature of 70℃ for 2 h using a Soxhlet device (Barn stead/Electrotbermae, UK).

b. Maceration Method

The maceration method, started with dispersing 40 g of the samples in 50 mL of 70% ethanol in D.W. (v/v). The samples were then kept in a dark place for two weeks, followed by shaking in the dark at room temperature for 24 h.

Also, the extracts were filtered using Whatman #3 paper filters, and solvent removal was carried out using a Rotary device (Heidolph Instruments, Germany) with a rotational speed of 136 rpm and a heating bath temperature of 30 °C for 20 min. For the extracts that were not completely dried, a freezer dryer [53] (Martin Christ, Alpha 2–4 LD plus, German) was used for 48 h (Table 3).

The solvent extraction processes for the maceration and Soxhlet methods are depicted schematically in Fig. 1.

Finally, the spectroscopic method was used to confirm the presence of some compounds of active ingredients in plant extracts [54]. Specifically, the absorption spectra of control samples were compared with the absorption spectra of the extracts, and each extract's spectroscopy was repeated several times to ensure accuracy. Tables 4 and 5 presents the measured absorption spectrum of the extracts and the spectral fingerprints of the extract compounds used in previous studies.

The spectroscopy of each extract was repeated several times to ensure accuracy.

GC/MS analysis

Gas chromatography-Mass spectroscopy (GC–MS) is an effective technique to detect the composition of volatile components of herbal samples origin. The GC–MS analysis of the selected great natural samples of *H. sabdariffa* [55], and Opuntia [56] were carried out on an Agilent Technologies, USA) GC model 7890 B equipped with a mass

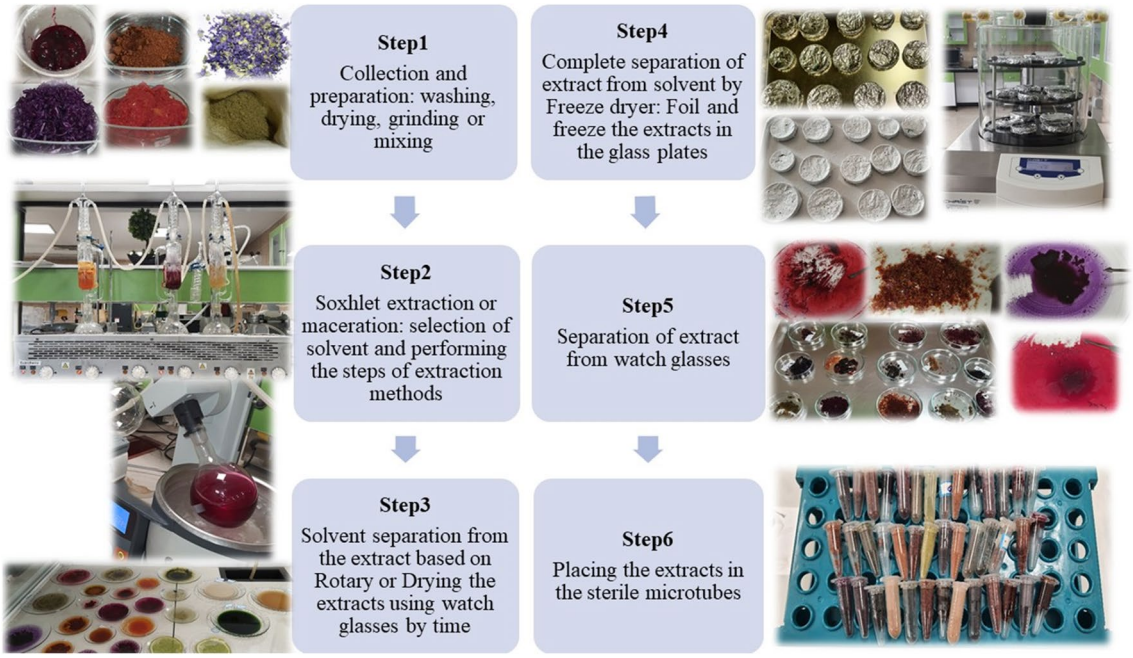


Fig. 1 The steps of the solvent extraction using soxhlet and maceration methods

selective detector model MSD model 5977 A in the electron ionization mode at 70 eV. All method was the same for the two samples. For both samples, 1 µl of each sample volume was used for injection, with a split ratio of 1:50 and operating temperature set at 260 °C. The carrier gas utilized was high-purity helium with a flow rate of 0.46 mL/min, and volatiles were separated using a capillary column on a DB5-MS column (30 m length, 0.25 mm inner diameter, and 0.25 µm film, J&W Scientific, Santa Clara, CA, USA). The column temperature was held at 50 °C for 4 min, followed by an increase to 240 °C with a 10 °C/min rate and a hold for 2 min. The GC analysis and the column temperature programming were in the same situation. Then, the GC column effluent was introduced into the MS source, while ion source and interface temperatures were set at 200 °C and 240 °C, respectively. MS parameters were set to 70 eV (E1), electron multiplier voltage 1800 eV, mass range 35–550 amu, the event time 0.15 s, and the scan speed 5000. Standard matters in the references revealed the quartz index, NIST, and WILEY library database was used to find out mass spectrum components.

Antibacterial activity evaluation

In this research, *Staphylococcus aureus ATCC 33591,* and *Acinetobacter baumannii ATCC 19606* were evaluated as two critical types of human pathogenic bacteria, classified as Gram-positive and Gram-negative, respectively.

Well diffusion method

To examine the antibacterial activity of the extracts, a diffusion test was used on Muller-Hinton Agar and Blood Agar mediums, according to Clinical and Laboratory Standards Institute (CLSI) guidelines [57]. Initially, a bacterial suspension with a turbidity of 0.5 McFarland in sterile normal saline was prepared and then plated. Sterile Pasteur pipettes were utilized to create the wells, into which 30 µL of each extract solution was added. The plates were then incubated at 37 °C for 20–22 h. In subsequent steps, the most potent plant extracts exhibiting a broad zone of inhibition around the wells were evaluated.

Determination of MIC and MBC without and with laser irradiation

The antimicrobial efficacy of the extracts was evaluated using the standard broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [58]. The experiment was conducted using Mueller–Hinton broth as the test medium. Two-fold serial dilutions of each extract were prepared, and a final volume of 100 µL was added to wells containing 100 µL of MHB. A bacterial suspension was prepared in sterile normal saline and added to each well to achieve a final concentration of -106 colony forming units (CFU)/mL. The microplate was incubated at 37 °C for 20–22 h. The minimum inhibitory concentration (MIC) was determined as the lowest

concentration of the extract that could inhibit visible growth of the bacteria being tested. The minimum bactericidal concentration (MBC) was determined by culturing 100 µL of the MIC well and all wells that showed no growth on Muller-Hinton agar. After 20–22 h of incubation at 37 °C, the MBC values were recorded as the lowest concentrations that could kill all bacterial cells, represented by the absence of bacterial colonies.

Selecting the good photosensitizers

We leveraged a spectrophotometer (Cecil Spectrophotometer (SE9500), UK) to determine the absorption spectra of the extracts within the 200–900 nm UV–Visible range in order to select the most effective photosensitizers.

Selecting the relevant light source and setup

The methodology in the article involves tailoring laser wavelengths based on the absorption peaks of plant wavelengths, optimizing absorption for maximal bacterial inactivation during Photodynamic Therapy (PDT). Low-power laser was used to minimize damage risks and conduct trials to identify the most effective inhibition strategies. Additionally, factors such as photosensitizer type and concentration, as well as light output power and duration, to select the ideal wavelength for photoinactivation were considered. This meticulous approach ensures both efficacy and safety in bacterial inactivation within antibacterial plant species. To leverage its therapeutic backgrounds and benefits, we utilized a low-level laser [59], which was specifically selected based on the photosensitizers' absorption peaks. The technical specifications for the laser entailed a wavelength of 532 nm, a bandwidth of laser: 4.16 nm, continuous mode of irradiation, a power output of 20 mW, irradiation durations of 6, 12, 18, and 20 min, a 5 cm distance to target, a laser spot size of 1 mm^2, a light intensity of 25 mW/cm^2, and energy for each times periods, 9, 18, 27, and 30 J/cm^2, relatively.

We fixed laser setups and aligned them vertically, covering the sub-microplate using a black mask to hinder reflected light from reaching the neighboring wells during laser irradiation. As the laser beam was divergent, we employed a plano-convex lens to focus it. Additionally, to maintain a sterile environment, we conducted laser irradiations under laminar flow hood conditions.

MIC determination

The minimum inhibitory concentration MIC test was done the same as the previous one using the standard broth dilution method according to CLSI [58]. Negative control (without bacteria) and positive control (without photosensitizer) wells were implemented in the sterile microplate's eleventh and twelfth positions, respectively, in each row. The exposure time of approximately 6, 12, 18 and 20 min was applied to the wells. Next, the microplates were incubated for 20–22 h after the laser exposure. The MBC values were estimated applying the process outlined in Sect. "GC/MS analysis".

Time-killing assay

To determine the colony-forming units (CFUs), 100 µL from the control well (which contained bacteria without extracts) and the two wells preceding the MBC well and the actual MBC well (which included both bacteria and herbal sample) were serially diluted in sterile normal saline into 9 consecutive tubes (0.1 mL of well content and 0.9 mL of diluent). Next, 100 µL from the tubes 7, 8, and 9 were transferred to Muller-Hinton agar plates, then incubated for bacterial growth overnight. After 18–24 h, the colonies were counted for CFU/mL using naked-eye observation. Each experiment was repeated thrice to ensure accuracy."

Data analysis

Tests were performed thrice to promote accuracy. The standard deviation (mean ± standard deviation) for each test were calculated using the descriptive statistics frequency method available in the SPSS (Statistical package for the social sciences) package. One-sample t-tests were carried out to compare antibiotic zone inhibition and medicinal plants effectiveness against bacteria. Similarly, the t-test was used to compare MIC tests outcomes for each sample in the presence and absence of laser irradiation. It's important to mention that a p-value of less than 0.05 was considered statistically significant.

Results and discussion

The results of spectroscopy measurements

There has been a lot of research on the healing properties of plants due to the presence of certain chemical structures. In this study, the selection of the plants was based on the presence of bioactive compounds including phenolic and non-phenolic compounds. The information about the color, bioactive compounds, fingerprints, and pharmacology of the

plants can be found in Table 3. Plant color is dependent on the type of pigments present, with carotenoids being responsible for yellows, oranges, and reds in plants such as the Capsicum annuum group, as indicated in Table 3. Bioactive compounds found in plants can lead to various pharmacological activities including antibacterial properties, as shown in the pharmacological section of Table 3. One of the most important groups of bioactive compounds found in plants is phenolic compounds. These compounds play critical roles in human health mainly as antioxidants, anti-allergic, anti-inflammatory, anticancer, antihypertensive, and antimicrobial agents [60].

They are secondary metabolites of plants are characterized by the presence of aromatic ring(s) bearing one or more hydroxyl moieties, and can be divided into different groups based on the number of phenolic rings [24–26]. For the extraction of both phenolic compounds and non-phenolic from the plants studied in this paper, the maceration and soxhlet methods were employed. Ethanol extraction was chosen due to its simplicity and effectiveness, as well as its boiling point of 78.37 °C which prevents the reduction of effective ingredients [52].

To select the best photosensitizers, the spectroscopy of most plant extracts was conducted in the range of 200–700 nm, with the rest being measured in the range of 400–700 nm, using a spectrophotometer. Additionally, the bioactive ingredients of the extracts were confirmed by comparing their absorption peaks of the extracts with their respective fingerprints, which are presented in Table 3.

As presented in Table 3, all extracts have high absorption in the ultraviolet region at 200–300 nm, indicating the presence of phenolic compounds such as phenols, tannins, and flavonoids. Non-phenolic compounds including alkaloids and coumarin, stachydrine, steroids, β-caryophyllene, allicin, and ascorbic acid, are listed in the phytochemical section of Table 3. Furthermore, the absorption peaks of the bioactive ingredients are directly correlated with the concentration of the non-phenolic compounds. The absorption peaks at about 535 nm for *Purple leaves of B. olrtacea, H. sabdariffa, Opuntia, C. mas,* and *Capsicum annuum group* have shown the presence of compounds such as anthocyanin and Carotenoid pigments. The presence of bioactive compounds in the examined plants was confirmed through spectral analysis, which revealed the presence of compounds such as anthocyanin and carotenoid pigments. These findings are consistent with the available fingerprints of the plants from previous studies, as presented in Table 3.

The selected plants possess therapeutic potential due to the presence of the bioactive compounds, especially phenolic structures [61], which exhibit significant antimicrobial properties.

The drying and extraction of the plant species used in this study were performed following the guidelines of valid references cited in this article. Extraction involves the separation of active ingredients of the raw plant [52], which depends on several factors, including temperature, particle size, extraction time, solvent type, solvent to solid ratio, and extraction methods [62–64]. Various extraction techniques, such as maceration, percolation, decoction, soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, pulsed electric field extraction, and enzyme-assisted extraction, hydro distillation, and steam distillation, have been utilized [52]. Different solvents have also been employed for the extraction of bioactive ingredients from plants [52], with each bioactive compound requiring a specific solvent.

GC/MS analysis

Gas chromatography-mass spectroscopy (GC–MS) was employed to analyze the chemical constituents of plant extracts *H. sabdariffa* and *Opuntia*, which exhibited potent antibacterial activity and had a greatly desired absorption peak. The results showed the presence of a range of phenolic and non-phenolic compounds with significant peak areas, as indicated in Table 4 and 5. These compounds were identified based on their retention time and area percentage, and are believed to be responsible for the observed antimicrobial effects against human pathogenic microorganisms. Overall, our findings highlight the potential of these plant extracts as natural sources of antimicrobial agents, and pave the way for further investigations into their therapeutic properties. Tables 4 and 5 provide detailed information on the chemical composition of extracts *H. sabdariffa,* and *Opuntia,* respectively. Gas chromatography-mass spectroscopy (GC–MS) analysis revealed the presence of various classes of bioactive compounds in both extracts, as evidenced by the 16 and 8 peaks detected in the GC–MS chromatograms of *H. sabdariffa* and *Opuntia*, respectively (Figs. 2a, b). Further studies are needed to explore the potential applications of these extracts and their bioactive components in the development of new therapeutic agents. In order to identify specific and non-specific compounds, we compared the identified compounds in our extracts with known compounds reported in the literature. This comparison involved analyzing absorption peaks and comparing them with fingerprints from previous valid references. Specific compounds *H.sabdariffa*, with antibacterial properties or can combination with that (as components of antibacterial formulations) are Anthocyanins, betacyanins, citric acid, malic acid, propanoic acid, Flavonoids, Hydroxycinnamic acids, 2(3H)-Furanone (γ-butyrolactone), 2-Furancarboxaldehyde (furfuraldehyde), 4-Benzoquinone, Tetradecanoic acid (myristic acid), n-Hexadecanoic acid (palmitic acid), Hexanoic acid (caproic acid), 9,12-Octadecadienoic acid (linoleic acid), 9-Octadecenoic

Table 3 information on phytochemical and pharmacology of plants

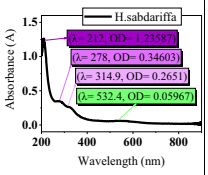
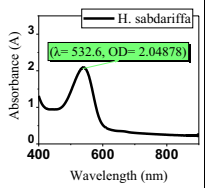
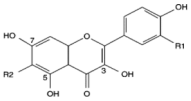
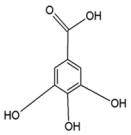
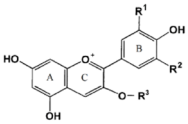
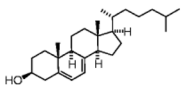
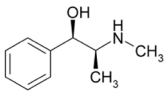
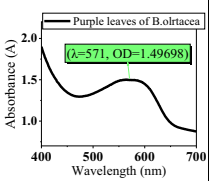
Plants and the absorption spectra	Color	Phytochemical: bioactive compounds and their fingerprints (absorption peaks)	Pharmacology	Ref
H. sabdariffa  The absorption spectrum of the ethanolic extract of *H. sabdariffa* is in the range of 200-900. The absorption peaks are remarked.  The absorption spectrum of the ethanolic extract of *H. sabdariffa* is in the range of 400—900 nm (the absorption peak is remarked).	Red	Phenolic compounds: Flavonoids (in the range of 240-285 nm and the other in the range of 300-400 nm) [67 -69]  Tannins (280nm) [70]  Anthocyanin and Anthocynadin (predominantly one between 270–290nm and the other in the visible **spectrum** at 500–550 nm) [69,71]  Non-phenolic compounds: Steroids [72]  Alkaloids [73] 	Antipyretic, antinociceptive activity Inflammatoy activities Anticancer activity Antibacterial, antifungal, and antiparasitic actions Anticholesterol effect, Renal effects	[61-62]
Purple leaves of B. olrtacea  The absorption spectrum of the ethanolic extract of *Purple leaves of B. olrtacea* is in the range of 400-700 nm. The absorption peak is identified on the fig. The phenolic compound has a high absorption before 400 nm, with a high dilution of the extracts.	Purple	Phenolic compounds: Flavonoids (in the range of 240-285 nm and the other in the range of 300-400 nm) [67 -69] Tannins (280nm) [70] Anthocyanin and Anthocynadin (predominantly one between 270–290nm and the other in the visible **spectrum** at (500–550 nm) [67,71] Non-phenolic compounds: Steroids [72] Alkaloids [73]	Antibacterial, anti-diabetic, neuroprotective, cardioprotective, anti-obesity, nephroprotective, hypocholesterolemic	[63]
Capsicum annuum group	Red\Orange	Phenolic compounds: Polyphenol [69]	Antimicrobial activity	[64]

Table 3 (continued)

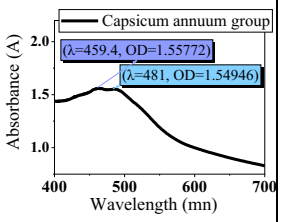
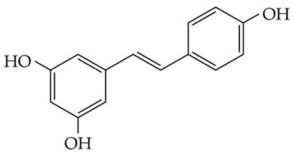
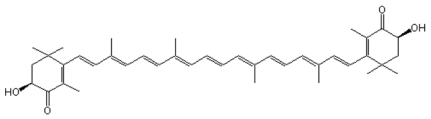
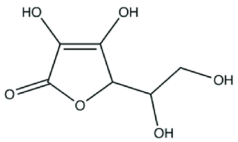
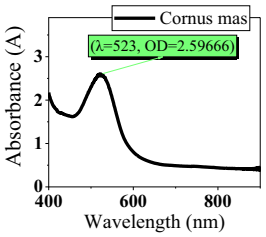
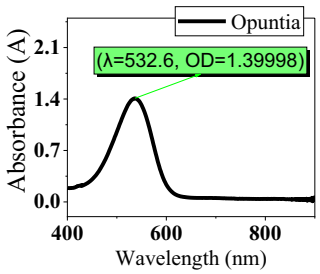
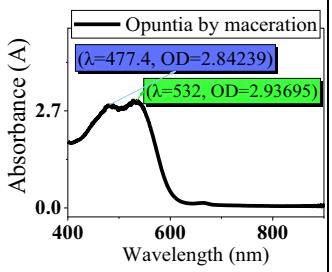
 The absorption spectrum of the ethanolic extract of the *Capsicum annuum group* is in the range of 400-700 nm. The absorption peaks are identified on the fig. The phenolic compound had a high absorption before 400 nm, with a high dilution of the extracts.		 Non-phenolic compounds: Carotenoid [74]  Chlorophyll a [74] vitamin c (ascorbic acid) (at 265 nm) [75] 		
Cornus mas  The absorption spectrum of the ethanolic extract of *Cornus mas* by the maceration method is in the range of 400-700 nm. The absorption peaks are identified on the fig. The phenolic compound had a high absorption peak before 400 nm, with a high dilution of the extracts.	Red	Phenolic compounds: Phenol [69] Anthocyanin and Anthocynadin (predominantly one between 270–290nm and the other in the visible **spectrum** at 500–550 nm) [69,71] Non-phenolic compounds: ascorbic acid [74]	Antimicrobial activity	[65]
Opuntia	Red	Phenolic compounds: Phenol [69] Anthocyanin and Anthocynadin (predominantly one between 270–290nm and the other in the visible **spectrum** at 500–550 nm) [69,71]	antiviral and antimicrobial action	[66]

Table 3 (continued)

 The absorption spectra of the ethanolic extract of *Opuntia* by the soxhlet method are in the range of 400-900 nm. The absorption peaks are identified on the fig. The phenolic compound had a high absorption peak before 400 nm, with a high dilution of the extracts.  The absorption spectrum of the ethanolic extract of *Opuntia* by the maceration method is in the range of 400-900 nm. The absorption peaks are identified on the fig. The phenolic compound had a high absorption peak before 400 nm, with a high dilution of the extracts.		Non-phenolic compounds: Amino acids [76], organic acids, sugars, vitamins, minerals, fibre		

acid (oleic acid). And about non-specific compounds of it, 9,9 Dimethyltetracycloundecan-2-one, Oceanic acid, Bis(2-ethylhexyl) phthalate. Specific compounds Opuntia, with antibacterial properties or can combination with that (as components of antibacterial formulations) are Anthocyanins, betacyanins, Tetradecanoic acid. And about non-specific compounds of it, silane, maleic anhydride, furfural, 2,5-furandicarboxaldehyde, n-Hexadecanoic acid. Some

Table 4 The components of *H. sabdariffa* ethanolic extract gained by GC–MC

No	Name of compounds	Retention Time (min)	Area	Area%
1	Furfural	2.489	27,666,055	1.52
2	5 Methyl Furfural	4.135	9,486,017	0.52
3	Propanoic acid	4.764	21,402,184	1.18
4	2(3H)-Furanone	5.797	19,787,603	1.09
5	Levoglucosenone	6.625	13,489,912	0.74
6	2-Furancarboxaldehyde	8.553	97,232,555	5.34
7	4-benzoquinone	8.597	34,333,357	1.89
8	9,9-dimethyltetracyclo[6.2.1.0(1,6).0(6,10)] undecan-2-one	14.552	42,112,651	2.31
9	Tetradecanoic acid	16.740	8,449,861	0.46
10	n-Hexadecanoic acid	19.340	156,546,913	8.60
11	Hexanoic acid	21.141	302,726,010	16.63
12	9,12-Octadecadienoic acid (Z,Z)	21.345	7,149,371	0.39
13	9-Octadecenoic acid	21.403	16,557,111	0.91
14	Octadecanoic acid	21.660	23,633,610	1.30
15	Octanoic acid	21.907	13,571,407	0.75
16	Bis(2-ethylhexyl) phthalate	22.161	8,021,896	0.44

Table 5 The components of *Opuntia* ethanolic extract gained by GC–MC

No	Name of compounds	Retention Time (min)	Area	Area %
1	Silane	3.454	583,671	1.38
2	Furfural	4.983	2,865,922	6.75
3	Maleic anhydride	5.478	7,857,041	18.51
4	2,5-Furandicarboxaldehyde	10.158	598,281	1.41
5	2-Furancarboxaldehyde	12.580	13,670,400	32.21
6	Phenol, 2-propyl-	14.635	2,228,623	5.25
7	Tetradecanoic acid	19.269	783,350	1.85
8	n-Hexadecanoic acid	21.358	9,205,739	21.69

non-specific compounds do not possess antibacterial properties themselves but can enhance and combine as components of antibacterial formulations as silane, maleic anhydride, Furfural, n-Hexadecanoic acid. Specific compounds with both antibacterial properties and being a good photosensitiser in desired wavelength (500–550 nm), anthocyanins, and betacyanins.

Antibacterial activity

One of the alternative approaches to antibiotics for killing or inhibiting the growth of bacteria is the usage of the antimicrobial properties of plant extracts, Since, antibiotics have lost their effectiveness in treating bacterial infections due to antibiotic resistance and side effects. One promising approach is the use of plant extracts with antimicrobial properties. To assess the antibacterial activity of the extracts, disk diffusion and dilution methods were used, both in the absence and presence of the light source. The extracts with better therapeutic performance against both Gram-positive and Gram-negative bacteria were selected for further experiments.

The results of the inhibition zone assay, presented in Table 6, demonstrated that the ethanolic extract of H. sabdariffa exhibited the highest antimicrobial activity against the tested bacteria. This activity can be attributed to the bioactive compounds identified in Table 3, which have been shown to inhibit cell wall synthesis, as well as protein and nucleic acid synthesis [52, 65]. Further analysis using the Spss package showed that all tested extracts, regardless of the extraction method, exhibited a significant reduction in the bacterial population due to their antimicrobial properties. Two extracts with both high zones of inhibition and good absorption peaks were chosen for further testing. Overall, these findings demonstrate the potential of plant extracts as natural sources of antimicrobial agents, and provide a basis for further investigations into their therapeutic properties.

Comparison of antimicrobial activity of extracts with standard antibiotics

The antimicrobial activity of the plant extracts was compared with the relevant antibiotics against the gram-positive and gram-negative bacterium**.** The antibiotics sensitivity of cyclophosphamide CTX(B), tetracycline TE(B), vancomycin V(B), penicillin P(A), erythromycin E(A), ciprofloxacin CFO(A), clindamycin CC(A) on some gram-positive and negative bacteria were confirmed according to CLSI 2020. The plant extracts with the best zone of inhibition were chosen for comparison. Figure 3 presents the results of comparing the zone of inhibition of antibiotics with the best extracts and its numerical results were shown in Table 7 and 8. For the gram-negative bacterium, A. baumannii, two antibiotics CTX(B) and TE(O) were more effective than the other plant extracts, but the extract of *H. sabdariffa* had the same therapeutic effect as the vancomycin (V(B)) antibiotic. For the gram-positive bacterium, S. aureus, some antibiotics p(A), E(A), CFO(A), and CC(A) exhibited higher antibacterial activity than the plant extracts, while others had similar zone of inhibition, approximately equal to that of the CC(A), CP(C), and T(B)antibiotic. The calculated *P*-values for A. baumannii and S. aureus were found to be less than 0.02 and 0.01,

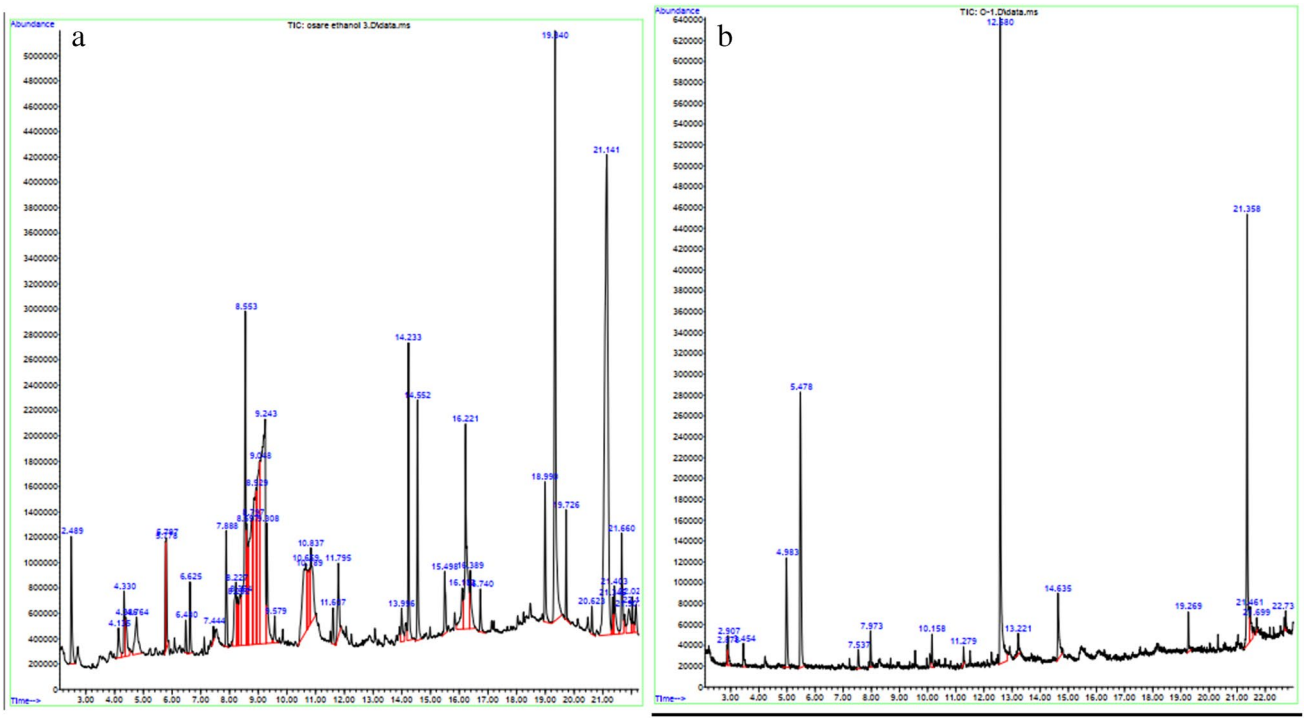


Fig. 2 (**a** and **b**) GC–MS Spectral Chromatogram of Ethanolic Extract of *H. sabdariffa* and *Opuntia* from Left to right respectively

Table 6 The zones of inhibition of antibacterial activity

Microorganisms	Gram-negative bacterium *A. baumannii*	Gram-positive *S. aureus*
Extract concentration $\left(\frac{30\mu g}{well}\right)$	Zone of inhibition (in mm) Mean ± Standard deviation*	
H. sabdariffa	17 ± 0.20*	17 ± 0.32*
Purple leaves of B. olrtace	0	0
Purple leaves of B. olrtace (macerate)	0	0
C. annuum group	0	0
C. annuum group (Macerate)	0	0
C. mas (Macerate)	0	13 ± 0.25*
Opuntia	10 ± 0.20*	11 ± 0.25*
Opuntia (macerate)	9 ± 0.30*	10 ± 0.20*

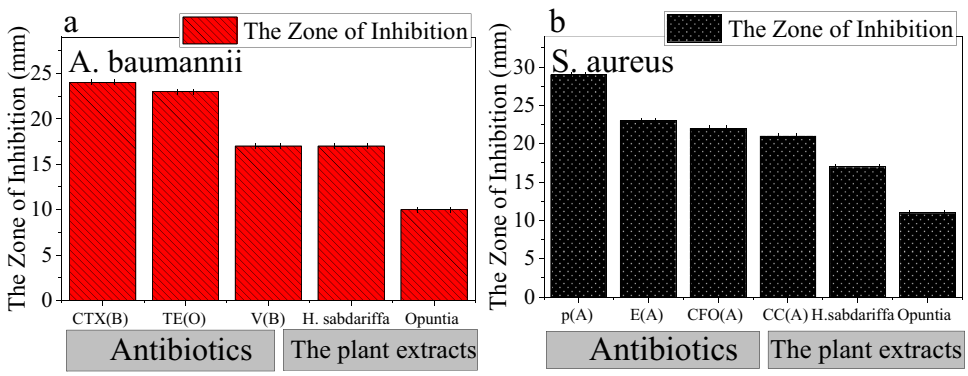


Fig. 3 Comparison of the inhibition zone of antibiotics with the best plant extracts against bacteria. In Fig. 3a, the statistical analysis showed a significant difference with a P value < 0.02, and in Fig. 3b, the difference was even more significant with a P value < 0.01, indicating the strong statistical significant of observed finding

Table 7 Numerical comparison of the inhibition zone of antibiotics with the best plant extracts against A. baumannii, *P* value < 0.02

Tested Samples against A. baumannii	The Inhibition Zones Mean ± Standard deviation*
CTX(B)	24 ± 0.1*
TE(O)	22 ± 0.5*
V(B)	17 + ± 0.5*
H. sabdariffa	17 ± 0.20*
Opuntia	10 ± 0.20*

Table 8 Numerical comparison of the inhibition zone of antibiotics with the best plant extracts against S. aureus

Tested Samples against S. aureus	The Inhibition Zones Mean ± Standard deviation*
P(A)	29 ± 0.1*
E(A)	23 ± 0.2*
CFO(A)	22 ± 0.15*
CC(A)	21 ± 0.1*
H. sabdariffa	17 ± 0.32*
Opuntia	11 ± 0.25*

P value < 0.01

respectively, indicating statistically significant differences the two groups.

The mechanism of action of antibiotics may vary for each bacterium. Beta-lactam antibiotics, including penicillin, cephalosporins (cephems), monobactams, and carbapenems, act as irreversible inhibitor of the enzyme transpeptidase, which bacteria use to make their cell walls [66]. Aminoglycosides inhibit protein production and affect cell membrane permeability by binding to the 30S subunit of rRNA, causing genetic code misread. Fluoroquinolone interrupts DNA breakage-reunion by binding to DNA-gyrase or topoisomerase II and topoisomerase IV, while tetracycline interferes with amino acid transfer by binding to the 30S subunit of rRNA, preventing protein production [67]. In conclusion, the tested plant extracts with antibacterial properties were found to be appropriate alternatives to antibiotics with fewer side effects for killing bacteria.

MIC and MBC assay

Based on the findings of the previous section, *H. sabdariffa* and *Opuntia* extracts exhibited a higher antibacterial activity than the antibiotics, as demonstrated in Fig. 3. Additionally, these extracts displayed strong absorption peaks at specific wavelengths, as illustrated in Table 3, indicating their potential as effective photosensitizers. Specifically, two extracts exhibited exceptional photosensitizing properties, with absorption at approximately 532 nm. As such, the present study aimed to evaluate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these two extracts under laser irradiation and non-irradiation conditions.

In the absence of laser radiation

As can be seen in Fig. 3a and 3b, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *H. sabdariffa* and *Opuntia* extracts were evaluated against Gram-positive and Gram-negative bacteria. Interestingly, the ethanolic extract of *H. sabdariffa* exhibited a higher suppressive effect on Gram-positive bacteria than Gram-negative bacteria. This observation may be attributed to the dissimilarities in the cell wall structure between the two bacterial types. The complex and multilayered cell wall structure of Gram-negative bacteria provides them with higher resistance compared to Gram-positive bacteria. Specifically, the cell wall of Gram-positive bacteria is composed of two layers, namely the cytoplasmic membrane and a thick peptidoglycan layer containing teichoic acid. Conversely, Gram-negative bacteria possess an additional membrane layer in their cell wall architecture, situated outside the peptidoglycan layer, that exhibits an asymmetric lipid structure comprising of strongly negatively charged lipopolysaccharides (LPS), lipoproteins, and proteins with porin function [68].

Moreover, the MIC and MBC results of *H. sabdariffa* and *Opuntia* extracts were found to be effective. It is evident that higher concentrations of the extracts led to greater antibacterial activity against the tested bacteria.

In the presence of laser irradiation

In this section, we investigated the therapeutic properties of two plant extracts against gram-positive and negative bacteria. Specifically, we determined the minimum inhibitory concentration (MIC) of the extracts in the presence of laser irradiation, using a photodynamic inactivation (PDI) method. Photosensitizers or photo acceptors are molecules that absorb specific wavelengths of light and can be used in PDI for antimicrobial activity. The combination of photosensitizers and light leads to a promising technique with antimicrobial activity that is called photodynamic inactivation of bacteria (PDI). While various pigments have been reported as good photosensitizers [14], plant extracts can also play this role due to their sharp absorption peak at

specific wavelengths, making them not only antibacterial agents but also excellent photosensitizers.

Two selected plant extracts (*H. sabdariffa* and *Opuntia* extracts) were found to have an absorption peak at around 532 nm, based on the spectra shown in Table 3. To increase the efficiency of their antibacterial action as photosensitizers, we determined the laser wavelength by their absorption spectra. We used a green (532 nm) low-level laser due to its safety, cost-effectiveness, and availability.

MIC testing was performed by the standard broth dilution method according to CLSI, and MBC testing was done in the same manner as before irradiation. Interestingly, a significant increase in the antibacterial effects of the photosensitizers (*H. sabdariffa* and *Opuntia*) against Gram-positive and Gram-negative bacteria was achieved in the presence of the green low-level laser beam. The laser was irradiated to each well of the microplate in 4-time steps of 6, 12, 18, and 20 min. It is worth noting that, each experiment was repeated three times. The results of laser irradiation were presented in Fig. 4, its numerical results in Table 9. The *P* value of MIC/MBC for *H. sabdariffa* and *Opuntia* on both A. baumannii and S. aureus was statistically significant (P value < 0.037), indicating the efficacy of the PDI method.

Since two tested extracts had significant antibacterial properties in the absence of laser, the result of increasing the antibacterial properties of these extracts was considered in the presence of the laser irradiation in consecutive periods of about 6, 12, 18, and 20 min. The MIC/MBC values showed that the antibacterial properties of two tested extracts were significantly increased in the presence of laser irradiation as indicated in Figs. 4a and 4b. This enhancement was attributed to the synergy between the laser and the photosensitizers in the PDI process, which produced singlet oxygen and free radicals that increased the antibacterial activities. The mechanism behind this enhancement was explained through the biophysical expression of an increase in antibacterial properties, where the laser radiation on photosensitizers increased and accelerated the number of reactions with oxygen molecules, which ultimately led to an increased antibacterial effect [69]. The study highlighted the potential of using photosensitizers and laser irradiation in the development of new antibacterial therapies.

Following the second, third, and fourth periods of irradiation, which had a mean duration of 12, 18, and 20 min, respectively, the antibacterial properties of the photosynthetic solution remained constant as it shown in Figs. 4a, 4b. However, this phenomenon can be attributed to the process of laser beam irradiation on wells containing photosynthetic solution and bacteria, which resulted in the generation of heat. As a low-level laser was employed in this study, the heat generated was insufficient to cause bacterial death but may have created a conducive environment for bacterial growth during prolonged irradiation periods [67].

As the laser irradiation continued, two competing processes emerged, the bacterial killing process related to the antibacterial activities and the heating process, which promoted bacterial growth. Consequently, with prolonged laser

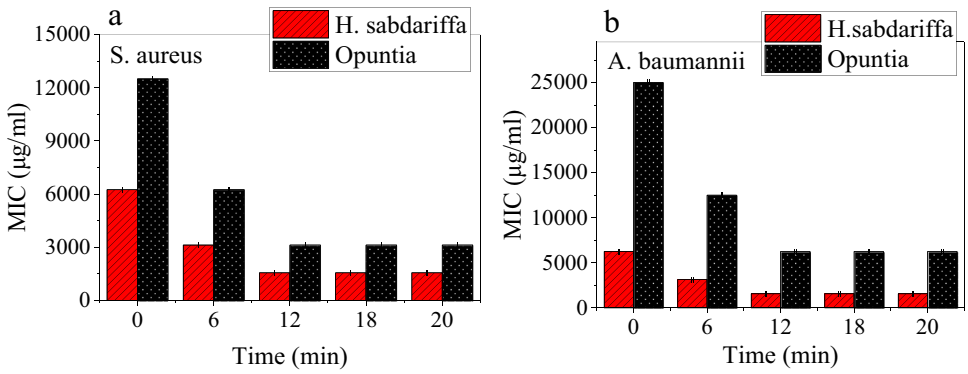


Fig. 4 The results of MIC and MBC testing for the absence of laser irradiation in 0 min and also, after 6 to 20 min of laser irradiation against **a**) gram-positive and **b**) gram-negative. MIC and MBC testing had the same results. The calculated *p* values of MIC/MBC of *H. sabdariffa* and *Opuntia* against both A. baumannii and S. aureus were found to be less than 0.037, indicating significant inhibitory effects

Table 9 Numerical results of the MIC/MBC test with the best plant extracts against bacteria

The MIC/MBC (mg/ml)								
Bacteria	Samples	t = 0	t = 6	t = 12	t = 18	t = 20	Standard Deviation	*P* Values
A.baumannii	H. sabdariffa	6250	3125	1562.5	1562.5	1562.5	0.001	0.037
A.baumannii	Opuntia	25,000	12,500	6250	6250	6250	0.001	0.037
S. aureus	H. sabdariffa	6250	3125	1562.5	1562.5	1562.5	0.001	0.037
S. aureus	Opuntia	12,500	6250	3125	3125	3125	0.001	0.037

P value < 0.037

irradiation, the antibacterial effect gradually subsided, and the bacteria continued to grow. Additionally, photosensitizers, including the synthetic pigments of plant extracts, that highly absorb laser light can potentially decompose and lose their antibacterial properties. Overall, the findings of this study suggest that the antibacterial efficacy of laser irradiation is dependent on several factors, including the type of laser used, the duration of irradiation, and the presence of photosensitizers. Therefore, future studies should consider these factors when investigating the antibacterial properties of laser irradiation on photosynthetic solutions.

The biophysical process can be described as follows, when the photosensitizers absorb the light, a higher number of molecules are excited compared to the absence of a laser. These molecules are excited to a singlet excited state, followed by rapid vibrational relaxation. The energy of the excited state decays through two pathways, fluorescence radiative decay, where the excited molecule returns to the ground state by emitting quantum, and non-radiative decay, such as intersystem crossing from the excited molecule transfers from the singlet excited state to the triplet excited state. Intersystem crossing occurs due to the different couplings between the nucleus and the electron in each molecule [69–71].

The absorption of laser light by photosensitizers increases the probability of their transition to the triplet excited state from the singlet excited state through intersystem crossing, resulting in more molecules being in the triplet excited state. The triplet excited state is more stable in terms of energy compared to the singlet excited state, as illustrated in Fig. 5. The energy level of the triplet excited state is lower than that of the singlet excited state, and the energy difference between the triplet excited and the ground state is smaller than in the case of the singlet excited state. However, the energy in the triplet excited state is also unstable and can decay through various mechanisms, including radiation decay, which releases energy similar to phosphorescence radiation. This leads to the occurrence of two types of photosensitized oxidation (PDI), referred to as type I and type II PDIs. The elevated absorption of energy during photosynthesis stems from the extended excitation phase of the photosensitizer. A prolonged singlet excitation phase results in heightened electronic energy conversion from the ground level to an excited triplet state. The surplus energy at the excited triplet state allows for the transfer of energy to neighboring oxygen molecules, leading to the generation of reactive oxygen species (ROS) [72].

Then, type I of PDI involved direct interaction between the photosensitizers in the triplet excited state and the biological substances, resulting in the transfer of an electron or a proton to them. This transfer can generate radical activated species, as well as react with oxygen molecules to produce reactive oxygen species (ROS) such as hydroxyl radicals ($HO^{\bullet}$) and superoxide anion radicals ($O_2^{\bullet}$).

In type II PDI, the reaction depends on the availability of oxygen molecules. If the energy difference between the excited state of the photosensitizer molecules and oxygen molecule's excitation energy is matched, the oxygen molecule becomes activated by accepting energy from the triplet excited state of photosensitizers, leading to the formation

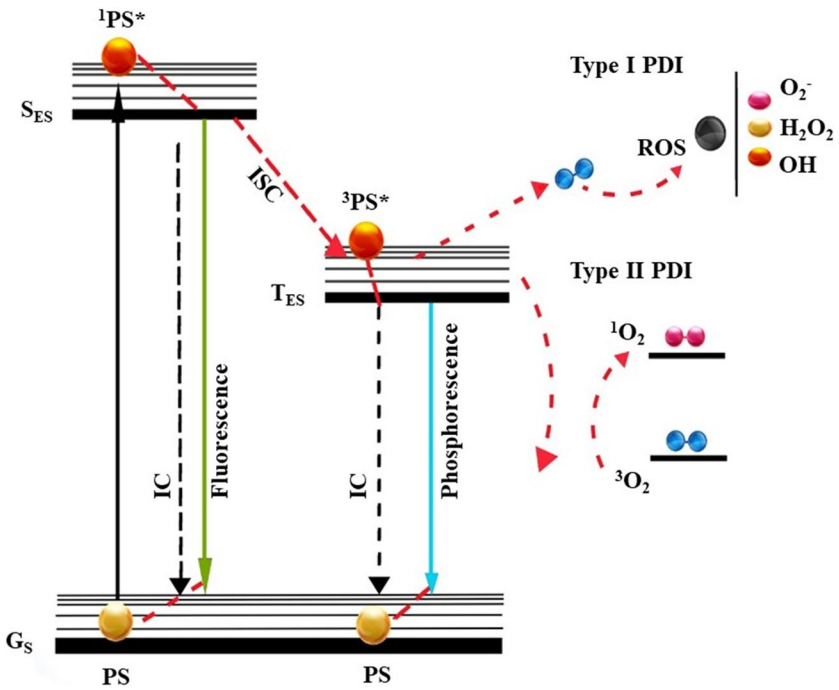


Fig. 5 Photophysical process of the photosensitizer excitations and production of the reactive species, $\mathbf{S_{ES}}$: Singlet excited state, $\mathbf{G_S}$: Ground state, $\mathbf{T_{ES}}$: Triplet excited state, **PS**: Photosensitizer, $^{\mathbf{1}}$**PS***: Excited-state (singlet), $^{\mathbf{3}}$**PS***: Excited-state (triplet), **ISC**: Inter-system crossing, **IC**: Internal conversion (vibrational decay), **ROS**: Reactive oxygen species

of singlet oxygen ($^3O_2$ to $^1O_2$). Single oxygen possesses an unoccupied π*2p orbital, which readily react with unsaturated lipids, alkenes (fatty acids), nitrogen and sulfur groups (amino acids), or nucleobases. The harmful effects of reactive oxygen species (ROS) include damaging cell membranes, disrupting cell division, and breaking DNA strands. Once cell membranes are compromised, photosensitizers can enter cells and harm organelles like lysosomes, mitochondria, and nuclei. ROS compounds interact with biomolecules within cells, leading to the breakdown of cellular structures and loss of function. Additionally, when ROS compounds react with phospholipids in cell membranes, they trigger lipid peroxidation, generating malondialdehyde and further damaging and rupturing cells. [73]. These reactions in bacteria cells lipids result in the formation of lipid hydroperoxides through photooxidation of unsaturated lipids, leading to cell death. Additionally, the acidification of the cell due to an oxidative burst of the endosomes and/or lysosomes can also contribute to cell death**.** Furthermore, photooxidation of DNA can result in genomic mutation or cell death [14, 69].

Time-killing assay

For more accuracy, time-killing assay was performed on both plant extracts that exhibited the significant antibacterial properties against tested bacteria in the MIC and the MBC assay in the absence and the presence of laser irradiation. Time-killing assay was performed using the colony count method [74]. As depicted in Fig. 6, the colony counts of all samples at 3.125 ppm in the seventh dilution tube were compared under different laser irradiation durations (the absence of laser irradiation (t = 0), the presence of laser irradiation (t = 6, 12, 18, and 20 min)). The results of colony counting demonstrated the effectiveness of our study in inhibiting

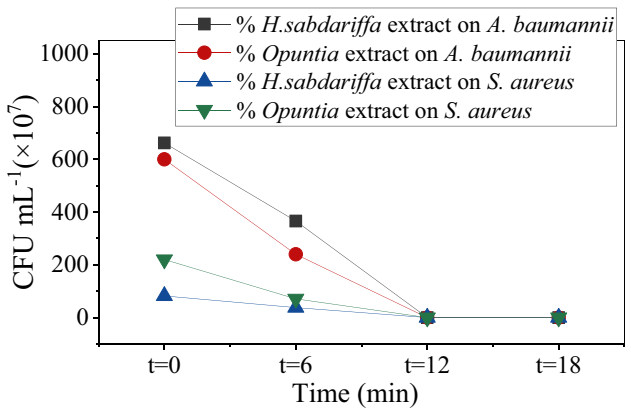


Fig. 6 The number of CFUs/ml of *A.baumannii* and *S.aureus* employing *H.sabdariffa and Opuntia* extracts separately in the absence and in the presence of laser irradiation at 3 times, 6, 12, and 18 min

Fig. 7 The absence of bacterial colony images in comparison with the colony study groups

bacterial growth and revealed the bactericidal properties of the herbal samples. Specifically, the graphs in Fig. 6 showed that all samples at 3.125 ppm exhibited excellent reduction in bacterial colony count, with a decrease from log7 to complete elimination as the irradiation time increased. The colony count of all bacteria was zero in the presence of laser irradiation, indicating the potent antibacterial effect of the photosensitizers at this concentration. As it shown in Fig. 7, the absence of bacterial colony images in comparison with the colony study groups is evident.

Conclusion

One of the significant threats to human life is antibiotic resistance which leads to serious illnesses. A safe alternative approach to antibiotics is the PDI method. To optimize the efficiency of this method, we have proposed using medicinal plant extracts as natural photosensitizers. The antibacterial effect of the proposed medicinal plant's extracts was tested and compared with antibiotics. To enhance the antibacterial effect, the absorption peak of the natural photosensitizers should be matched with the wavelength of laser light. So, the spectroscopy of the plant extracts was utilized to find excellent photosensitizers.

Our results demonstrated that two plant extracts, *Hibiscus sabdariffa*, and *Opuntia*, had inhibitory effects against both Gram-positive and Gram-negative bacteria at low concentration. The ethanolic extracts had excellent absorption peaks at 532 nm, and gas chromatography-mass spectrometry (GC/MS) analysis presented some excellent details of the chemical composition of the selected great natural photosensitizers include of *H. sabdariffa* and *Opuntia*.

The MIC and MBC tests were conducted in the absence and presence of laser irradiation, and the results showed that a 12 min exposure of the natural photosensitizers to low-level laser led to maximum inhibition of bacteria. The MIC/MBC values of *H. sabdariffa* and *Opuntia* against *A. baumannii* were 1562 and 6250 ppm, respectively, and 1562 and 3125 ppm against *S. aureus*. Moreover, colony counting revealed a significant reduction in the number of colonies in the presence of herbal samples and laser irradiation, with a decrease in bacterial count from log107.

The experiments showed a significant increase in the antibacterial effect of medicinal natural photosensitizers against both bacteria after laser exposure, which was attributed to increased production of reactive oxygen species through biophysical phenomena. Our findings suggest that the simultaneous use of plant extracts and laser irradiation can provide a safe and cost-effective alternative to antibiotics, particularly for the treatment of bacterial skin infections.

In conclusion, our study highlights the potential of medicinal plant extracts as natural photosensitizers to enhance the antibacterial effect of PDI. Further investigations are warranted to fully understand the underlying mechanisms and optimize the conditions for bacterial inhibition. Nonetheless, our findings provide promising evidence for the use of medicinal plants as low-cost antibacterial agents.

Author contribution Z. Aghaebrahimi: methodology, data collection and analysis, design, and writing the first draft of the manuscript.

M. Ranjbaran: helping to write the final manuscript, and assisting in setting up the laser setup.

J. Sabaghzadeh: methodology, design, Supervision.

S. Soudi, M. Tanhayi Ahary, S. H. Nabavi, and all authors revised and approved the final manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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