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Research Article
Evaluation of the Antibacterial Activity of Essential Oil of *Dysphania ambrosioides* (L.) Mosyakin and Clemants Against Clinical Multidrug-Resistant Bacteria

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Abstract

Background and Objective: The discovery of new antibacterial agents is of high priority particularly given the global threat of bacterial resistance. A potential source to improve the current antibacterial arsenal may be in exploiting natural compounds such as essential oils (EOs). The present *in vitro* study investigated the chemical composition and the antibacterial activity of EO extracted from *Dysphania ambrosioides*. **Materials and Methods:** The EO was obtained by hydrodistillation using a clevenger-type apparatus and analyzed by Gas Chromatographic-Mass Spectrometry (GC-MS). The evaluation of the antibacterial activity was performed by the agar diffusion method and the broth microdilution method on six bacteria. Four clinical multidrug-resistant bacteria encompassing Extended-Spectrum β-Lactamase-Producing *Escherichia coli* (*ESBL-EC*), Carbapenem-Resistant *Acinetobacter baumannii* (*CRAB*), Ceftazidime-Resistant *Pseudomonas aeruginosa* (*CRPA*) and Methicillin-Resistant *Staphylococcus aureus* (*MRSA*) and two sensitive reference bacterial strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213). **Results:** The EO yield was 0.65%. The chemical analysis revealed the presence of 10 compounds including *p*-cymene (31.72%), 4-carene (27.34%) and α-cyclogeraniol acetate (16.90%). The EO had antimicrobial activity against all the tested bacteria. The minimum inhibitory concentration (MIC) recorded for *E. coli* ATCC was 90 and 120 µg mL^{-1} for *S. aureus* ATCC 29213, 120 µg mL^{-1} for *CRPA*, 140 µg mL^{-1} for *CRAB*, 150 µg mL^{-1} for *ESBL-EC* and 230 µg mL^{-1} for *MRSA*. **Conclusion:** The EO investigated in this study showed an interesting antibacterial activity against the tested bacteria. This might be due to the diversity of its chemical compounds. Promisingly, current results may have potential applications in the drug discovery process of new antibacterial agents. However, further research is needed to depict the mechanisms involved in this observed antibacterial activity.

Key words: Antibiotic resistance, *Dysphania ambrosioides*, essential oil, antimicrobial activity, minimum inhibitory concentration (MIC), inhibition diameter, GC/MS analysis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Currently, human health is threatened by the emergence of global antibiotic resistance[1]. Multi-drug resistant bacteria (MDR) are responsible for an increased mortality rate with a considerable economic cost to health authorities[1]. Indeed, annual deaths from MDR infections are expected to rise from 700,000-10 million by 2050, at a cumulative cost of 100 trillion US$[2]. The discovery of novel therapeutics against multi-drug resistant bacteria (MDR) is an urgent need that requires a global action plan[3]. The exploitation of natural products for medicinal purposes is a promising field as they are considered potential sources for therapeutic properties as they can pharmacologically interact with a wide variety of cell targets[4]. Thus, plant extracts and their active compounds may be effective against MDR and may also have synergistic effects when combined with conventional antibiotics[4-8]. The EOs exhibit different biological properties such as antimicrobial, anti-inflammatory, antiviral, sedative, digestive, antioxidant and cytotoxic activities[6-8]. These potential activities are associated with their significant chemical and structural variability[6-9]. Therefore, EOs can contribute to the development of new therapeutic drugs to combat the issue of MDR[10]. Dysphania ambrosioides (*D. ambrosioides*) (previously known as *Chenopodium ambrosioides*), is a plant belonging to the Amaranthaceae family, popularly called in Morocco "Mkhinza"[11] and which is characterized by a disagreeable odour[12]. *Dysphania ambrosioides* is native to Central and South America[12,13]. According to the WHO, this herb has several pharmacological activities such as antibacterial, antirheumatic, anti-inflammatory, antipyretic, anthelmintic, antifungal, anti-ulcer and for wound treatment[13]. These properties are associated with its richness in chemical compounds present in the extracts and EOs[14]. The present study aims to investigate the chemical composition of the EO of *D. ambrosioides* using Gas-Chromatography Coupled to Mass Spectroscopy (GC-MS) and its potential antibacterial activity on different MDR clinical strains.

MATERIALS AND METHODS

Study area: This study was conducted in December, 2021 at the Faculty of Medicine and Pharmacy, University Mohammed First Oujda, Morocco.

Plant material: *Dysphania ambrosioides* plant was collected from the Gafaït Region (Eastern Morocco, Coordinates 34°14'24.0"N, 2°24'28.8"W). The plant species were identified by a group of professional botanists and a specimen was deposited at the herbarium of Mohamed First University, Oujda, Morocco, under voucher number (HUMPOM1055).

Extraction of the essential oil of *D. ambrosioides*: *Dysphania ambrosioides* samples were deposited in a dark room to be naturally dried until their weight and stabilized for approximately 10 days. After that, 100 g of the aerial parts were subject to hydrodistillation using a Clevenger apparatus for about 2 hrs until the stabilization of the EO level. Finally, the obtained EO were stored in sealed glass bottles at 4°C for further use.

Qualitative analysis using Gas Chromatography-Mass Spectrometry (GC-MS): Gas chromatography (Shimadzu GC-2010, Kyoto, Japan) was used for the analysis of the obtained EOs. The apparatus is characterized by the presence of a capillary column RTX-5 (5% diphenyl, 95% dimethylpolysiloxane, 30×0.25 mm, 0.25 µm film thickness). The GC was linked to a mass spectrometer detector (QP2010-MS). The carrier gas used was helium, which was adjusted to a constant pressure of 100 KPa. After setting the oven temperature at 50°C for about 1 min. A gradient of +10°C until reaching 250°C and then maintained for 1 min. To perform qualitative and semi-quantitative analysis, a solution of 1 µL of the sample prepared in hexane (50 mg g^{-1}) was injected in split mode (split ratio = 50-80) and the GC-MS system was operated in scan mode. The identification of the chemical composition of samples was assessed based on the comparison process between their mass spectra and the data stored at the level of the National Institute of Standards and Technology (NIST147)[15]. Regarding the data collection, the LabSolutions of 2.5 was used.

Antibacterial activity

Bacterial strains: To determine the antibacterial activity of extracted EO, we used two reference bacterial strains including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and four clinical strains were used including Extended-Spectrum β-Lactamase-Producing *Escherichia coli* (*ESBL-EC*), Carbapenem-Resistant *Acinetobacter baumannii* (*CRAB*), Ceftazidime-Resistant *Pseudomonas aeruginosa* (*CRPA*) and Methicillin-Resistant *Staphylococcus aureus* (*MRSA*).

Antimicrobial susceptibility testing: Identification of bacterial strains was performed by the PHOENIX 100 automaton (BD)™. Determination of the resistance profile of the different bacteria was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Agar diffusion method: A standardized inoculum of tested bacteria at a density of 0.5 McFarland was plated into the surface of the Mueller-Hinton agar (MHA) plate. Then sterilized Whatman papers (6 mm) were loaded with 20 µL of the EO already prepared in 2% DMSO and placed on the surface of the agar plate and left incubated for about 24 hrs at 37°C. After incubation of the different plates, the zone of inhibition around each disc was measured. All the measurements were carried out in triplicates[16].

Microdilution method: The minimal inhibitory concentration (MIC) was determined by the microdilution method using a 96-well plate was adopted. Different concentrations ranging from 5-1000 µg mL^{-1} of the EO of *D. ambrosioides* dissolved in 2% DMSO were prepared in Mueller-Hinton broth (MHB). From each concentration, a 180 µL was pipetted and added to the plate wells. After that 20 µL of the prepared bacterial solution at 0.5 Mcfarland was added to each well. To facilitate the determination of the MIC value, the resazurin was added to each well and the plates were incubated at 37°C for about 24 hrs. The wells with no colour variation were considered MIC. Positive control contains MHB and bacterial suspension and resazurin. Negative control contains MHB, essential oil dissolved in 2% DMSO and resazurin without the microorganism. To facilitate MIC determination, resazurin has been used as a coloured indicator of bacterial growth as previously described by Dalli *et al.*[17]. Resazurin is a non-fluorescent blue/purple coloured marker that turns into pink when reduced to resorufin by the oxidoreductase enzymes of viable bacteria[18]. As for the minimal bactericidal concentration (MBC), a 20 µL was taken from each well with no colour and seeded on the agar surface plate and incubated at 37°C for 24 hrs. The plates with no subculture were considered the MBCs.

Statistical analysis: All the experiments were reproduced in triplicates. Values of each were expressed as Mean±Standard Deviation (SD). The statistical analysis was performed using Excel (Microsoft Office, V16).

RESULTS

Chemical composition and yields of *D. ambrosioides* essential oil: The obtained EO was characterized by an unpleasant odour and dark yellow colour. The EO yields obtained in this study were 0.65% (w/w). The chemical analysis using the GC-MS revealed the presence of 10 compounds. A total of 95.17% of global EO composition was identified in the samples. Among these compounds, hydrocarbon monoterpenes constituted the highest level (60.75%), followed by oxygenated monoterpenes (34.42%). As reported in Table 1, current findings showed that the main identified compounds are *p*-cymene (31.72%), 4-carene (27.34%) and α-cyclogeraniol acetate (16.90%). In addition, D-limonene, eucalyptol, γ-terpinene, 2-undecanone, thymol and carvacrol were also detected. The chromatogram of identified EO by hydrodistillation was presented in Fig. 1.

Determination of the antibacterial activity: The obtained EO was tested to evaluate their antibacterial potential against two reference strains and various pathogenic strains *ESBL-EC*, *CRAB*, *CRPA* and *MRSA*. The *ESBL-EC* was the most sensitive bacteria toward the tested EO with an inhibition zone of (49.2±3.9 mm), followed by the *MRSA* and *CRAB* with an inhibition diameter of (31.5±0.5) and (31±1.63), respectively, while the tested EO has a weak activity on *CRPA*. Regarding the MIC, all the obtained results indicated that the tested EO demonstrated an important antibacterial activity against all the tested strains. The lowest MIC value was recorded for *EC*

Table 1: Chemical constituents identified in the essential oils of *D. ambrosioides* collected from Gafaït Region of Morocco

Compounds[a]	RT[b] (min)	Relative content (%)	Type[c]
4-carene ($C_{10}H_{16}$)	6.47	27.34	MT
p-cymene ($C_{10}H_{14}$)	6.60	31.72	MT
D-limonene ($C_{10}H_{16}$)	6.67	1.24	MT
Eucalyptol ($C_{10}H_{18}O$)	6.74	0.36	OM
γ-terpinene ($C_{10}H_{16}$)	7.17	0.45	MT
α-cyclogeraniol acetate ($C_{12}H_{20}O_2$)	10.12	16.90	OM
2-undecanone ($C_{11}H_{22}O$)	10.80	5.04	OM
Thymol ($C_{10}H_{14}O$)	10.83	7.76	OM
Carvacrol ($C_{10}H_{14}O$)	10.99	4.36	OM
UC	11.12	4.83	MT
Monoterpenes (%)		60.75	
Oxygenated monoterpenes (%)		34.42	
Unidentified compounds (%)		4.83	
Yield of extract (%)		0.65	

No.: Number of compounds, [a]UC: Unidentified Compound, [b]RT: Retention time, [c]MT: Monoterpenes and [c]OM: Oxygenated monoterpenes

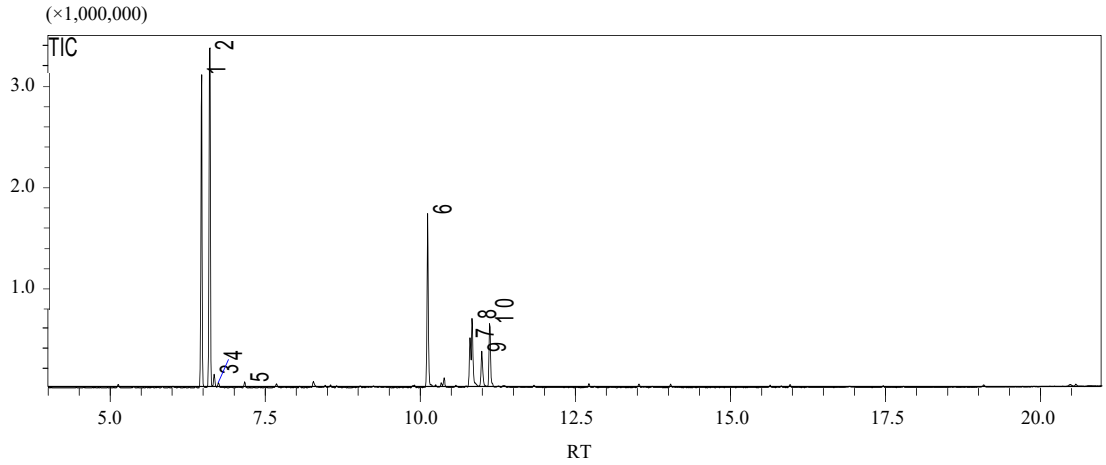

Fig. 1: GC-MS chromatogram of the essential oil of *D. ambrosioides*

Table 2: Antimicrobial activity evaluation of the EO of *D. ambrosioides*

Bacterial strains	*EC* ATCC 25922	*SA* ATCC 29213	*ESBL-EC*	*CRPA*	*CRAB*	*MRSA*
Inhibition diameter (mm)	ND	ND	49.2±3.9	14.3±0.47	31±1.63	31.5±0.5
MIC (µg mL^{-1})	90	120	150	120	140	230
MBC (µg mL^{-1})	ND	ND	400	400	400	400

ND: Not determined, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *ESBL-EC*: Extended-spectrum β-lactamase-producing *Escherichia coli*, *CRAB*: Carbapenem-resistant *Acinetobacter baumannii*, *CRPA*: Ceftazidime-resistant *Pseudomonas aeruginosa* and *MRSA*: Methicillin-resistant *Staphylococcus aureus*

ATCC 25922 (90 µg mL^{-1}). The *SA* ATCC 29213 and *CRPA*' MIC values were 120 µg mL^{-1} followed by *CRAB* which had a MIC value of 140 µg mL^{-1} and *ESBL-EC* which had a MIC value of 150 µg mL^{-1} and *MRSA* had the highest MIC value of 230 µg mL^{-1}, while the MBC value was 400 µg mL^{-1} for all strains of MDR bacteria tested. The results of the antibacterial activity were reported in Table 2.

DISCUSSION

In this study, *D. ambrosioides* EO yielded 0.65%. Several reports showed that the yields of the hydrodistillation EO ranged from 0.12-1% (w/w) which was in line with current findings of Chekem *et al.*[19] and Ávila-Blanco *et al.*[20]. The main constituents of *D. ambrosioides* EO were *p*-cymene, 4-carene, α-cyclogeraniol acetate, thymol, carvacrol and 2-undecanone. These compounds were also present in several EOs as reported elsewhere by other authors[20-22]. These compounds were known for their antimicrobial activity [21-23]. Current results displayed a great variability with the literature. The EOs obtained by hydrodistillation of the leaves showed a richness with α-terpinene followed by thymol and cymene, while another study showed the presence of the ascaridole in the *D. ambrosioides* EO (35%) which was absent in the EO extracted in this study[24,25]. This variability is principally associated with the large influence of the climate and the geographical distribution on the chemical composition of EOs[8].

The EOs are known to exert their antibacterial effect either by inhibiting the synthesis of functional and structural molecules[1]. Moreover, they can also increase the membrane permeability of the bacteria because of toxic effects on membrane structure and function[2] or by damaging the proton pump which induces an interruption of the energy production into the bacterial cell[26-28]. Other EOs possess antibiofilm activity against *Pseudomonas* spp. and *Staphylococcus aureus*[29]. The current study showed that *D. ambrosioides* EO has a significant antibacterial effect against all tested bacteria. Notably, the antibacterial activity of *D. ambrosioides* against susceptible strains and MDR strains was rarely reported in the literature[9,30].

The *p*-cymene is an important monoterpene compound with a substituted methyl and isopropyl group in the benzene ring[31]. It is a precursor of carvacrol and it is present in several plant species, belonging to Lamiaceae, Myrtaceae, Burseraceae and Asteraceae families[32]. This compound has many biological activities including antimicrobial, antioxidant, antinociceptive, anti-inflammatory, anxiolytic and remarkably anti-biofilm properties[31]. The antimicrobial power of *p*-cymene is due to the fact it distorts the cytoplasmic membrane, thus

facilitating the transport of the active compounds of the essential oil across the lipid bilayer[23]. It may also have a synergistic effect on the efficacy of phenolic monoterpenes such as thymol and carvacrol[8]. The *p*-cymene has also been shown to decrease cell motility through an effect on membrane potential and affected protein synthesis in *E. coli* bacteria[33,34]. Thymol and carvacrol are widely studied phenolic monoterpenoids. They have several activities including a remarkable antimicrobial activity[35]. They alter the structure and function of the cytoplasmic membrane and modify its fatty acid composition[36-39]. They alter membrane fluidity and permeability which in turn is responsible for potassium ions (K+) leakage[40,41]. They have an action on bacterial metabolism through an intracellular action on the energy generation process by altering the enzymes involved in ATP synthesis and its intracellular depletion[21]. Thymol is involved in the up-regulation of genes coding for outer membrane protein synthesis. It also interacts with membrane proteins which disrupt outer and inner membrane and intracellular targets[42,43]. Carvacrol is responsible for the inhibition of flagellin which damages bacterial motility[34]. It was also noticed that the morphology of Gram-negative cells was much more affected by carvacrol than that of Gram-positive cells[23]. Promisingly, the activity of carvacrol is potentiated by *p*-cymene[44] which further supports their possible synergistic combination for therapeutic use.

Analysis of the chemical composition of the EO of *D. ambrosioides* showed that α-cyclogeraniol acetate was one of the dominant compounds. Previous studies have deeply investigated the chemical composition of *D. ambrosioides* EO but none of them has reported the presence of α-cyclogeraniol acetate. This compound is derived from geraniol, a known antibacterial molecule that was widely reported by Maczka *et al*.[45].

The chemical structure affects the antibacterial activity. For 4-carene, which is a monoterpene hydrocarbon, its antibacterial activity could be attributed to the methylene group[45]. Likewise, for 2-undecanone, a dialkylated ketone with two alkyl groups, methyl and nonyl may have potential antimicrobial properties[46]. The 2-undecanone has an antibacterial activity against both Gram-negative and Gram-positive bacteria associated with its richness in long-chain methyl ketones[47,48]. It was found that the hydroxyl group present on the thymol, cymene and carvacrol are responsible for the observed antibacterial effects[28]. In addition to this, other factors that could influence the antibacterial activity of EOs are the nature and concentration of the compounds, the functional groups, the structural configuration and the possible interaction between the different compounds[46]. The presence of multiple compounds in EOs may be more potent than the action of a single compound, thereby enhancing and prolonging antimicrobial activity. Some studies have noted a synergy between EO components, which supports the holistic use of EO as anti-infective agents[47]. Current findings revealed that the EOs are promising exploratory new and effective antimicrobials. Further research to understand the mechanisms of action of the essential oil is needed. Bacterial resistance to antibiotics is an ancient and evolutionary phenomenon[48]. Despite the introduction of new antibiotics, they are not without side effects and have a limited spectrum of activity against resistance mechanisms[49]. Therefore, their high cost[49,50] limits their use, especially in developing countries. Appropriate use of antibiotics is the cornerstone to fighting this issue[50].

Dysphania ambrosioides (L.) is commonly used in Moroccan traditional medicine for its different properties. This study demonstrated the antibacterial property of *Dysphania ambrosioides* (L.) EO on multidrug-resistant strains. However, there are some limitations, such as *in vivo* study on a murine model, toxicity study and exploration of the antibacterial activity mechanism. The study recommended further research to evaluate the *in vivo* antibacterial activity of this EO, its safety and the isolation of the different chemical compounds to understand the mechanisms of action of antibacterial activity. This will promote the use of *Dysphania ambrosioides* (L.) EO is an innovative source in the pharmaceutical and medical industries.

CONCLUSION

The EO of *D. ambrosioides* demonstrated interesting antibacterial activity against the MDR bacteria tested. This might be due to the diversity of its chemical compounds and it will be promising for the development of innovative antibiotics. However, further research is needed to understand the mechanisms involved in the EO of *D. ambrosioides*.

SIGNIFICANCE STATEMENT

This study discovered the antibacterial effect of EO of *D. ambrosioides* against clinical MDR bacteria. It could be promising for the development of new antibiotics. Through our study, we will encourage researchers to explore other plants and participate in the efforts to control bacterial resistance.

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