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Antibacterial activity of selected plant (Aqueous and methanolic) extracts against some pathogenic bacteria

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Abstract
The development of new antibacterial agents from natural origins is of increasing interest. Therefore, the aim of this study is to evaluate 8 selected plants for their *in vitro* antibacterial activity against pathogenic bacteria isolated from infected wounds and burns. The plants were extracted with hot water and methanol. The antibacterial activity was determined using the disc and agar-well diffusion methods. All the plant extracts demonstrated activity against the tested bacteria, with inhibition zone diameters ranging from 3-24.5 mm. The best results were obtained with those from aqueous extract of *Carissa edulis*. The phytochemical screening of the aqueous and methanol extracts indicated the presence of flavonoids, tannins, alkaloids, glycosides, and terpenoids, which might explain the activity of the extracts whereby they can be offering additive activity against the test bacterial strains. In conclusion, the results indicate the plant extracts contain important metabolites in the search for new effective antibacterial agents.

Keywords: Antibacterial activity, Yemeni plants, pathogenic bacteria

1. Introduction
Infectious diseases are still one of the main causes of death in the world, in spite of the great advances in chemotherapy (Da Rosa *et al*. 2010) [11]. The World Health Organization states that infectious and parasitic diseases accounted for nearly 11 million out of the 57 million deaths in 2003 (WHO, 2003) [39]. Bacteria are considered as a group of the microorganisms that cause the most deadly diseases and widespread epidemics of human civilization. Infectious diseases caused by pathogenic bacteria, have prevalence rate and morbidity more than other pathogenic microorganisms **(**CDC, 1999**)** [9]. *Staphylococcus aureus* (*S. aureus*) is a major human pathogen that causes a wide range of clinical infections. It is a main cause of bacteremia, infective endocarditis, toxic shock syndrome, osteomyelitis, post-operative wound infections, and food poisoning (Tong *et al*. 2015) [35]. Gram-negative bacteria such as *Escherichia coli* (*E. coli*), which found in human intestine, causes enteric infections, neonatal meningitis, lower urinary tract infections, coleocystis, and septicaemia **(**Bannister *et al*. 2006) [4]. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a common cause of nosocomial infections such as pneumonia, urinary tract infections, and bacteremia (Veesenmeyer *et al*. 2009) [38]. Due to the poor sanitary conditions, the infections of superficial and deep wounds as well as burns are common in developing countries.
Generally, pathogenic bacteria are controlled by antibiotics; however, these antibiotics are not always possible due to their high cost (Camporese *et al*., 2003) [7]. Different antibiotics exercise their inhibitory activity on different pathogenic bacteria **(**Kohanski *et al*., 2010**)** [18]. Multiple-drug resistance in human pathogenic bacteria has been developed due to indiscriminate use of commercial antibiotics commonly used in the treatment of bacterial diseases (Andersson and Hughes, 2011) [3].The development of bacterial resistance against available antibiotics has necessitated the need to search for new antibacterial agents. The development of antibiotic resistance is multifactorial, including the specific nature of the relationship of bacteria to antibiotic, the usage of antibiotic, host characteristics, and environmental factors. In general, bacteria have the genetic ability to transmit and acquire resistance to antibiotics **(**Jouda, 2013**)** [17]. To overcome this problem, researchers studied on the antibacterial properties of various plants against antibiotic-resistant bacteria. Medicinal plants may offer a new source of antibacterial agents for use. Biologically, active compounds from natural sources has always been of great interest to scientists working on infectious diseases. Plant-derived drugs serve as a prototype to develop more effective and less toxic medicines (Samy and Ignacimuthu, 2000) [30]. Recently, there has been a growing interest to evaluate Yemeni plants having antibacterial activity for various diseases (Mothana and

Lindequist, 2005; Al-Fatimi *et al*., 2005; Al-Fatimi *et al*., 2007; Mothana *et al*., 2011) [25, 1, 24]. Therefore, the aim of this study was to evaluate the antibacterial activity of the selected parts of eight Yemeni plants against some pathogenic bacteria, namely *S. aureus*, *E. coli*, and *P. aeruginosa* isolated from wounds and burns infections.

2. Materials and methods

2.1 Plants collection and identification

Fresh plant materials of *Adenium obesum*, *Carissa edulis*, *Calotropis procera*, *Kanahia laniflora*, *Origanum majorana*, *Syzygium aromaticum*, *Lepidium sativum*, and *Capparis spinosa* were collected in January 2016 from different localities in Ibb governorate, Yemen. The plants were identified by Associate Prof. Dr. Naji Ebrahim, Department of Plant Production, Ibb University. The scientific names of the plants, local names, plant families, and the parts used are shown in Table 1.

Table 1: Plant species screened for antibacterial activity.

Plant species	Local name	Family	Part used
Adenium obesum	Addana	Appocynaceae	Barks
Carissa edulis	Arm	Appocynaceae	Leaves
Calotropis procera	Aoshar	Asclepiadaceae	Leaves
Kanahia laniflora	Kanah	Labiatae	Leaves
Origanum majorana	Albardakosh	Lamiaceae	Leaves
Syzygium aromaticum	Zer	Myrtaceae	Buds
Lepidium sativum	Hab el-rashaad	Brassicaceae	Seeds
Capparis spinosa	Allasaf	Capparidaceae	Leaves

2.2 Preparation of plant extracts

The plant materials were processed and naturally dried, according to traditional procedures, grinded by mortar and pestle, and finally sieved to obtain the plant materials in powder form to be used for extraction.

2.2.1 Aqueous extract

Aqueous extraction was prepared according to the method reported by Parekh and Chand (2006) [29], with some modifications. 50 g of the powdered sample was added to 300 ml of distilled water and heated for 15 min, with continuous stirring. Then the extract was allowed to cool for 24 h, at room temperature (~26 °C), and then filtered by Whitman filter paper (No. 1), with the help of vacuum pump. The filtrate was taken and concentrated to dryness at 40 ºC until the solvents evaporated completely. The dried samples were stored at 4 °C until use.

2.2.2 Methanol extract

Methanol extraction was conducted based on the method described by Benlafya *et al*. (2014) [5], with some modifications. 50 g of the powdered sample was extracted with 250 ml methanol 96% at room temperature for 48 h, with continuous stirring. The filtrate was filtered, dried, and preserved as the same method mentioned above in section 2.2.1.

2.3 Bacterial strains: isolation and identification

Three bacterial strains were clinically isolated from infected wounds and burns of patients attending different hospitals located in Ibb city during the period May to July, 2016. They were *E. coli*, *S. aureus*, and *P. aeruginosa*. The isolated strains were cultivated at 37 °C on nutrient agar medium (HiMedia) and maintained at 4 ºC for further experiments. The strains were identified and characterized by conventional biochemical methods according to Forbes *et al*. (2002) [14].

2.4 Antibiotic sensitivity test

The sensitivities of the isolated bacterial species against 8 different antibiotics were tested based on the disc diffusion (Kirby–Bauer) technique, which conforms to the recommended standards of CLSI (2015) [10]. For preparation of bacterial inoculum, bacterial strain was suspended in sterile distilled water and the turbidity adjusted to ~10^6 CFU/ml (corresponding to 0.5 McFarland standards). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Müeller-Hinton Agar (MHA) plates. The plates were allowed to dry for 5 min. Thereafter, antibiotic discs were then placed on the plates and pressed gently to ensure complete contact with agar, and the plates were incubated at 37 °C for 24 - 48 h. After the incubation period, the zones of growth inhibition of the bacterium around the discs were measured and expressed in millimeters. The diameter of inhibition zone for each disc was classified as sensitive (S), intermediate (I), or resistant (R) according to the standardized Table supplied by the manufacturer (Hi-Media).

2.5 Antibacterial activity of plant extracts

The plant extracts were screened for their antibacterial activity by using disc and agar-well diffusion methods described by CLSI (2015) [10].

2.5.1 Disc diffusion method

For the disc diffusion assay, 1 ml of each bacterial suspension was evenly spread on a solidified 20 ml MHA. Discs (6 mm in diameter) were punched from a sheet of Whatman filter paper, sterilized, and impregnated with 20 µl each of 0.5 g/ml plant extract or solvent alone and dried at room temperature for 48 h. Thereafter, the discs were placed on the surface of inoculated MHA plates, and then incubated at 37 °C for 24 - 48 h to observe formation of inhibition zones around the discs.

2.5.2 Agar-well diffusion method

In this method, MHA plates were prepared and inoculated as in the disc diffusion method. 4 holes of 6 mm in diameter were made on the inoculated MHA plates using a sterile corn borer and agar discs were removed. Holes were aseptically filled with 20 µl of 0.5 g/ml of plant extract by means of automatic microliter pipette, and allowed to diffuse at room temperature for 2 h, and thereafter incubated at 37 °C for 24 - 48 h. For comparative purposes, standard gentamicin (30 µg), vancomycin (10 µg), and ciprofloxacin (30 µg) were used as positive controls. Antibacterial activity was assessed by the appearance of inhibition zone around the hole, without bacterial growth.

2.6 Phytochemical screening of plant extracts

The qualitative screening of the phytochemical constituents of the tested plants was performed on the crude aqueous and methanol extracts by using chemical methods based on Harbone (1998) [16].

2.6.1 Test for flavonoids

The presence of flavonoids was determined by taking 5 ml of plant extract that was mixed with 1 ml of diluted ammonia (NH_4OH). A yellow coloration indicates the presence of flavonoids.

2.6.2 Test for tannins
The presence of tannins was determined by adding 3 drops of ferric chloride ($FeCl_3$) solution (5%) to 5 ml of plant extract. Development of brownish green or blue black coloration indicates the presence of tannins.

2.6.3 Test for alkaloids
In this test, few drops of diluted hydrochloric acid (HCl; 0.1 M) were added to 5 ml of plant extract. Then, few drops of Dragendorff's reagent were added to the mixture. A positive reaction for the presence of alkaloids was recorded when an orange precipitate was observed.

2.6.4 Test for glycosides
The presence of glycosides was determined by adding 2 ml of glacial acetic acid (CH_3COOH) to 2 ml of plant extract. Then, 1 ml of diluted sulphuric acid (H_2SO_4) was added to the mixture. A brown ring coloration indicates the presence of glycosides.

2.6.5 Test for terpenoids
In this test, 1 ml of plant extract was added to 2 ml of chloroform, and then 2-3 ml of concentrated H_2SO_4 was added to the mixture by carefully pouring on the inner side of test tube to form two different layers. A reddish violet coloration of the interface formed indicates the presence of terpenoids.

3. Results and discussion
3.1 Antibiotic sensitivity test
Antibiotic sensitivity was assessed by streaking the bacterial isolate on MHA plates. The commercial antibiotic discs used in this study were amoxyclav, gentamicin, cefotaxime, vancomycin, ciprofloxacin, co-trimoxazole, ceftriaxone, and ampicillin. The results presented in Table (2) and figure (1), showed various degrees in antibiotic resistance. All bacterial species showed resistance to amoxyclav, cefotaxime, vancomycin (except *S. aureus*), and ampicillin. Among the tested bacterial isolates, the strongest antibacterial activities of antibiotics were obtained by vancomycin and gentamicin against *S. aureus*, with inhibition zones of 22 and 19 mm, respectively.

Bacterial resistance against antibiotics is increasing worldwide in both outpatients as well as hospitalized patients. This resistance varies according to geographic locales and is directly proportional to the use and misuse of antibiotics (Orhan *et al*., 2010) [27]. Recent reviews on the application of antibiotics in human medicine have documented the development of antibiotic resistance by bacteria (Andersson and Hughes, 2011; Cars *et al*., 2008; Lestari *et al*., 2012) [3, 8, 20]. This phenomenon has forced scientists around the world to search for new antibacterial agents from various sources of natural origin including medicinal plants as novel antibacterial agents without or minimal side effects.

Table 2: Antibacterial sensitivity pattern of the test bacteria against 8 antibiotics using disc diffusion technique.

Antibiotic	Diameter of inhibition zone of antibiotic discs (mm)		
	E. coli	***S. aureus***	***P. aeruginosa***
Amoxyclav (10 µg)	7 (R)	9 (R)	0 (R)
Gentamicin (10 µg)	15 (I)	19 (S)	16 (S)
Cefotaxime (10 µg)	5 (R)	5 (R)	7 (R)
Vancomycin (10 µg)	0 (R)	22 (S)	0 (R)
Ciprofloxacin (30 µg)	11 (R)	12 (R)	8 (R)
Co-trimoxazole (30 µg)	10 (R)	6 (R)	9 (R)
Ceftriaxone (30 µg)	9 (R)	12 (R)	18.5 (I)
Ampicillin (10 µg)	(0) R	(0) R	(6.5) R

S, sensitive; R, resistant; I, intermediate

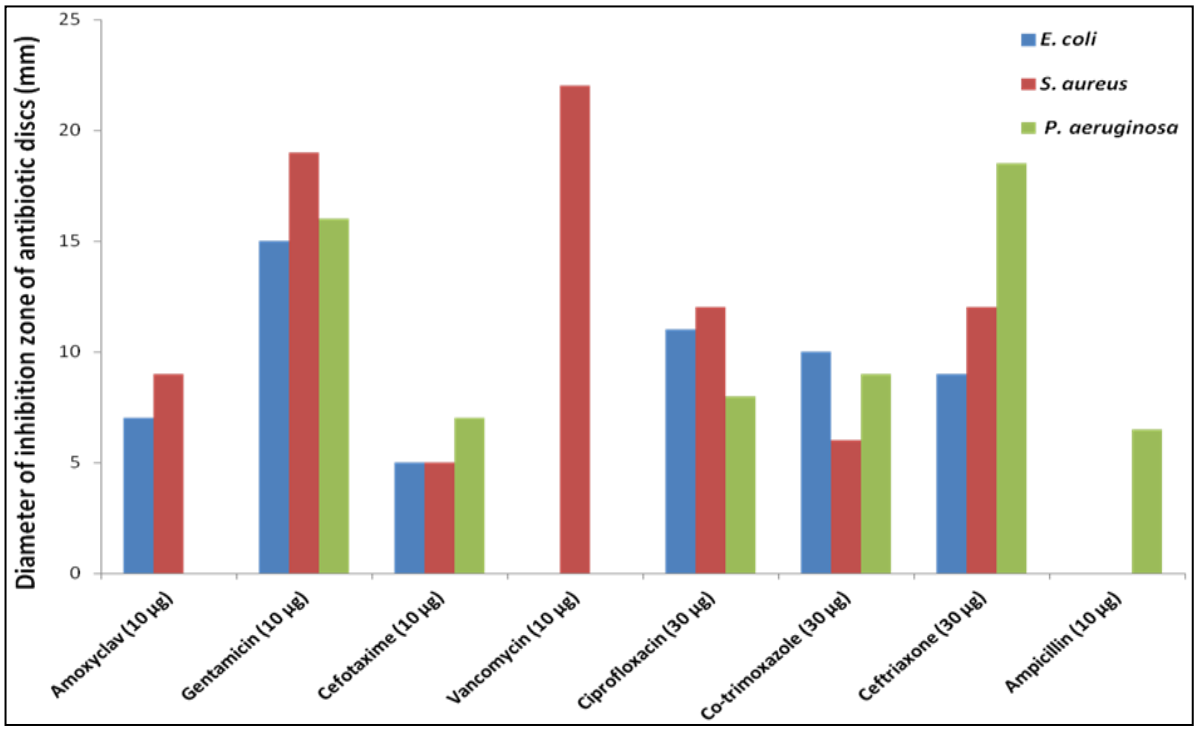


Fig 1: Antibacterial sensitivity pattern of the test bacteria against 8 antibiotics using disc diffusion technique.

3.2 Antibacterial activity of plant extracts

The antibacterial activities of aqueous and methanol extracts of eight plants were screened against three bacterial isolates, namely *E. coli*, *S. aureus*, and *P. aeruginosa*. From the results presented in Table (3), Figure (2), Figure (3) and Figure (4), it was observed that all extracts were active against the tested bacteria, either Gram-positive or Gram-negative bacteria, with inhibition zone diameters ranging from 3 - 24.5 mm. In general, the inhibitory effects of plant extracts using well diffusion method were found to be higher than that of disc diffusion method. This result was consistent with the previous studies on other plants (Essawi and Srour, 2000; Valgas *et al*., 2007) [14, 37]. Also, the aqueous and methanol extracts from the same plants showed different activities. There are no common rules for this, but in most cases, the organic extracts showed the same or greater activity than the aqueous extracts (Olano *et al*., 1996) [26].

In this study, some of the antibacterial inhibition zone diameters were more than 20 mm. The highest antibacterial activity was achieved by the aqueous extract of *Carissa edulis* against *P. aeruginosa***,** with inhibition zone of 24.5 mm using agar-well diffusion method. Because of its high efficacy against *P. aeruginosa*, the extract of *C. edulis* may be effective in the treatment of infections resulted from this multi-resistant pathogenic bacterium. *P. aeruginosa* is known to develop resistance against multiple classes of antibacterial agents, even during the course of treating an infection (Lister *et al*., 2009) [21].

The lowest antibacterial activity was recorded for aqueous extract of *Adenium obesum* against *S. aureus* (using agar-well diffusion method) and methanol extract of the same plant against *P. aeruginosa* (using disc diffusion method), with inhibition zone of 3 mm for each. In general, the aqueous extract of this plant showed the lowest antibacterial activities against all tested bacteria.

By using agar-well diffusion method, the highest antibacterial activities against *E. coli*, *S. aureus*, and *P. aeruginosa* were achieved by using aqueous extract of *C. edulis*, methanol extract of *Kanahia laniflora*, and aqueous extract of *C. edulis*, respectively, and the antibacterial values were 22.5, 19.5, and 24.5 mm, at the same order.

In comparison with agar-well diffusion method, the highest antibacterial activity against *E. coli* using disc diffusion method was 17.5 mm when aqueous extract of *Calotropis procera* was applied. For *S. aureus* and *P. aeruginosa*, the highest antibacterial activities using disc diffusion method were 20 and 22 mm, respectively, when methanol extract of *Syzygium aromaticum* was used.

3.3 Phytochemical screening of the plant extracts

The phytochemical analysis of the crude aqueous and methanol extracts of the tested plants revealed that flavonoids, tannins, and alkaloids are found in all tested plants as shown in Table (4). Glycosides and terpenoids were also found to be present in all tested plants, but glycosides were absent in *K. laniflora*, and terpenoids were absent in *C. procera*.

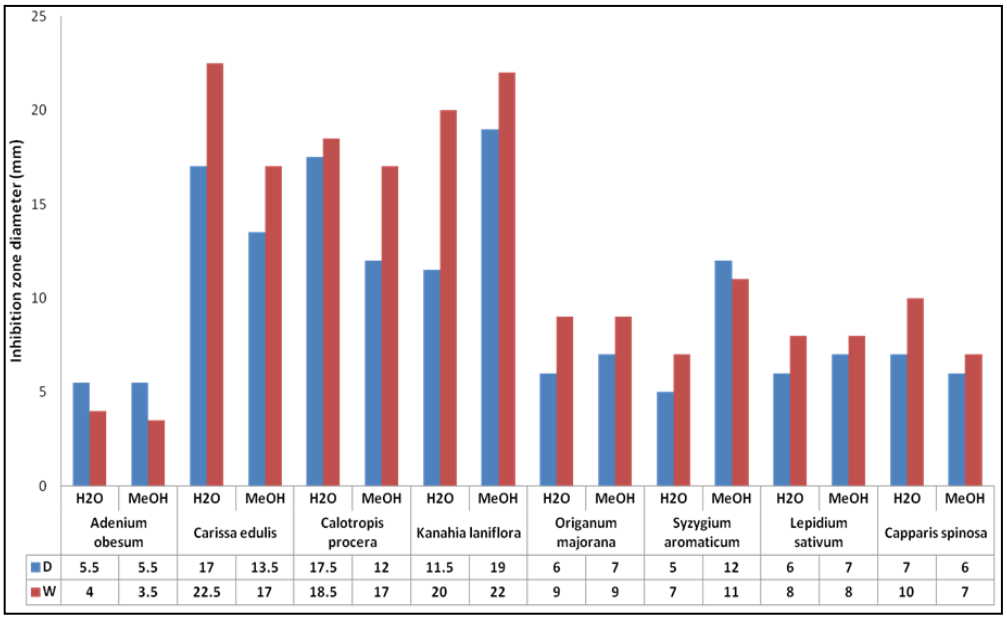
These five phytochemicals, which are naturally occurring in most plants, are known to be biologically active and have bactericidal and fungicidal activities, conferring the antibacterial property to the tested plants. They exert antibacterial activity through different mechanisms.

Flavonoids are polyphenolic compounds that are ubiquitous in plants, and their antimicrobial activity is probably due to their ability to inhibit nucleic acid synthesis and/or energy metabolism (Tim Cushnie and Lamb, 2005) [33]. Lipophilic flavonoids may also disrupt microbial cell membranes (Mishra *et al*., 2009) [23].

Table 3: Antimicrobial activity of plant extracts against tested by using disc diffusion and well diffusion methods.

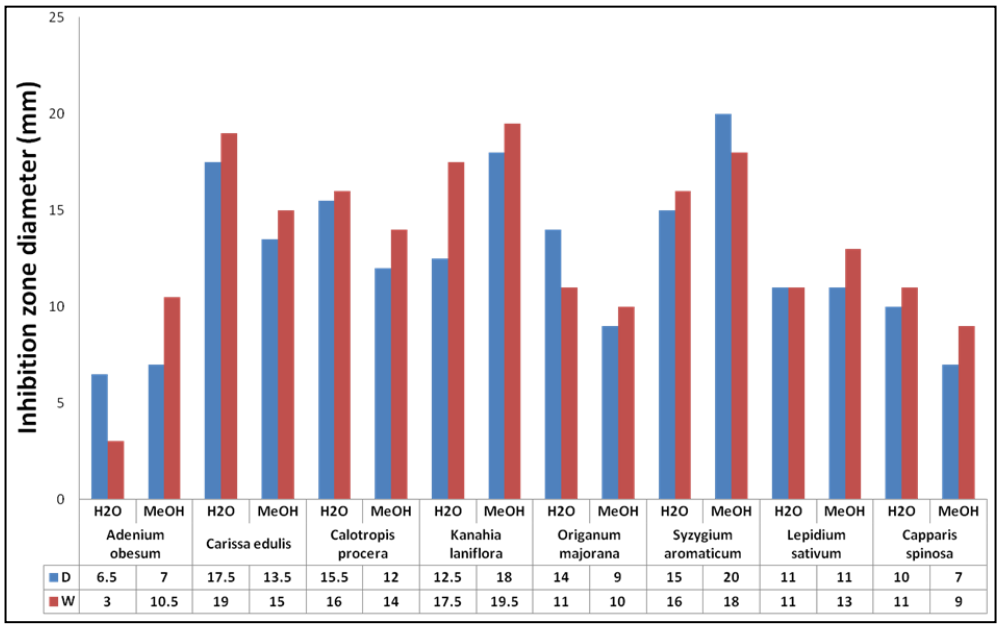
Plant species	Extract (0.5 g/ml)	Inhibition zone diameter (mm)					
		E. coli		*S. aureus*		*P. aeruginosa*	
		D	W	D	W	D	W
Adenium obesum	H_2O	5.5	4	6.5	3	8.5	4
	MeOH	5.5	3.5	7	10.5	3	6
Carissa edulis	H_2O	17	22.5	17.5	19	13.5	24.5
	MeOH	13.5	17	13.5	15	12	18
Calotropis procera	H_2O	17.5	18.5	15.5	16	16	19
	MeOH	12	17	12	14	11.5	17
Kanahia laniflora	H_2O	11.5	20	12.5	17.5	11	17.5
	MeOH	19	22	18	19.5	18	21.5
Origanum majorana	H_2O	6	9	14	11	15	17
	MeOH	7	9	9	10	14	15
Syzygium aromaticum	H_2O	5	7	15	16	11	13
	MeOH	12	11	20	18	22	20
Lepidium sativum	H_2O	6	8	11	11	10	14
	MeOH	7	8	11	13	10	11.5
Capparis spinosa	H_2O	7	10	10	11	10	12
	MeOH	6	7	7	9	9	12
Gentamicin (30 µg)	-	15	-	19	-	16	-
Vancomycin (10 µg)	-	0	-	22	-	0	-
Ciprofloxacin (30 µg)	-	11	-	11	-	8	-

H_2O, aqueous extract; MeOH, methanol extract; D, disc diffusion method; W, agar-well diffusion method



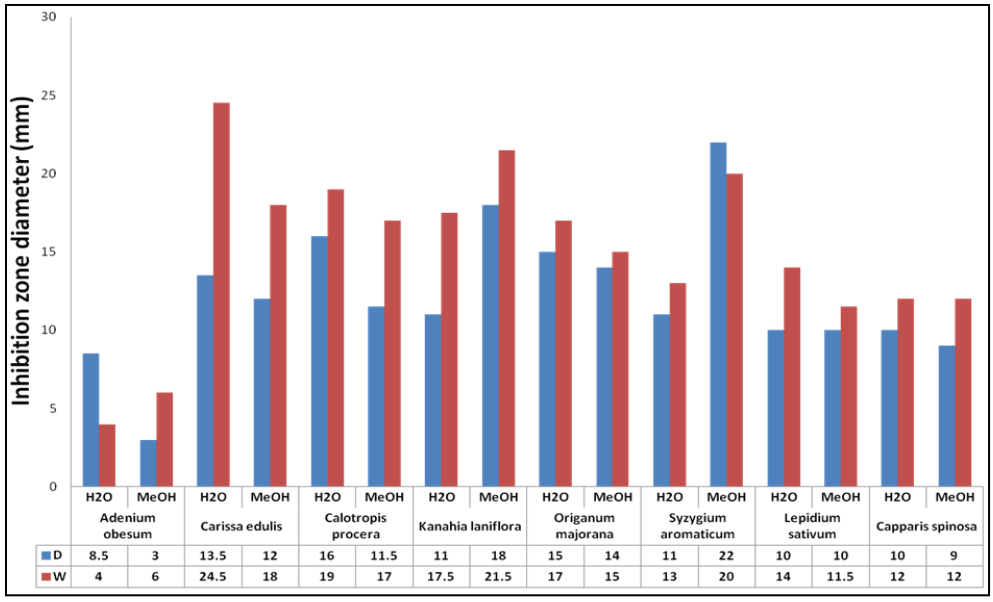
	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH
	Adenium obesum		Carissa edulis		Calotropis procera		Kanahia laniflora		Origanum majorana		Syzygium aromaticum		Lepidium sativum		Capparis spinosa	
D	5.5	5.5	17	13.5	17.5	12	11.5	19	6	7	5	12	6	7	7	6
W	4	3.5	22.5	17	18.5	17	20	22	9	9	7	11	8	8	10	7

Fig 2: Antimicrobial activity of plant extracts against *E. coli* by using disc diffusion and well diffusion methods.



	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH
	Adenium obesum		Carissa edulis		Calotropis procera		Kanahia laniflora		Origanum majorana		Syzygium aromaticum		Lepidium sativum		Capparis spinosa	
D	6.5	7	17.5	13.5	15.5	12	12.5	18	14	9	15	20	11	11	10	7
W	3	10.5	19	15	16	14	17.5	19.5	11	10	16	18	11	13	11	9

Fig 3: Antimicrobial activity of plant extracts against *S. aureus* by using disc diffusion and well diffusion methods.



	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH	H2O	MeOH
	Adenium obesum		Carissa edulis		Calotropis procera		Kanahia laniflora		Origanum majorana		Syzygium aromaticum		Lepidium sativum		Capparis spinosa	
D	8.5	3	13.5	12	16	11.5	11	18	15	14	11	22	10	10	10	9
W	4	6	24.5	18	19	17	17.5	21.5	17	15	13	20	14	11.5	12	12

Fig 4: Antimicrobial activity of plant extracts against *P. aeruginosa* by using disc diffusion and well diffusion methods.

Tannins are water-soluble polyphenols that are present in nearly all plants. They have been known to display different biological activities, including antibacterial and antifungal (Lanchoti Fiori *et al*., 2013; Scalbert, 1991) [19, 32]. Tiwari *et al*. (2011) [34] reported that the antimicrobial activity of tannins is attributed to its ability to inhibit enzymes activity, deprive substrates, complex with the cell wall, and disrupt the cell membrane of bacteria.

Alkaloids derived from plants are important bioactive compounds, and their antimicrobial activity has been reported (Deng *et al*., 2011; Ozçelik *et al*., 2011) [12, 28]. The activity of aromatic planar quaternary alkaloids is ascribed to their ability to intercalate with microbial DNA, resulting in impaired cell division and cell death (Savoia, 2012) [31].

Plant glycosides are secondary metabolites produced in plants by the condensation of a sugar with other organic molecules (aglycone or genina) (Brito-Arias, 2007) [6]. Glycosides have been known to display different biological activities, including antibacterial (Da Rosa *et al*., 2010) [11].

Terpenoids derived from plants known to produce antibacterial activity (Drewes *et al*., 2005; Mathabe *et al*., 2008) [13, 22], through promotion bacterial lysis and disruption of the cell membrane as suggested by Urzúa *et al*. (2008) [36].

Plant species	Flavonoids		Tannins		Alkaloids		Glycosides		Terpenoids	
	H_2O	MeOH	H_2O	MeOH	H_2O	MeOH	H_2O	MeOH	H_2O	MeOH
Adenium obesum	+	+	+	+	+	+	+	+	+	+
Carissa edulis	+	+	+	+	+	+	+	+	+	-
Calotropis procera	+	+	+	-	+	+	+	+	-	-
Kanahia laniflora	+	+	+	+	+	+	-	-	+	-
Origanum majorana	+	+	+	+	+	+	+	+	+	+
Syzygium aromaticum	+	+	+	+	+	+	+	+	+	+
Lepidium sativum	+	+	+	+	+	+	+	+	+	+
Capparis spinosa	+	+	+	-	+	+	+	+	+	+

H_2O, aqueous extract; MeOH, methanol extract

4. Conclusion

Extracts of natural origin are a common starting point in the search for new antibacterial agents. From the results presented in this study, it is possible to conclude that some extracts of studied plants were have antibacterial activity against pathogenic bacteria isolated from burns and wounds infections higher than standard antibiotics using in this study. Furthermore, the obtained results demonstrate that these plants could represent a new source of antibacterial agents, less expensive than the imported antibiotics. In this respect, it will be very interesting to conduct a bioassay-oriented fractionation of the active extracts to isolate the pure compounds responsible for the antibacterial activities.

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