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Antioxidant activity and evidence for synergism of *Cannabis sativa* (L.) essential oil with antimicrobial standards

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ABSTRACT

The present work was conducted to study the antioxidant activity and to determine for the first time the antimicrobial potential of Moroccan *Cannabis sativa* essential oil (EO) singly or in combination with two conventional antibiotics (fluconazol and ciprofloxacin) against some resistant and pathogenic microorganisms. The chemical composition analyzed by means of GC/MS showed that *C. sativa* EO was characterized by the dominance of (*E*)-caryophyllene (35.0%), α-humulene (12.8%) and caryophyllene oxide (10.6%). Results from antioxidant tests showed that *C. sativa* EO exhibit a moderate potency (IC_{50} = 1.6 ± 0.1 mg/m L for 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, IC_{50} = 1.8 ± 0.2 mg/mL for β-carotene/linoleic acid assay, and IC_{50} = 0.9 ± 0.1 mg/m L for ferric reducing power assay). Regarding antimicrobial assays, the results demonstrated that *C. sativa* EO displayed interesting antimicrobial activities with minimum inhibitory concentration (MIC) values in the range from (1.2 to 37.8) mg/m L for tested microorganisms. The combination of the studied EO with fluconazol and ciprofloxacin showed a significant decrease in their individual MICs. In fact, at sub-inhibitory concentrations, *C. sativa* EO contributed to the decrease of ciprofloxacin MICs of bacterial strains by 2- to 64-fold and by16-fold regarding fluconazol MICs against *Candida spp*. These findings suggest that *C. sativa* EO can be considered as a potential source of natural antioxidant and antimicrobials, and constitute in combinational treatments a promising strategy to overcome the intense use of antibiotics against some infectious diseases.

1. Introduction

The use of medicinal and aromatic plants to treat human infectious diseases dates back thousands of years, because of their preservative and pharmacological properties. Many secondary metabolites extracted from these plants have been demonstrated to possess important biological activities and are sorely needed (Brooks and Brooks, 2014). Among these compounds, volatile fractions (also known as essential oils (EOs) are attracting more attention from different segments of the industry, because of their biological actions, especially as plant-based antimicrobials and antioxidants amongst others (Andrade et al., 2013). Many reports have described the beneficial effects of using EOs of plant origin in the treatment of different infectious diseases (Astani et al., 2009; Chouhan et al., 2017; Safaei-Ghomi and Ahd, 2010). Regarding the antimicrobial efficiency of some of these EOs, many research works explored their combinational effect with antibiotics in order to overcome the microbial resistance and to diminish the minimum effective dose of these conventional antimicrobial agents (Aleksic and Knezevic, 2014; Knezevic et al., 2016; Kwiatkowski et al., 2017). *Cannabis sativa L.*, locally known in Morocco as “El kif” is an annual angiosperm belonging to the *Cannabaceae* family. This medicinal plant is widely cultivated in regions with temperate climates and found in the north of Morocco (Rif region) (Bellakhdar, 1986). In traditional medicine, leaves of *C. sativa* are well known for their bitter, intoxicating, tonic, analgesic and aphrodisiac properties (Isahq et al., 2015). They are also used to treat hysteria, insomnia, diarrhea, abdominal disorders and skin diseases. Hundreds of biologically active substances (*e.g.* cannabinoids) have been extracted from this plant and some recent research works

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demonstrated that they have several pharmacological properties, such as antimicrobial, insecticidal and anti-inflammatory activities (Appendino et al., 2008; Benelli et al., 2018; Nissen et al., 2010). As far as our literature survey could ascertain, no reports are published concerning the possible synergistic interaction between standard antimicrobial drugs and *C. sativa* EO. In this context, and in continuation to our research work to discover new interesting synergistic effect of many Moroccan EOs with several conventional antibiotics, the purpose of the present study was to characterize the EO of Moroccan *C. sativa*, to evaluate its antioxidant and antimicrobial properties, and to evaluate for the first time the possible synergistic combination of this EO with two conventional antibiotics (ciprofloxacin and fluconazol) against some resistant bacteria and clinic pathogenic yeasts.

2. Material and methods

2.1. Plant material and chemical characterization of EO

Aerial parts of *Cannabis sativa* were collected from Rif region (34° 54′ 57″ N, 4° 34′ 07″ W) in northern Morocco. The voucher specimen (CASA-39) has been deposited at the Laboratory of Biology and Biotechnology of Microorganisms, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco. The air-dried aerial parts were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus and the essential oil obtained was stored in darkness at 4 °C. The calculated yield was 2.7% (v/w), based on dry plant material. Gas chromatographic coupled to mass spectrometric (GC–MS) analysis was performed on a 1300 GAZ gas chromatograph equipped with a TG-5MS column (30 m length; 0.25 mm i.d.; 0.25 μm film thickness) and coupled to mass selective detector "ISQ Single Quadrupole Mass spectrometer" (70 eV). The analytical conditions were: carrier gas, helium; injection volume was 1 μL; injector temperature 260 °C, temperature program was 1 min at 100 °C ramped from 100 to 260 °C at 4 °C/min and 10 min at 246 °C. Components were identified based on their retention indices (RI), determined with respect to a homologous series of *n*-alkanes (C_9-C_{24}), and comparing their mass spectra with reference libraries (Adams, 2007).

2.2. Antimicrobial activity

2.2.1. Microorganism strains

Antimicrobial activity of the EO was tested against six pathogenic bacteria: *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (DSM 50090), clinically isolated *Klebsiella pneumoniae*, *Bacillus subtilis* (ATCC 9524), *Micrococcus luteus* (ATCC 10240), and *Staphylococcus aureus* (CCMM B3), and four pathogenic clinically isolated *Candida* strains provided by the Moroccan coordinated collection of microorganisms : *Candida albicans* CCMM-L4 and *Candida glabrata* CCMM-L7 (from vaginal sampling), *Candida krusei* CCMM-L10 (from human blood) and *Candida parapsilosis* CCMM-L18 (human skin) (Nafis et al., 2018).

2.2.2. Antimicrobial screening

The antibacterial and antifungal activities of *C. sativa* EO were determined using the disk diffusion agar method as previously described by the National Committee for Clinical Laboratory Standards (NCCLS) (1997) Petri dishes containing 20 m L of Mueller Hinton (MH) and Sabouraud agar media were seeded with an inoculum of bacteria and yeasts, respectively. Sterile disks (Whatman, 6 mm in diameter) were loaded with 10 μL of *C. sativa* EO, and placed on the surface of the inoculated Petri dishes. The activity was determined by measuring the inhibition zone diameters (in mm). Discs of fluconazol (10 μg) and ciprofloxacin (5 μg) were used as positive controls.

2.2.3. Determination of minimal inhibitory concentration and synergistic interaction

The minimum inhibitory concentration (MIC) of *C. sativa* EO and antibiotics (ciprofloxacin and fluconazol) was performed by the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines M07-A10 for bacteria Clinical and Laboratory Standards Institute (CLSI), 2015 and M27-A3 for yeasts (Clinical and Laboratory Standards Institute (CLSI), 2008). The EO and antibiotics were analyzed using two-fold dilution series prepared in dimethyl sulphoxide (DMSO) (4%). Each microwell was contained 100 μ L of oil dilution and 100 μ L of cell suspension prepared by dilution (1/100) an overnight culture in MHB and Sabouraud media for bacteria (10^6 CFU/m L) and yeasts (1–2 × 10^3 cells/m L), respectively.

The synergistic interactions between *C. sativa* EO and conventional antibiotics (ciprofloxacin, fluconazol) were determined as described by checkerboard assay method (Fadli et al., 2014). The combination of EO (CMI/4) and antibiotics was studied using the microdilution assay. Aliquots (50 μ L) of *C. sativa* EO were added separately to microwells containing 50 μ L of antibiotic dilutions, and inoculated with 100 μ L of cell suspension. Then, the 96-microwell plates were incubated at 37 °C and 28 °C for bacteria and yeasts, respectively. The fraction inhibitory concentration index (FICI) and the gain was calculated by the method reported by Didry et al. (1993).

2.3. Antioxidant activity of C. sativa EO

To determine the antioxidant activity of *C. sativa* EO, we used the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Şahin et al. (2004). The reductive potential was determined by the Fe^{+3} to Fe^{+2} transformations in the presence of the oil, using the method of Gülçin et al. (2010). The essential oil ability to prevent bleaching of β-carotene, by its oxidation in the presence of O_2 molecule, was performed according to Miraliakbari and Shahidi method (Miraliakbari and Shahidi, 2008).

3. Results and discussion

3.1. Chemical composition determination

The quantitative and qualitative composition of *C. sativa* EO analyzed by means of GC–MS are shown in Table 1. The total number of chemical constituents identified in *C. sativa* EO was 24, representing 97.1% of the total oil content. Sesquiterpene hydrocarbons (57.1%) constituted the principal compound class, followed by oxygenated monoterpenes (16.0%) and oxygenated sesquiterpenes (13.2%). The predominant components in *C. sativa* EOs were (E)-caryophyllene (35.0%), α-humulene (12.8%) and caryophyllene oxide (10.6%). Many investigations have reported the chemical composition of *C. satica* EOs. The compared studies revealed that the chemical profile of our Moroccan EO is quite different of what has been previously reported in other provenances of *C. sativa* EOs. Benelli et al. (2018) reported that the major volatile constituents of *C. sativa* EO from fresh industrial hemp inflorescences were (*E*)-caryophyllene (23.8%), α-pinene (16.4%) and myrcene (14.2%). Bertoli et al. (2010) demonstrated that oil extracted from the inflorescences of *C. sativa* cultivated in Italy was dominated essentially by α-pinene (20.3–20.4%), (*E*)-caryophyllene (19.4–19.5%), terpinolene (15.0–19.1%) and myrcene (12.3–13.6%). α-pinene (7.21–14.61%), myrcene (21.08–35.02%), (*E*)-β-ocimene (7.33–9.04%), α-terpinolene (7.02–16.61%), (*E*)-caryophyllene (12.19–18.93%), and α-humulene (6.10–8.71%) were found as the predominant compounds in five cultivars of *C. sativa* cultivated in Austria (Novak et al., 2001). From these data, it appears that the volatile oil composition of *C. sativa* presented a large intra-specific variability, which seems to be linked to the conditions under which the plants were grown. In fact, many extrinsic (such as, geographical origin, collection time, plant age, soil composition…*etc.*) and/or intrinsic factors (genetic characteristics) can affects the oil composition (Elhidar et al., 2019; Hajdari et al., 2016).

Table 1
Chemical composition of volatile constituents extracted from leaves of *C. sativa*.

RT	RI	Compounds	Relative Concentration (%)
2.78	980	α-Pinene	1.2
3.17	990	Myrcene	4.5
3.29	994	Mesitylene	1.0
3.35	1028	β-Pinene	1.2
3.67	1030	Limonene	1.9
3.74	1069	1,8-Cineole	1.3
4.53	1090	Linalool	3.0
4.92	1120	Fenchol	1.7
5.31	1140	Ipsdienol	1.0
5.5	1153	(E,E)-2,6-Dimethyl-3,5,7-octatriene-2-ol	1.2
5.87	1169	Borneol	1.1
6.02	1173	Carveol	1.3
6.3	1182	α-Terpineol	2.1
6.64	1226	Coumaran	1.4
7.37	1241	Pulegone	2.9
12.0	1410	(E)-Caryophyllene	35.0
12.31	1412	α-Bergamotene	1.4
12.77	1458	α-Farnesene	2.4
12.9	1460	α-Humulene	12.8
13.11	1465	Aromadendrene	1.7
13.79	1482	β-Selinene	1.7
15.17	1497	cis-α-Bisabolene	2.1
16.42	1589	Caryophyllene oxide	10.6
17.12	1612	Humulene epoxide-II	2.6
		Monoterpene hydrocarbons	10.8
		Oxygenated monoterpenes	16.0
		Sesquiterpene hydrocarbons	57.1
		Oxygenated sesquiterpenes	13.2
		Total (%)	97.1

RT: Retention time, RI: Retention index measured relative to *n*-alkanes (C-9 to C-24) on a non- polar TG-5MS column.

Table 2
Antioxidant activities (IC_{50}) of *C. sativa* essential oil, quercetin and butylated hydroxytoluene (BHT).

Antioxidant tests	EO (mg/m L)	BHT (μg/m L)	Quercetin (μg/m L)
DPPH assay	1.6 ± 0.1	4.2 ± 0.1	1.1 ± 0.0
Reducing power assay	1.8 ± 0.2	7.1 ± 0.1	2.3 ± 0.1
β-Carotene/linoleic acid assay	0.9 ± 0.1	4.3 ± 0.3	0.9 ± 0.0

Table 3
Inhibition zone diameters and MIC of Moroccan *C. sativa* essential oil using the disc diffusion and micro-well dilution assays.

Microorganisms	Essential Oil		Ciprofloxacin		Fluconazol	
	IZ	MIC	IZ	MIC	IZ	MIC
M. luteus	11.4 ± 0.1	4.7	26.3 ± 0.8	0.015	–	–
S. aureus	13.0 ± 0.2	4.7	27.5 ± 0.4	0.031	–	–
B. subtilis	13.0 ± 0.3	1.2	35.1 ± 1.2	0.015	–	–
E. coli	11.1 ± 0.3	1.2	12.0 ± 0.8	0.062	–	–
P. aeruginosa	12.6 ± 0.2	1.2	9.3 ± 0.2	1	–	–
K. pneumoniae	8.5 ± 0.2	37.8	8.2 ± 0.8	0.250	–	–
C. albicans	12.0 ± 0.7	9.5	–	–	20.0 ± 0.5	1
C. glabrata	13.0 ± 0.2	9.5	–	–	13.0 ± 0.0	1
C. krusei	12.5 ± 0.2	9.5	–	–	24.0 ± 0.8	1
C. parapsilosis	15.0 ± 0.3	9.5	–	–	28.2 ± 0.4	1

IZ: Diameter of inhibition zone including disc diameter of 6 mm, by the agar disc diffusion method at a concentration of 10 μL of oil/disc and a concentration of 10 μg/disc and 5 μg/disc for fluconazol and ciprofloxacin, respectively. MIC: minimum inhibitory concentration in mg/mL.

3.2. Antioxidant activity evaluation

The results of the antioxidant activity are presented in the Table 2. The data showed that *C. sativa* EO exhibited interesting antioxidant potency with IC_{50} values of 1.6 ± 0.1 mg/m L for free radical-scavenging activity (DPPH), 1.8 ± 0.2 mg/m L for β-carotene/linoleic acid assay, and 0.9 ± 0.1 mg/m L for reducing power assay. However, these activities were lower than that found for the synthetic antioxidant butylated hydroxytoluene (BHT) with IC_{50} from (4.2 ± 0.1 to 7.1 ± 0.1) μg/m L and quercetin with IC_{50} from (0.9 ± 0.0 to 2.3 ± 0.1) μg/m L. To our knowledge, the results obtained in this study are the first published data concerning the antioxidant activity of *C. sativa* EO. This antioxidant effectiveness of our EO may be attributed primarily to the presence of (*E*)-caryophyllene and caryophyllene oxide in high concentrations. In fact, it has been previously shown that species rich in these compounds possessed appreciable antioxidant activity (Figueiredo et al., 2019; Salleh et al., 2015; Sarikurkcu et al., 2018). It is known that the antioxidant properties of EOs cannot be related to only their major constituents (Bakkali et al., 2008). Other minor components, such as, myrcene, linalool and pulegone could also contribute by synergistic effects to the obtained antioxidative activity (Kasrati et al., 2015; Pereira et al., 2018; Ruberto and Baratta, 2000; Wen-Bing and Zhang, 2016).

3.3. Antimicrobial activity and synergistic effect of EO

The results of the antimicrobial potential of *C. sativa* EO and antibiotics against reference and clinical strains used in the present work are presented in Table 3. The data showed that *C. sativa* EO exhibited a moderate antimicrobial efficiency against all tested strains, with inhibition zone (IZ) diameters ranging from (8.5 ± 0.2 to 15.0 ± 0.3) mm. The MIC values of the EO are in the range of (1.2–37.8) mg/mL. The bacterial strains tested were found to be more sensitive to the effect of EO with MIC values ranging from (1.2 to 4.7) mg/m L, except for the Gram-negative *K. pneumoniae*, which appeared as the most resistant bacteria to the action of *C. sativa* EO (MIC = 37.8 mg/m L). Remarkably, *P. aeruginosa*, one of the well-known resistant Gram-negative bacteria, expressed the same sensitivity to this oil compared with the antibiotic ciprofloxacin (Table 3). *Candida* strains displayed the same sensitivity to *C. sativa* EO, with MIC value of 9.5 mg/m L. The antimicrobial potency observed in our EO is comparable to what has been previously reported for the same species originated from other regions (Calzolari et al., 2017; Nissen et al., 2010; Novak et al., 2001), and highlights the considerable potential of this plant as an antimicrobial agent. This antimicrobial potency observed in our EO may be related to the high content of (*E*)-caryophyllene, α-humulene and caryophyllene oxide, three sesquiterpenes with well documented antimicrobial activity (Ali et al., 2017; C. Matasyoh et al., 2007; Dahham et al., 2015).

According to the results of FICI values (Tables 4 and 5), of the 10 combinations performed between *C. sativa* EO and ciprofloxacin and fluconazol, 8 (80%) showed total synergism and 2 (20%) presented partial synergistic interaction. Interaction of *C. sativa* EO with ciprofloxacin demonstrated total synergistic effect against *S. aureus*, *B. subtilis*, *E. coli* and K. *pneumoniae* (FICI = 0.5), and partial synergistic effect against *S. aureus* and *P. aeruginosa* (FICI = 0.75). The combination of *C. sativa* EO and fluconazol gave great synergistic effect against all yeast strains tested, with FICI values of 0.313. The gains expressed by the MIC of antibiotics in the presence of *C. sativa* EO (MIC/4) have been also determined. *C. sativa* EO has reduced the antibiotics MICs by 2 to 64-fold depending on the tested microorganism. The highest decrease was observed in the case of *B. subtilis* with gain of 64-fold. Interestingly, the EO have greatly reduced the sensitivity of the Gram-negative bacteria *E. coli* to ciprofloxacin, with gain of 32-fold. Concerning the pathogenic yeasts, we found that the combination of our EO and fluconazol gave a gain of 16-fold on all strains used.

Synergistic interactions of EO plant in combination with antibiotics

Table 4
Synergistic interaction between *C. sativa* EO and ciprofloxacin against some resistant bacteria.

	M. luteus			*S. aureus*			*B. subtilis*			*E. coli*			*P. aeruginosa*			*K. pneumoniae*		
	FIC	FICI	Gain	FIC	FICI	Gain	FIC	FICI	Gain	FIC	FICI	Gain	FIC	FICI	Gain	FIC	FICI	Gain
C. sativa EO	0.25	–	–	0.25	–	–	0.25	–	–	0.25	–	–	0.25	–	–	0.25	–	–
Ciprofloxacin	0.5	0.75[b]	2	0.125	0.375[a]	8	0.016	0.266[a]	64	0.031	0.0.281[a]	32	0.5	0.75[b]	2	0.25	0.5[a]	4

FIC of oil = MIC of oil in combination with antibiotic/MIC of oil alone; FIC of antibiotic = MIC of antibiotic in combination with oil/MIC of antibiotic alone; FIC index = FIC of oil + FIC of antibiotic.
[a] Total synergism.
[b] Partial synergism.

Table 5
Synergistic interaction between *C. sativa* EO and fluconazol against some clinical pathogenic yeasts.

	C. albicans			*C. glabrata*			*C. krusei*			*C. parapsilosis*		
	FIC	FICI	Gain	FIC	FICI	Gain	FIC	FICI	Gain	FIC	FICI	Gain
C. sativa EO	0.25	–	–	0.25	–	–	0.25	–	–	0.25	–	–
Fluconazole	0.063	0.313[a]	16	0.063	0.313[a]	16	0.063	0.313[a]	16	0.063	0.313[a]	16

FIC of oil = MIC of oil in combination with antibiotic/MIC of oil alone; FIC of antibiotic = MIC of antibiotic in combination with oil/MIC of antibiotic alone; FIC index = FIC of oil + FIC of antibiotic.
[a] Total synergism.

are one of the novel ways to overcome drug resistance. To our knowledge, we are the first to study the synergistic effects of EO extracted from species of the genus *Cannabis* with standard antibiotics. So, the results from this study cannot be compared to others. However, several research works on the interaction between sesquiterpene rich EOs and known antibiotics indicated a synergistic interaction (Elhidar et al., 2019; Luna-Herrera et al., 2007; Pinto et al., 2017). In fact, *Lantana caatingensis* EO containing a high proportion of sesquiterpenes showed significant synergism with aminoglycoside antibiotics against *S. aureus* (de Aguiar et al., 2015). In the same way, the combination of *Geophila repens* EO with streptomycin against *Escherichia coli* revealed a prominent synergistic effect (Rao et al., 2017). These synergistic effects can be produced by some accepted mechanisms including sequential inhibition of protective enzymes, inhibition of common biochemical pathways and use of membranotropic agents to enhance the diffusion of other antimicrobials (Aleksic and Knezevic, 2014).

4. Conclusion

The obtained results showed that the Moroccan *C. sativa* EO is characterized by the dominance of sesquiterpenes compounds, namely (E)-caryophyllene, α-humulene and caryophyllene oxide. The antioxidant and antimicrobial assays, showed that our EO displayed interesting activity. The data showed also the capacity of *C. sativa* EO to enhance fluconazol and ciprofloxacin actions toward some pathogenic microorganisms. It should be noted that *C. sativa* EO contributed to the decrease of ciprofloxacin MICs of bacterial strains by 2 to 64-fold, and by16-fold regarding fluconazol MICs in *Candida spp*. Given the results described above, *C. sativa* EO may be useful in combination with antibiotics in the clinical management of some infectious diseases. However, further investigations should be undertaken in the future to have a complete view on the toxicity of this oil and the determination of optimal concentrations for clinical applications.

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