EVALUATION OF ANTIBACTERIAL ACTIVITY OF ETHANOLIC LEAF EXTRACTS OF THREE MEDICINAL PLANTS AGAINST PATHOGENIC BACTERIAL SPECIES

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Abstract

The present investigation was carried out to evaluate the antibacterial activity of *Conium maculatum* L., *Pistaciaatlantica* Desf. and *Calendula suffruticosa* Vahl. Dried leaf powder of these plants was successively extracted with ethanol using Soxhlet apparatus. All extracts were screened for its antibacterial activity using an agar well diffusion method. The bacteria species used for antibacterial were *Staphylococcus aureus, Enterococcus faecalis, Acinetobacter boumannii, Serratiamarcescens, Klebsiella pneumoniae, Pseudomonas aeruginosa*. Gentamicin, Imipenem and Colistin were used as standards. The obtained results revealed that all plant extracts were potentially effective in inhibiting bacterial growth of all tested species at concentrations (100 and 200 mg/ml). These extracts were most effective against the highly susceptible species *S. aureus, E. faecalis, K. pneumoniae* and *P. aeruginosa*. Results of the MIC and MBC against these highly susceptible bacterial species were as follows: the MIC of *C. maculatum* and *P. atlantica* extracts started at 12.5 mg/ml and the MBC of 25mg/ml against *S. aureus, E. faecalis, K. pneumonia*. *P. aeruginosa* was less sensitive and its MIC and MBC reached to 25 and 50 mg/ml, respectively, while MIC of *C. suffruticosa* extract reached to 25 mg/ml and MBC of 50mg/ml against *S. aureus, E. faecalis, K. pneumoniae* and *P. aeruginosa*. These plant extracts can be supplemented in the treatment of infectious diseases, and leaf extracts of the plants may be potential antibacterial agents.

Keywords: Medicinal plants; Antibacterial activity; agar well diffusion method; MIC; MBC.

Introduction

Since ancient times medicinal plants have continued to be an essential therapeutic aid for alleviating the ailments of humankind (Nair and Chanda, 2007). These medicinal herbs constitute indispensable components of the traditional medicine practiced worldwide due to their low cost, easy accessibility, and ancestral experience (Natarajan et al., 2005). There are several advantages to using herbs or natural products as a source for the development of antibiotics.

Medicinal plants are known to produce very complex compounds which are still beyond the capability of synthetic chemists. However, there is minimal risk of side effects associated with medicinal plants as compared to synthetic drugs, as edible plants or traditional medicine is reported to have very less or no side effects (Ekor, 2014). Several studies are currently focusing on natural medicinal herbs, which are regularly used in folk medicine and have a high probability of developing as bactericidal or bacteriostatic agents (Verma and Singh, 2008).

Conium maculatum L. (*C. maculatum*), poison hemlock, (Apiaceae) is a poisonous plant well known since antiquity, with high toxicity for animals and humans (Al-Snafi, 2016; Vetter, 2004; Lopez et al., 1999). The plant grows preferentially on uncultivated land, and on loamy and nitrogen rich soils, which is sparsely distributed throughout Europe and the Mediterranean region. In the aerial parts piperidine alkaloids as coniine and coniceine are the toxic compounds. A further novel alkaloid, named conmaculin, has been described by Radulovic et al., (2012).

Pistaciaatlantica Desf. (*P. atlantica*) belongs to the Anacardiaceae family, which is distributed in the Mediterranean and Middle Eastern areas (Kamrani et al., 2007). There are several pharmaceutical studies on composition and antioxidant and antimicrobial activities of essential oil and extracts obtained from the different parts of *P. atlantica* species including leaves, fruits, hull and gum (Hatamnia et al., 2016; Farhoosh et al., 2011; Hosseini et al., 2013; Peksel, 2008; Salimi et al., 2011). The aerial part of this plant has traditionally been used as a stimulant for its diuretic properties. It has also been used to treat hypertension, coughs, sore throats, eczema, stomachaches, kidney stones, and jaundice (Palevitch and Yaniv, 2000). The chemical composition of the essential oil of this plant reveals the presence of several main compounds: myrcene (19 - 25%), a_pinene (16%), terpinen-4-ol (22%), d-3-carene (65%), myrcene, limonene, terpinen-4-ol, a-pinene, b -pinene, a -phellandrene, sabinene, *para*-cymene and g-terpinene (Castola et al., 2000).

Calendula suffruticosa Vahl. (*C. suffruticosa*) belongs to the Asteraceae family. The genus is native to the Mediterranean countries (Naguib et al., 2005). *Calendula* exhibits antimicrobial activity (Arora et al., 2013), and it has been used for the treatment of burns, abrasions, skin inflammations, ulcers, wounds and eczema (Schulz et al., 2004). It has been used internally for the treatment of gastiritis, bleeding of duodenal ulcers and colitis (Bone, 2003). The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world (Srinivasa Reddy et al., 2001). Plants are rich in a wide variety of secondary metabolites such as Flavonoids, triterpenoids, which have been found in vitro to have antimicrobial properties (Al-Traboulsi and Alaib, 2021; Cowan, 1999).

Mahin et al., (2011) investigated the antibacterial activity of *Pistaciaatlantica* and *Pistaciakhinjuk* on three species of bacteria *Escherichia coli*, *Staphylococcus aureus*

and *Staphylococcus epidermidis*, using the disk diffusion method; the results showed that extract of two plants had a beneficial drug property. Mohamed (2009) investigated the antimicrobial activity of *Pistaciaatlantica* essential oil against the growth of clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pyogenes* using agar disc diffusion and dilution broth methods. Results revealed that essential oil of *P. atlantica* has antimicrobial activity against gram-positive and negative bacteria, which are resistant to commonly used antimicrobial agents, and they were considerably dependent on concentration. Essassi et al., (2014) studied antibacterial activity of *Calendula aventis* flower extracts using 6 Gram positive and 7Gram negative bacteria (sensitive and multidrug resistant). Antibacterial activity screening was conducted by the well diffusion method. The results indicated that the methanolic extracts of *C.aventis* flowers generally showed high inhibitory activity against Gram-positive and Gram negative bacteria except Acinetobacter baumanii, Proteus mirabilis and Listeria monocyte genes. The *C. Aventis* flowers hexanolic extract inhibited all bacteria of Gram-positive and Gram negative bacteria except, *Staphylococcus aureus* MRSA, *Streptococcus agalactiae* and *Acinetobacter baumanii.* There were no inhibitory effects of the aqueous extracts against all tested bacterial strains except *Rhodococcussequi* and *Morganellamorganii*; MICs values of methanolic extracts were between 12.5-25µg/ml while MICs of values of hexanolic extracts were between 6.25- 12.5 µg/ml.

This study is to investigate the antibacterial activity of ethanolic leaf extracts of three medicinal plants, namely, *Conium maculatum* L., *Pistaciaatlantica* Desf. and *Calendula suffruticosa* Vahl. against pathogenic bacterial species.

Material and Methods

Collection and Preparation of Plant Samples

Fresh samples were collected from the leaves of the selected plants in the middle of the spring month of 2022 from Al-jabal al-Akhdar (a mountain), Libya. The collected plants were washed with water and dried in the shade for 2 weeks. The dried leaves of each plant species were crushed into a fine powder using an electric blender, transferred into a glass container, and preserved until the extraction procedure was performed in the laboratory (Miloud and Senussi, 2021).

Preparation of Extracts

Adapting Mohammadi *et al.,* (2015) method, 50 g of the powder of *Conium maculatum, Pistaciaatlantica, and Calendula suffruticosa* were filled in the thimble and extracted successively with 200 ml each of ethanol using a Soxhlet apparatus for 24 hours. Extracts were evaporated using a rotary evaporator. The crude extracts were dissolved in 10 ml of dimethyl sulfoxide (DMSO). In airtight bottles,100 and 200 mg/ml concentrations of extracts were prepared and stored at 4 °C for further use.

Collection of Bacteria

Bacterial species were collected from the microbiology laboratory of Benghazi Medical Centre (BMC). In total, six bacterial species were collected: two Gram positive (*S. aureus*, *E. faecalis*) and four Gram negative (*A. boumannii*, *S. marcescens*, *K. pneumoniae*, *P. aeruginosa*). The bacterial species were maintained on nutrient agar slants at 4 °C.

Preparation of Bacterial Suspension

Bacteria stock cultures 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 of 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, approximately1.5×10^6 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).

Assay of Antibacterial Activity

The obtained crude extracts were tested against six bacterial species by Mueller Hinton agar (MHA) medium. Anagar well diffusion method was used for evaluating the antibacterial activity (Athanassiadis et al., 2009). Gentamicin, Imipenem and Colistin were used as the standard antibacterial agents. The media was poured into the sterile Petri plates and allowed to solidify to make a base layer. The bacterial suspension of each test was evenly spread over the media by sterile cotton swabs. After allowing the plates to dry, a sterile cork borer (6 mm in diameter) was used to punch wells (four wells) in the agar media. Subsequently, wells were filled with 100 µl of each extract at concentrations of 100 and 200 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. The DMSO solvent was used as a negative control. The resulting diameters of inhibition zones were measured using a ruler in millimeters. To maintain the consistency of measurements, each zone of inhibition was measured twice (one vertical and one horizontal measurement) and the average value was taken. The experiments were conducted three times, and 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 MIC test was prepared according to the method of Mostafa *et al.*, (2018), with minor modifications. The crude plant extracts that exhibited a highest antibacterial activity at 200 mg/ml against the most sensitive bacterial species were tested to

determine their MIC using an 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, 100 and 200 mg/ml) were used. A sterilized cork-borer (6 mm in diameter) was used to punch wells (seven wells) in the seeded Mueller-Hinton agar (MHA) with bacterial suspensions of the tested species. The wells were filled with 100µl of each of the various concentrations of the 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 MBC is the concentration that caused growth inhibition by 99.9%. This was confirmed by cultivating a swab from the zones of inhibition on a MHA medium again to make sure that the bacteria were eliminated. 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

Using a agar well diffusion method, three plant species were investigated to evaluate their antibacterial activity against *S. aureus* and *E. faecalis*, *A. boumannii*, *S. marcescens*, *K. pneumoniae* and *P. aeruginosa* which that are known to cause common infectious diseases. The inhibition zone diameter was observed for extracts and positive control, but not for the negative control. Evaluation of antibacterial activity of these plant extracts was recorded in Table 1. The obtained results revealed that all plant extracts were potentially effective in inhibiting bacterial growth of all tested species at concentrations (100 and 200 mg/ml). These extracts were the most effective against *S. aureus*, *E. faecalis*, *K. pneumoniae* and *P. aeruginosa* with inhibition zone diameters (IZDs) of 8-21mm. The antibacterial effect of the three plant extracts was less than the standard antibiotics effect. However, *P. aeruginosa* was less effective to Gentamicin.

Experiments were conducted to determine their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against the highly susceptible bacterial species (*S. aureus*, *E. faecalis*, *K. pneumoniae* and *P. aeruginosa*). The concentrations effect of the plant extracts were reported in Table 2and illustrated in figures 1 and 2. The MIC of *C. maculatum* and *P. atlantica* extracts started at 12.5 mg/ml with inhibition zones of 8-9 mm against *S. aureus*, *E. faecalis*, *K. pneumonia*. *P. aeruginosa*, which was less sensitive, minimal inhibitory concentration reached 25 mg/ml. MIC of *C. suffruticosa* extract reached to 25 mg/ml with inhibition zones of 8-9 mm against *S. aureus*, *E. faecalis*, *K. pneumoniae* and *P. aeruginosa*.

The MBC was confirmed by the absence of bacterial growth of the tested strains streaked form inhibition zone corresponding to their lowest MIC. *C. maculatum* and *P. atlantica* extracts showed potentially bactericidal activity against *S. aureus*, *E. faecalis*, and *K. pneumoniae* with MBC of 25 mg/ml. On the other hand, *P. aeruginosa* was less sensitive and its minimal bactericidal concentration reached to 50 mg/ml. The

same MBC of *C. suffruticosa* extract, which was 50 mg/ml, was noticed against all the tested bacterial species.

The selected plants in this study contain several chemical compositions that have clear antimicrobial activity against pathogenic microbes. *C. maculatum* consists mainly of piperidine alkaloids such as coniine, coniceine, conmaculin (Radulovic et al., 2012), germacrene D, β-caryophyllene, and EE-α-farnesene (Masoudi et al., 2006) and flavone glycosides (Al-Traboulsi and Alaib, 2021). *P. atlantica* consists of α-Pinene, β-Pinene, Myrcene, Limonene and Terpineol (Raeisi et al., 2016; ElyasiGhahfarrokhi et al., 2022). Finally, *C. suffruticosa* consists mainly of Undecanoic acid, α-Bisabolol, β-sitosterol, α –Amyrin, Myristic, Palmitic acid (Ismaheneet al., 2018), Flavonoids, and triterpenoids (Al-Traboulsi and Alaib, 2021).

Several studies have been conducted on ethanolic leaf extracts of medicinal plants that had potent antimicrobial activity (Chakraborthy, 2008; Rigane et al., 2017; Nabila et al., 2008; EL-Kamali and EL-Amir, 2010; Ababutain, 2011; Valle et al., 2015). Ethanol extracts exhibited greater antibacterial activities against Gram-positive bacteria than Gram-negative bacteria. This difference could be because of the different cell surface structure of Gram-positive bacteria and Gram-negative bacteria; the outer membrane of Gram-negative bacteria possesses lipopolysaccharides and lipoproteins. The lipopolysaccharides are amphipathic compounds that comprise hydrophilic polysaccharide at the core that makes up a more rigid outer membrane. This membrane slows down the diffusion of hydrophobic compounds through the Gram negative bacteria cell, and consequently acts as a barrier of permeability (Bayoub et al., 2010; Helander et al., 1998; Puupponen-Pimia et al., 2001; Lopez et al., 2005; Zgurskaya et al., 2015).

Table (1): Antibacterial Activity of the Ethanolic Extracts of Selected Medicinal Plants

No.	Microbes sp.	The zone of Inhibition is Measured in Millimeter									
		Plant Species						Standard Antibiotics			
		C. maculatum		*P. atlantica*		*C. suffruticosa*		Gentamicin	Imipenem	Colistin	DMSO (10ml)
		100	200	100	200	100	200				
1	***S. aureus***	17	20	17	21	18	21	23	R	R	R
2	***E. faecalis***	18	20	17	20	17	19	22	R	R	R
3	***A. boumannii***	10	11	10	12	8	8	R	R	13	R
4	***S. marcescens***	11	12	9	12	10	12	R	22	R	R
5	***K. pneumoniae***	15	17	14	17	15	17	23	R	R	R
6	***P. aeruginosa***	14	16	15	18	15	17	14	R	R	R
R: Resistant											

Table (2): MIC of the Plant Extracts Against the Highly Susceptible Bacterial Species

No.	Plant Species	The Zone of Inhibition is Measured in Millimeter				
		Bacterial Species				
		Concentrations in mg/ml	*S. aureus*	*E. faecalis*	*K. pneumoniae*	*P. aeruginosa*
1	*C. maculatum*	**3.1**	-	-	-	-
		6.2	-	-	-	-
		12.5	8	9	9	-
		25	11	12	10	8
		50	13	15	12	11
		100	17	18	15	14
		200	20	20	17	16
2	*P. atlantica*	**3.1**	-	-	-	-
		6.2	-	-	-	-
		12.5	9	9	8	-
		25	11	10	11	9
		50	15	14	12	13
		100	17	17	14	15
		200	21	20	17	18
3	*C. suffruticosa*	**3.1**	-	-	-	-
		6.2	-	-	-	-
		12.5	-	-	-	-
		25	9	9	8	9
		50	13	12	12	12
		100	18	17	15	15
		200	21	19	17	17
		-		No activity		

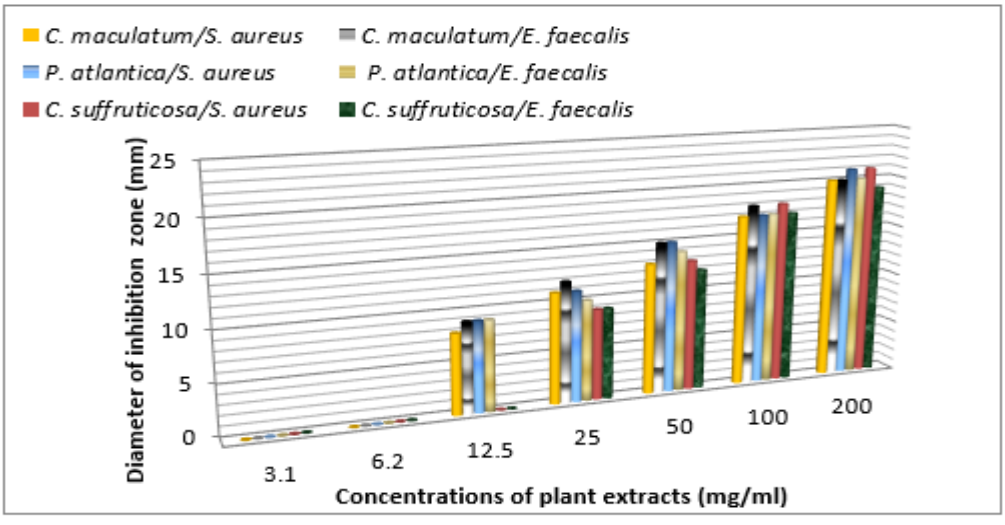

Figure (1): MIC of the Plant Extracts Against *S. aureus* and *E. faecalis*.

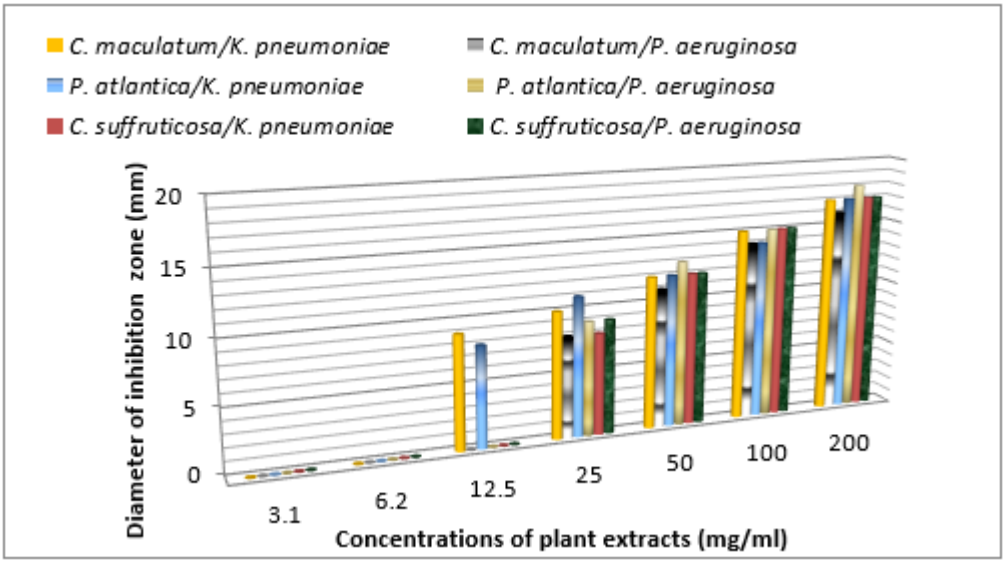

Figure (2): MIC of the Plant Extracts Against *K. pneumoniae* and *P. aeruginosa.*

Conclusion

The efficient antibacterial activity of *C. maculatum*, *P. atlantica* and *C. suffruticosa* from the present investigation revealed that the ethanol leaf extracts of the selected plants have significant potential to inhibit the growth of pathogenic bacteria species. In conclusion, these plant extracts can be supplemented in the treatment of infectious diseases and leaf extracts of these plants may have the potential to be used as antibacterial agents for new medicines.

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