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Research Article

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Antimicrobial activity of leaf extracts of *Euphorbia paralias* L. and *Melilotus sulcatus* Desf. against some pathogenic microorganisms

Miloud M. Miloud[1]*, Najma A. Senussi[2]

[1]Department of Botany, Faculty of Science, Benghazi University (Al-abyar Branch), Libya, [2]Department of Botany, Faculty of Science, Ajdabiya University, Libya

ABSTRACT

The present study was aimed to investigate the antimicrobial potential of leaf extracts of *Euphorbia paralias* and *Melilotus sulcatus* against four bacterial species *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella sp.* and two fungal species *Asperigillus niger* and *Aspergillus flavus*. The agar well diffusion assay was used to evaluate the antimicrobial activity. The effect of these extracts was most effective against the bacterial species compared to the fungal species at a used concentration (100 mg/ml). Methanolic extracts of selected plants displayed good antimicrobial activity against all tested microorganisms species, while, no activity for aqueous extracts against tested fungal species. Methanolic extracts were the most effective plant extracts against all tested bacterial species, with MIC and MBC reached 6.2 and 12.5 mg/ml, except *Klebsiella sp.* which was less sensitive to *M. sulcatus* methanolic extract and its MIC and MBC reached 12.5 and 25 mg/ml, respectively. These plant extracts which proved to be potentially effective can be used as bioactive agents to control microorganisms caused for diseases and they can be used naturally in the human and veterinary healthcare systems.

KEYWORDS: *Euphorbia paralias L.*, *Melilotus sulcatus Desf.*, Antimicrobial activity, agar well diffusion assay, MIC, MBC.

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***Corresponding Author:**
Miloud M. Miloud,
Miloud.elagaily@uob.edu.ly

INTRODUCTION

The pathogenic microorganism species have been increased their resistance to many of the chemically synthesized antibiotics that were previously used to resist them. In 2011, the WHO called for increased research on new drugs as antibiotic resistance increases dramatically (Abedini *et al.*, 2013; Abreu *et al.*, 2012). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Izzo *et al.*, 1995; Schapoval *et al.*, 1994; Kubo *et al.*, 1993). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils (Janssen *et al.*, 1987). *Euphorbia paralias* L. (*E. paralias*), belongs to the family Euphorbiaceae. It is a perennial herb. The stem and leaves produce a white or milky juice when cut. This species distributed in coastal areas of Europe & North Africa. A species, growing in the sand along the coastal area of Libya, easily recognized by its small densely compact appressed leaves (Jafri & El-Gadi, 1982). The *Euphorbia* includes many species that containing tannins, terpenes, anthocyanins, alkaloids, steroids like ßsito-sterol, ß-amyrin and glycosides (Scalbert, 1991; Gupta & Gupta, 2019), it includes also many species that are used medically in the treatment of many diseases such as pulmonary tuberculosis, ascites, edema, asthma, stomach, liver and uterine cancer, It also treats worm infestations in children and for gonorrhea, jaundice, pimples, digestive problems (Kirtikar & Basu, 1991; Feng *et al.*, 2010).

Melilotus sulcatus Desf. (*M. sulcatus*) belongs to the family Fabaceae. It is an annual herb. This species distributed in the Mediterranean region "South Europe & North Africa" (Jafri & El-Gadi, 1980). The *Melilotus* includes many species that contains coumarins, melilotin, phenolic acids, flavonoids, carbohydrates, glycosides, saponins, volatile oils, steroids, triterpenes, tannin, alcohols, bishydroxycoumarin, choline, uric acid and many other chemical groups (Al-Snafi, 2020; Sonju *et al.*, 2017), it includes also many species that are used medically in the treatment of osteoporosis, high blood pressure, cancer, kidney and liver

complaints (Akhtar *et al.*, 2016). The aim of this study is to evaluate the activity of aqueous and methanolic crude extracts of *E. paralias* and *M. sulcatus* against some pathogenic bacteria and fungi species.

MATERIALS AND METHODS

Collection and Preparation of Plant Samples

Fresh samples were collected from the leaves of the selected plants in the late spring month of 2020 from the Zuwetina region, northwest of Benghazi for *E. paralias* and *M. sulcatus* from the southeast of Benghazi in Libya. The leaves were placed in plastic bags. Then leaves were washed gently under tap water to remove the dust and left in the air under shade to dry for 2 weeks, then cut into small pieces and crushed into powder using an electric blender, transferred into a glass container, and preserved until the extraction procedure was performed in the laboratory.

Preparation of Extracts

According to the method of Mohammadi *et al.*, (2015), with minor modifications, 50 gm of the powder of *Euphorbia paralias* and *Melilotus sulcatus* were filled in the thimble and extracted successively with 300 ml each of sterile distilled water and methanol using a Soxhlet apparatus for 24 hours. All the extracts were evaporated using a rotary evaporator. At last, 2.3g of dried extracts were obtained, all the extracts were dissolved in dimethyl sulfoxide (DMSO). One concentration of extracts was prepared, which is 100 mg/ml, and stored at 4 °C in airtight bottles until further use.

Test Microorganisms

Bacterial species were obtained from the microbiology laboratory of Benghazi Medical Centre (BMC). Fungal species were obtained from the Botany Department, Benghazi University, Al-Abyar branch. In total, six microorganisms, four bacterial species: *S. aureus*, *P. aeruginosa*, *E. coli* and *Klebsiella sp.* and two fungal species: *A. niger* and *A. flavus*. The bacterial species were maintained on nutrient agar slants and the fungal species maintained on Potato dextrose agar slants at 4 °C.

Preparation of Culture Media

Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) media were prepared by suspending 38 g and 39.1g in 1000 ml of distilled water. The media was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50 °C, then pour into sterile Petri plates.

Preparation of the Microbial Suspension

Bacteria stock cultures (*S. aureus*, *P. aeruginosa*, *E. coli*, and *Klebsiella sp.*) were sub-cultured onto Nutrient Agar (NA) plates and incubated overnight at 37°C (bacterial cultures are 24 hours old). The next day, three to four discrete bacterial colonies with similar morphology were inoculated into 10 ml sterile Mueller Hinton broth (MHB) and incubated overnight at 37 °C. The overnight bacterial suspensions were adjusted to 0.5 McFarland Standard with sterile MHB broth, approximately 1.5×10^6 cell/ml. To aid comparison, the adjustment of bacterial suspensions to the density of the 0.5 McFarland Standard was done against a white background with contrasting black lines (Teh *et al.*, 2017).

The fungal inoculum (spores) was prepared according to the method of Surapuram *et al.*, (2014) with some modification, by suspending five representative colonies, obtained from fresh, mature (3 to 7 days-old) cultures grown at 27°C on PDA medium, in potato dextrose broth (PDB). Then the inoculum was adjusted to 0.5 McFarland standard, approximately $1\text{-}5 \times 10^6$ spores/ml, by measuring the absorbance in a spectrophotometer at a wavelength of 625 nm.

Assay for Antimicrobial Activity

The aqueous and methanolic crude extracts were tested against four bacterial species and two fungal species by MHA medium and PDA medium. This study was carried out using the agar well diffusion assay (Athanassiadis *et al.*, 2009). Tetracycline and Fluconazole were used as the standard antibacterial and antifungal agents. The media was poured into the sterile Petri plates and allowed to solidify. The microbial suspension of each test was evenly spread over the media by sterile cotton swabs. The plates have been kept to dry and a sterile cork borer (6 mm in diameter) was then used to punch wells (four wells) in the agar media. Subsequently, wells were filled with 100µl of each extract at a concentration of 100 mg/ml and allowed to diffuse at room temperature for 1 hour, then the plates were placed in an incubator at 37 °C for 24 hours in the case of bacteria and at 27 °C for 48-72 hours in the case of fungi. The DMSO solvent was used as a negative control. The resulting diameters of inhibition zones were measured using a ruler in millimeters. The experiment was performed in triplicate for each tested microorganism and plant extract, the mean zone of inhibition was calculated for each crude extract and standard antibiotic.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) of the Effective Plant's Extract

The MIC test was prepared according to the method of Mostafa *et al.*, (2018), with some modifications. The MIC is defined as the lowest concentration of the antimicrobial agent that completely inhibits visible growth as judged by the naked eye, disregarding a single colony. The most effective plant extracts which exhibiting a strong antimicrobial activity at 100 mg/ml were tested to determine their MIC using a well diffusion assay and evaluate their efficiency in controlling bacterial species causing diseases. Different concentrations of the effective plant extracts (3.1, 6.2, 12.5, 25, 50, and 100 mg/ml). A sterile cork borer (6 mm in diameter) was then used to punch wells (six wells) in the seeded Mueller-Hilton agar (MHA) with bacterial suspensions of the pathogenic species. Subsequently, wells were filled with 100µl of each various concentrations of the effective plant extracts and

allowed to diffuse at room temperature for 1 hour then the plates were incubated in the incubator at 37 °C for 24 h. The zones of inhibition were measured by a ruler in millimeters. While, the MBC is the concentration that causes growth inhibition by % 99.9, and this was confirmed by taking a swab from the zones of inhibition and cultivate it on MHA medium again to make sure the bacteria are killed. The concentration of the plant extract that did not show any bacterial growth on the freshly inoculated MHA medium was determined as the MBC.

RESULTS AND DISCUSSION

The obtained results through this study were recorded in (Table 1 and shown in Figures 1-3). The antimicrobial activity experiments were carried out using the concentration of 100 mg/ml obtained by dissolving the aqueous and methanolic crude extracts in DMSO solvent. The antimicrobial activity was tested against four bacteria and two fungi species. Tetracycline and Fluconazole were used as a positive controls for the antibacterial and antifungal assays, respectively, the DMSO solvent was used as a negative control. Through obtained results, the effect of the *E. paralias* and *M. sulcatus* extracts was most effective against the bacterial species, compared to the fungal species, especially methanolic extracts exhibited a clear inhibitory effect against all tested microorganisms species and order of inhibition was found to be *S. aureus* > *P. aeruginosa* > *E. coli* > *Klebsiella sp.* > fungal species. While aqueous extracts did not show any activity against the fungal species.

Table 1: Inhibition Zones diameter of *E.paralias* and *M.sulcatus* extracts against tested microorganisms species

Microorganisms			The zone of inhibition is measured in millimeter					
			Concentration 100 mg/ml					
			Bacterial species		Fungal species			
No.	Plant extracts		*S. aureus*	*P. aeruginosa*	*E. coli*	*Klebsiella sp.*	*A. niger*	*A. flavus*
1	*E. paralias*	Aqueous	12	10	10	R	R	R
		Methanol	16	15	13	13	9	9
2	*M. sulcatus*	Aqueous	12	9	9	R	R	R
		Methanol	15	14	12	10	9	9
3	C^+Tetracycline/Antibacterial *Fluconazole/Antifungal		15	14	13	13	*14	*14
4	C^- (DMSO)		R	R	R	R	R	R

C^+: Positive control, C^-: Negative control, R: Resistant

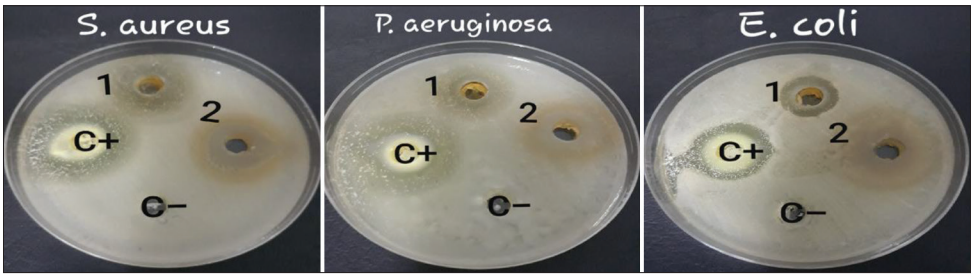

Figure 1: Effect of the aqueous extracts of 1- *E. paralias* and *2- M. sulcatus* on bacterial species at 100 mg/ml concentration, compared with a positive and negative controls.

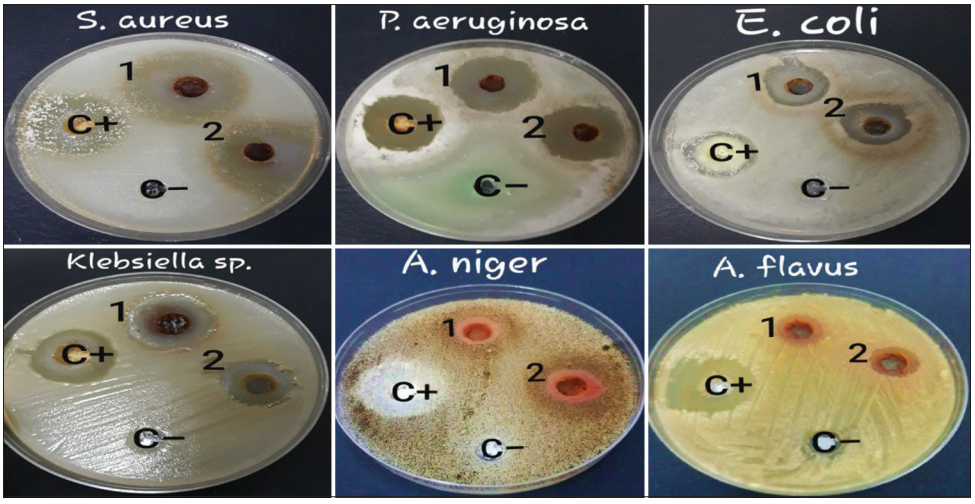

Figure 2: Effect of the methanolic extracts of 1- *E. paralias* and *2- M. sulcatus* on microorganisms species at 100 mg/ml concentration, compared with a positive and negative controls

By comparing the obtained results from these extracts of both plants with positive controls (Tetracycline and Fluconazole), the results of Tetracycline were fairly closed. Whereas, the results of Fluconazole have a higher effect than extracts.

The results listed in (Table 1) exhibited that the methanolic extracts were the most effective extracts and showed a good antibacterial activity against all tested pathogenic bacteria, therefore, to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against these bacterial species, experiments on methanolic crude extracts were conducted.

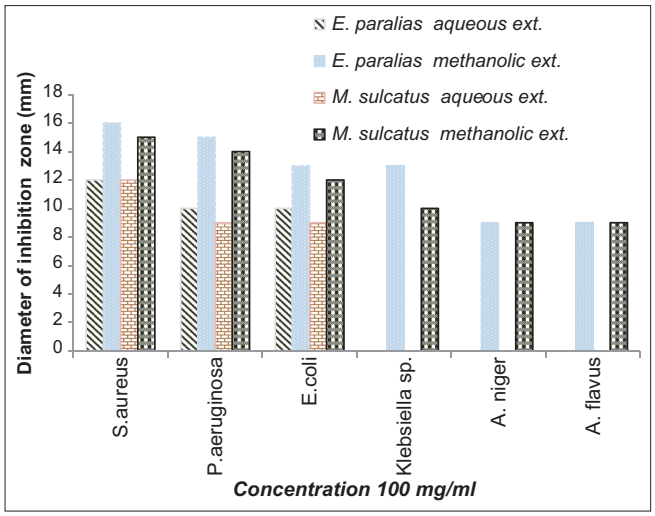

Figure 3: Effect of selected plant extracts on pathogenic microorganisms species.

The MIC results have been reported in (Table 2 and can be seen in Figures 4-6). The inhibitory effect of the methanolic extract of *E. paralias* reached 6.2 mg/ml, with inhibition zones of 11, 9, 8 and 7 mm against all tested bacterial species *S. aureus*, *P. aeruginosa*, *E.coli* and *Klebsiella sp.*, while the methanolic extract of *M. sulcatus* was given an inhibitory effect reached 6.2 mg/ml with inhibition zones of 10, 10, 7 mm against *S. aureus*, *P. aeruginosa* and *E. coli* except *Klebsiella sp.* reached 12.5 mg/ml with inhibition zone of 7 mm. The MBC of *E. paralias* and *M. sulcatus* extracts reached 12.5 mg/ml against all tested bacterial species, except *Klebsiella sp.* the MBC value reached 25 mg/ml to a methanolic extract obtained of *M. sulcatus*. The concentrations of methanolic extract of the selected plant species were similar through the obtained results to determine the MIC and MBC values, which showed antimicrobial activity against all pathogenic bacteria, except *Klebsiella sp.* which was less sensitive to *M. sulcatus* methanolic extract and its MIC and MBC reached 12.5 and 25 mg/ml, respectively.

For many years people around the world have healed the sick with herbal derived remedies and handed down through

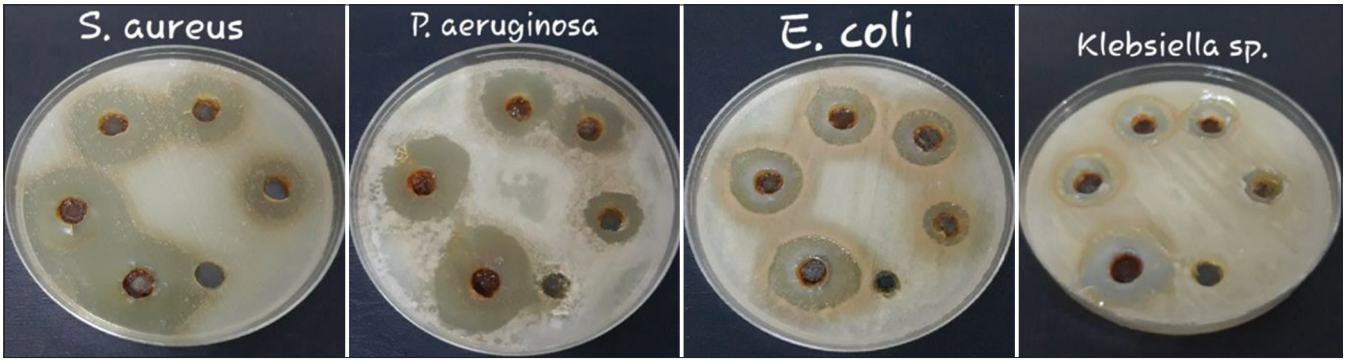

Figure 4: MIC of the methanolic extract of *E. paralias* against bacterial species at 3.1, 6.25, 12.5, 25, 50 and100 mg/ml concentrations, respectively (from right to left).

Table 2: MIC of the methanolic extract against pathogenic bacterial species

No.	Plant species	The zone of inhibition is measured in millimeter				
			Bacterial species			
		Concentrations in mg/ml	*S. aureus*	*P. aeruginosa*	*E. coli*	*Klebsiella sp.*
1	*E. paralias*	3.1	R	R	R	R
		6.2	11	9	8	7
		12.5	12	10	10	9
		25	13	11	10	10
		50	15	14	12	11
		100	16	15	13	13
2	*M. sulcatus*	3.1	R	R	R	R
		6.2	10	10	7	R
		12.5	11	11	9	7
		25	13	11	10	8
		50	13	13	12	9
		100	15	14	12	10

R: Resistant

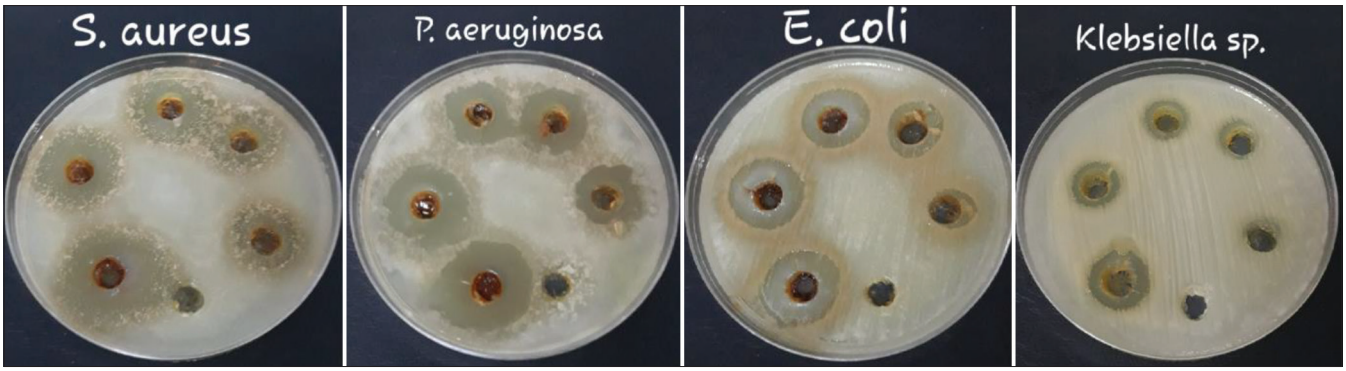

Figure 5: MIC of the methanolic extract of *M. sulcatus* against bacterial species at 3.1, 6.25, 12.5, 25, 50 and100 mg/ml concentrations respectively (from right to left).

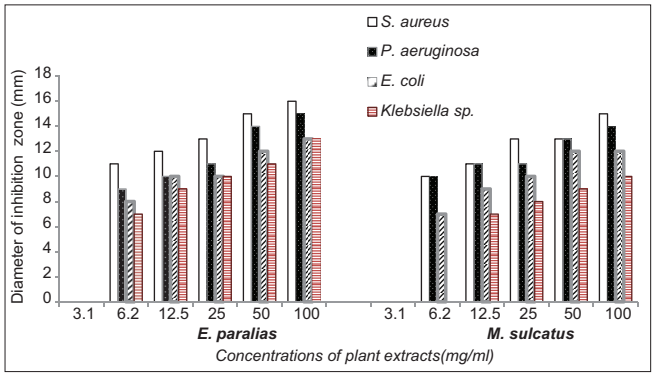

Figure 6: MIC of the methanolic extract against pathogenic bacterial species.

generations. Traditional medicine has an old history in health maintenance, as well as to prevent, diagnose, improve or treat physical and mental illness (Dhivya & Kalaichelvi, 2017). The agar well diffusion assay has been used in this research because it is more sensitive than the agar disc diffusion assay (Valgas *et al.*, 2007). Many studies have shown that extracts obtained from the *Euphorbia* and *Melilotus* genera inhibit the growth of different microorganisms at various concentrations (Annapurna *et al.*, 2004; Singh & Kumar, 2013; Sonju *et al.*, 2017; Sisay *et al.*, 2019). Other studies on the species of genus *Euphorbia* and *Melilotus* have confirmed the efficacy of the methanolic extracts compared with the aqueous extracts. The methanolic extracts showed high antimicrobial activity against various pathogenic microorganisms, also, the methanolic extracts displayed larger inhibition zones against Gram-positive bacteria compared to Gram-negative bacteria (Naz *et al.*, 2017; Lone *et al.*, 2013; Sonju *et al.*, 2017). The results of the present study are consistent with the above studies conducted on species belong to the selected genera in this study. In plants, alkaloids, tannins, flavonoids and many aromatic compounds or secondary metabolites which is involved in defense mechanism against invading microorganisms and various predators like herbivores and insects (Lin *et al.*, 1999). Some researchers have indicated that plant extracts antimicrobial components (terpenoid, alkaloid and phenolic compounds) interact with enzymes and proteins of the microbial cell membrane, causing their disruption to disperse a flux of protons to the outside of the cell that causes cell death or may inhibit enzymes required for the biosynthesis of amino acids (Burt, 2004; Gill & Holley, 2006).

CONCLUSION

The degree of antimicrobial activity may be suggested to be based on the extraction process, the type, amount of extracts and the chemical properties of the compounds. According to the results of this study on *Euphorbia paralias* L. and *Melilotus sulcatus* Desf., it can be concluded that the antimicrobial potential of these plants is confirmed and their extracts are suitable to control microorganisms caused for diseases and it can be used naturally in human and veterinary healthcare systems.

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