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Antimicrobial Activity of *Cistus* Plant Extracts Against Some Pathogenic Microbes Species

Miloud M. Miloud

Department of Botany, Faculty of Science, Benghazi University-Al-Abyar branch, Benghazi, Libya

Corresponding author: (Miloud.elagaily@uob.edu.ly)

(ميلود مرعي ميلود)

الملخص

الهدف من الدراسة الحالية هو تقييم النشاط المضاد للميكروبات لمستخلصات أوراق الميثانول من نباتات *Cistus incans* L.، *Cistus salvifolius* L. و *Cistus parviflorus* Lam. ضد ستة أنواع من الميكروبات الممرضة وهي *Staphylococcus aureus* ، *Serratia marcescens* ، *Acinetobacter boumannii*، *Aspergillus flavus* ، *Aspergillus niger* و *Cladosporium cladosporioides* باستخدام طريقة الانتشار بواسطة الآجار Agar well diffusion. أظهرت النتائج المتحصل عليها من المستخلصات النباتية نشاط مضاد للميكروبات جيد ضد جميع أنواع الميكروبات المختبرة. هذه المستخلصات كانت أكثر فعالية وأظهرت نشاط مثبط bacteriostatic وقاتل bactericidal ضد الأنواع الأكثر حساسية من البكتيريا الممرضة (*S. aureus* and *S. marcescens*) مع التركيز المثبط الأدنى MIC الذي بدأ من 12.5 ملغم/مل و التركيز قاتل الأدنى MBC من 25 ملغم/مل، باستثناء بكتيريا *S. aureus* التي كانت أكثر حساسية لمستخلص نبات *C. salvifolius* و سجلت 6.2 و 12.5 ملغم/مل للتركيزين على التوالي. يمكن استخدام هذه المستخلصات النباتية التي أثبتت فعاليتها كعوامل وقائية بديلة طبيعية للسيطرة على الأمراض المتسببة بواسطة هذه الأنواع الميكروبية وتجنب المخاطر الصحية من استعمال العوامل الكيميائية المضادة للميكروبات.

ABSTRACT

The objective of the present study is to evaluate the antimicrobial activity of the methanolic leaf extracts of *Cistus incans* L., *Cistus salvifolius* L., and *Cistus parviflorus* Lam. against six pathogenic microbial species (*Staphylococcus aureus*, *Serratia marcescens*, *Acinetobacter boumannii, Aspergillus flavus, Aspergillus niger and Cladosporium cladosporioides*) using the agar well diffusion method. The obtained results from the plant extracts showed good antimicrobial activity against all tested microbes species. These extracts were most effective. It showed bacteriostatic and bactericidal activities against the highly susceptible species of pathogenic bacteria (*S. aureus* and *S. marcescens*) with MIC, which started at 12.5 mg/ml and MBC of 25 mg/ml, except *S. aureus* that was more sensitive to *C. salvifolius* extract. Its MIC and MBC reached to 6.2 and 12.5 mg/ml, respectively. These plant extracts, which proved to be

potentially effective, can be used as natural alternative preventives to control diseases caused by this microbial species and avoiding health hazards resulting from antimicrobial chemical agents application.

Keywords: *Cistus* species, Antimicrobial activity, well diffusion assay, MIC, MBC.

INTRODUCTION

In recent years, considerable academic researches has been focused on medicinal plants due to their biological potential, including antioxidant and antimicrobial activities (Krishnaiah *et al.,* 2011). Aromatic and medicinal plants (AMPs) have long been a part of man's everyday existence for a variety of purposes. AMPs play a crucial and fundamental role in traditional medicine and play a very significant role in drug discovery, notably for antibiotics (Es-Safi *et al.,* 2021; Saleem *et al.,* 2010). This is of paramount importance, given the worldwide problems of healthcare-associated infections (Ho¨gberg *et al.,* 2010) and because of a lack of novel antibiotics currently available (Cheng *et al.,* 2009). *Cistus* plant is one of the medicinal plants, belonging to the Cistaceae family, the genus *Cistus* encompass 20 species particularly distributed in the Mediterranean region (Zohary, 1987; Tomas-menor *et al.,* 2013), this kind is widespread in Portugal, Spain, Italy, Algeria, Morocco and Libya (Mariotti *et al.,* 1997; El-Mokassbi, 2022) . The plant, which in Libyan local names called Birbish (El-Mokassbi, 2022). The leaves of all *Cistus* species secrete essential oils (Bonnier et al., 1990). Plants essential oil consist mainly of terpenoids (Demetzos *et al.,* 1994), flavonoid aglycons (Demetzos *et al.,* 1990), and glycosides (Vogt *et al.,* 1987). Essential oils from different species of therapeutic plants possess antimicrobial propriety with strong activity against Gram-negative, Gram-positive bacteria and fungi (Di Pasqua *et al.,* 2005). In Mediterranean folk medicine, *Cistus* species have been used as general remedies (Barrajon-Catalan *et al.,* 2010), and are reputed for their uses as antimicrobial (Demetzos *et al.,* 1999), antiviral, anti-tumor (Dimas *et al.,* 2000) and cytotoxic (Ben jemia *et al.,* 2013) properties. The literature describes some studies on the biological activity of *Cistus* species. Extracts from different species of *Cistus* reported to have antioxidant, antimicrobial, and antifungal properties. Benbelaïd *et al.,* (2017) investigated the antimicrobial activity of *Cistus munbyi* essential oil against nine pathogens using the disc diffusion and broth micro-dilution methods and their results showed that *C. munbyi* essential oil possesses strong antimicrobial activity against all strains. Rebaya *et al.,* (2016) studied antibacterial and antifungal activities of ethanol extracts of *Cistus salviifolius* and *Cistus monspeliensis*

against some tested microbial strains using the disc diffusion method. Ethanol extracts of different parts of the plant exhibited good activity against all microorganisms tested. Mahmoudi *et al.,* (2016) investigated the antimicrobial activity of *Cistus monspeliensis* and *Cistus salvifolius* against seven pathogenic microbial strains. Leaf ethanol extracts from both species were active against each microbial species, but the *C. monspeliensis* leaf ethanolic extract was much more active against several microbial species than that of *C. salvifolius*. The first study on the *Cistus* genus, published by R. Hegnauer, determined the constitution of diterpenes in the aerial parts of *C. monspeliensis* (Hegnauer & Hegnauer, 1962). The aim of this study is to evaluate the activity of methanolic leaf extracts of *Cistus incans*, *Cistus salvifolius*, and *Cistus parviflorus* 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 *C. incans*, *C. salvifolius* and *C. parviflorus* in spring month of 2022 from northeast region Al-Jabal Al-Akhdar of Libya. The leaves were cleaned using tap water to remove the dust and left in the air under shade to dry for 2 weeks, then grinded into powder using an electric blender, transferred into a glass container, and preserved until the extraction procedure performed in the laboratory (Miloud & Senussi, 2021).

Extracts preparation

According to the method of Mohammadi *et al.,* (2015), with minor modifications, 50 g of the powder of *C. incans*, *C. salvifolius* and *C., parviflorus* filled in the thimble and extracted successively with 300 ml of methanol using a Soxhlet apparatus for 24 hours. All the extracts were evaporated using a rotary evaporator and were dissolved in 10 ml of the same used solvent. One concentration of crude extracts was prepared, which is 100 mg/ml, and stored at 4 °C in airtight bottles until further use.

Microbial species

Bacterial species were obtained from the microbiology laboratory of Benghazi Medical Centre (BMC). Also, Fungal species were obtained from the Botany Department, Benghazi University, Al-Abyar branch. In total, six microorganisms, three bacterial species: *S. aureus, S. marcescens* and *A.*

boumannii and three fungal species: *A. Niger, A. flavus* and *C. cladosporioides.* 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. Cooled to 45-50 °C, and then poured into sterile Petri plates (Miloud & Senussi, 2021).

Preparation of the microbial suspension

Bacteria stock cultures were sub-cultured onto Nutrient Agar (NA) plates and incubated overnight at 37°C. Three to four discrete bacterial colonies with similar morphology inoculated into 10 ml sterile Mueller Hinton broth (MHB) and incubated overnight at 37 °C. The bacterial suspensions adjusted to 0.5 McFarland Standard with sterile MHB broth, approximately1.5×10^6 cell/ml. 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 suspensions were prepared according to the method described by Surapuram *et.al.* (2014), with minor modifications. Five representative colonies, obtained from fresh and mature cultures on PDA media, suspended in potato dextrose broth (PDB). Then the suspensions adjusted to 0.5 McFarland standard, approximately $1\text{-}5\times10^6$ spores/ml, by measuring the absorbance in a spectrophotometer at a wavelength of 625 nm.

Antimicrobial activity assay

The antimicrobial activity assay was determined by the agar well diffusion method (Athanassiadis *et al.,* 2009). Gentamicin, Imipenem, Colistin and Fluconazole used as the standard antibacterial and antifungal agents. Petri plates were poured with MHA and PDA media and allowed to solidify. The microbial suspension of each test was evenly spread over the media by sterile cotton swabs. The plates kept to dry and a sterile cork borer (6 mm in diameter) then used to punch wells (five wells) in the agar media. Subsequently, wells filled with 100µl of each extract at a concentration of 100 mg/ml and allowed to diffuse at room temperature for 1 hour, and then the plates 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 methanol solvent was used as a negative control. The resulting diameters of inhibition zones measured using a ruler in

millimeters. The experiment performed in triplicate for each tested microbes 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)

The MICs test was prepared according to the method of Mostafa *et al.,* (2018), with some modifications. The crude plant extracts, which exhibited a highest antimicrobial activity at 100 mg/ml, were tested to determine their MIC using agar well diffusion method and evaluate their efficiency in controlling tested bacterial species. Different concentrations of the tested plant extracts (3.1, 6.2, 12.5, 25, 50, and 100 mg/ml). A sterile cork borer (6 mm in diameter) then used to punch wells (six wells) in the seeded Mueller-Hinton agar (MHA) with bacterial suspensions of the tested species. Subsequently, wells filled with 100µl of each various concentrations of the plant extracts and allowed to diffuse at room temperature for 1 hour then the plates incubated in the incubator at 37 °C for 24 h. While, the MBC is the concentration that causes growth inhibition by % 99.9. The concentration of the plant extract that did not show any bacterial growth on the freshly inoculated MHA medium determined as the MBC.

RESULTS AND DISCUSSION

Gentamicin, Imipenem, Colistin and Fluconazole used as positive standards for antibacterial and antifungal activities respectively, whereas methanol solvent was used as negative control. All extracts showed good antimicrobial activity (Table 1 and Figure 1) against the microbes and were most effective against *S. aureus* and *S. marcescens* with zones of inhibition range of 12-16mm. The comparison between the obtained results from the plant extracts and the selected standard antibiotics in this study showed that the antibiotics have higher antimicrobial activity than the plant extracts against the tested microbes species. Hence, experiments on obtained crude extracts conducted to determine their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against the most susceptible microbial species (*S. aureus* and *S. marcescens*). The MIC and MBC results have been reported in (Table 2 and can be seen in Figures 2 and 3). The MIC of *C. incaus* and *C. parviflorus* started at 12.5 mg/ml with inhibition zones of 10 and 9 mm against *S. aureus* and *S. marcescens*, while *C. salvifolius* given an inhibitory effect reached to 6.2 mg/ml with inhibition zone of 9 mm against *S. aureus*. The MBC of *C. incaus* and *C. parviflorus* extracts started at 25 mg/ml against *S. aureus* and *S.*

marcescens, while *C. salvifolius* extract reached to 12.5 mg/ml against *S. aureus*. The agar well diffusion method has been used in this study because it is more sensitive than the agar disc diffusion method (Valgas *et al.,* 2007). The present study showed that the effectiveness of *Cistus* species against the tested microbial species and the bacterial species were more sensitive. These results are in accordance with that of Lahcen *et al.,* (2020), El Karkouri *et al.,* (2021), Mahmoudi *et al*., (2016), Karim *et al.,* (2016) and Bouamama *et al.,* (2006). The methanolic extracts showed high antimicrobial activity against various pathogenic microbes, 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), due to the presence of hydrophobic lipopolysaccharide in the outer gram-negative membrane (which provides protection against several agents) (Nikaido & Vaara, 1985). Some studies have indicated that plant extracts antimicrobial components (terpenoid, alkaloid, glycosides 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).

Table 1: Antimicrobial screening test of methanolic plant extracts against microbes species

Microorganisms				**The zone of inhibition is measured in millimeter**					
				Concentration 100 mg/ml					
				Bacterial species			***Fungal species***		
No.	***Cistus* Species**			***S. aureus***	***S. marcescens***	***A. boumannii***	***A. flavus***	***A. niger***	***C. cladosporioides***
1	***C. incaus***			**13**	**12**	**9**	**9**	**9**	**9**
2	**C. salvifolius**			**16**	**14**	**10**	**11**	**11**	**12**
3	**C. parviflorus**			**14**	**13**	**10**	**10**	**10**	**11**
4	**C^+**	**Gentamicin**	**Anti-bacterial**	**19**	**-**	**-**	**-**	**-**	**-**
		Imipenem		**-**	**23**	**-**	**-**	**-**	**-**
		Colistin		**-**	**-**	**12**	**-**	**-**	**-**
		Fluconazole	**Anti-fungal**	**-**	**-**	**-**	**14**	**14**	**13**
5	**C^- (Methanol)**			**-**	**-**	**-**	**-**	**-**	**-**
				C^+		**Positive control**			
				C^-		**Negative control**			

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-	**No activity (there was no zone of inhibition)**

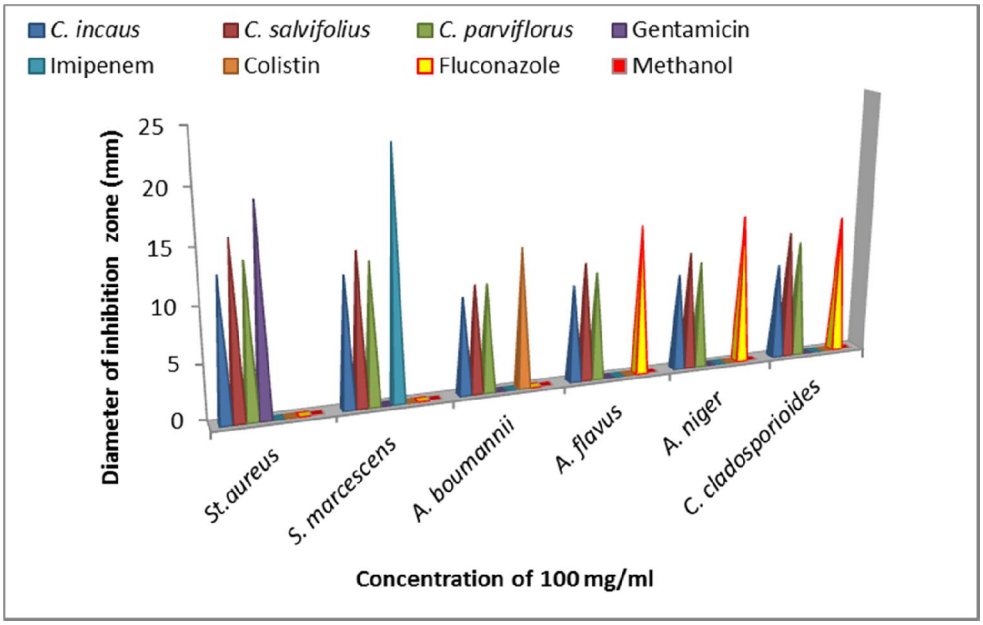

Figure 1: Effect of methanolic extracts of *Cistus* species against microbes species

Table 2. MIC of the methanolic plant extracts against microbes species.

No.	Plant species	The zone of inhibition is measured in millimeter		
		Bacterial species		
		Concentrations in mg/ml	*S. aureus*	*S. marcescens*
1	*C. incaus*	3.1	-	-
		6.2	-	-
		12.5	10	9
		25	10	10
		50	11	10
		100	13	12
		3.1	-	-
		6.2	9	-

2	*C. salvifolius*	12.5	10	9
		25	12	10
		50	14	12
		100	16	14
3	*C. parviflorus*	3.1	-	-
		6.2	-	-
		12.5	10	9
		25	11	10
		50	11	11
		100	13	13
		-		No activity

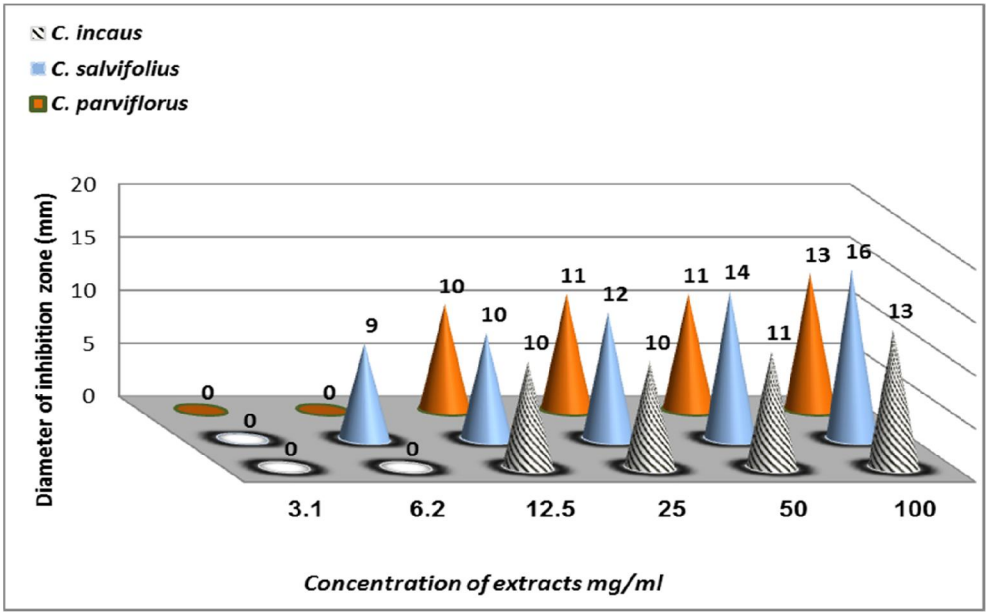

Figure 2: MIC of the methanolic plant extracts against *S. aureus*

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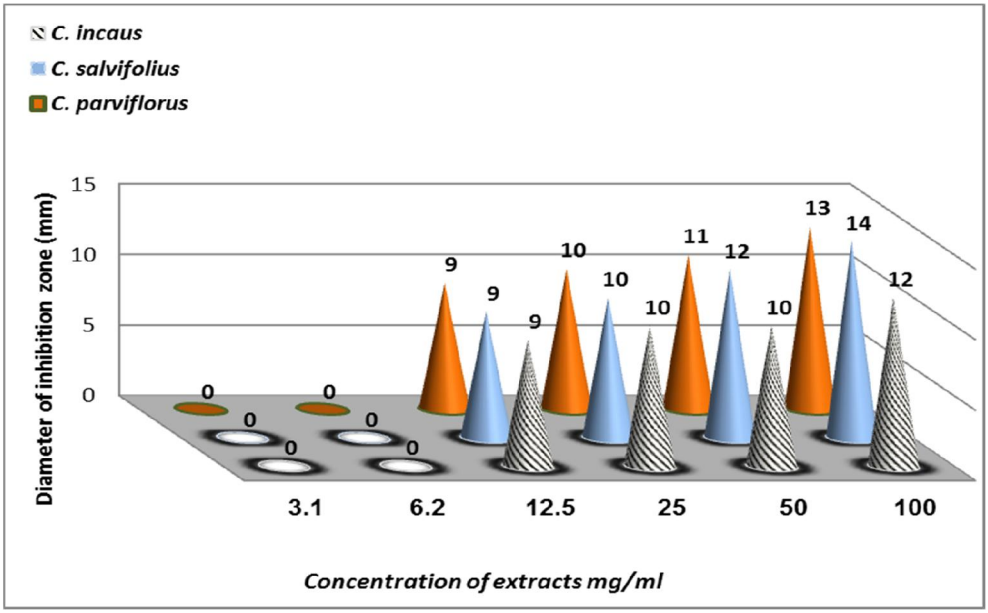

Figure 3: MIC of the methanolic plant extracts against *S. marcescens*

Conclusion

According to the results of this study and other studies that performed on different species of *Cistus*, it could be concluded that the antimicrobial potential of this plant is confirmed and its extractions are suitable to control pathogenic microbes and avoiding health hazards resulting from antimicrobial chemical agents application.

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