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Anticlostridial activity of the dill seed essential oil (*Anetum graveolens* L.): antibiofilm activity and antisporulation potential

Original Article

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Abstract:

Clostridioides difficile is an anaerobic, spore-forming pathogen that causes serious toxin-mediated enteric disease in humans. In addition to antimicrobial resistance, biofilm and spore formation play key roles in the persistence of *C. difficile* in the gut, as well as in the transmission and relapse of the disease. In this study, the antimicrobial potential of dill seed essential oil on the planktonic growth of *C. difficile* clinical strains (isolated from stool specimens of hospitalized patients with diarrhea and confirmed *Clostridioides difficile* infection (CDI)) was investigated, along with its effect on biofilm and spore formation. The results showed varying degrees of antimicrobial activity, ranging from strong to weak, depending on the strain, with concentrations ranging from 0.08 to 40 mg/ml. The essential oil (EO) at concentrations of 2xMIC and MIC significantly reduced biofilm production in 89% and 84% of the tested strains, respectively. Spore formation was also significantly reduced when treated with 0.5xMIC and MIC of EO. Considering the anticlostridial activity of the dill seed EO, along with its inhibition of biofilm production and sporulation, this natural product is an excellent candidate for supplementary treatment of CDI.

Key words:
dill, essential oil, *Clostridioides difficile*, antibiofilm activity, antisporulation activity

Apstrakt:

Antiklostridijska aktivnost etarskog ulja semena mirođije (*Anethum graveolens* L.): antibiofilmska aktivnost i antisporulativni potencijali

Clostridioides difficile je anaerobni sporogeni patogen koji je uzročnik ozbiljnih crevnih, toksinima uzrokovanih, bolesti kod ljudi. Pored antimikrobne rezistencije, formiranje biofilma i spora može igrati ključnu ulogu u perzistenciji *C. difficile* u crevima, kao i u prenosu i recidivu bolesti. U ovoj studiji, ispitivan je antimikrobni potencijal etarskog ulja semena mirođije na rast kliničkih sojeva *C. difficile* (izolovanih iz uzoraka stolice hospitalizovanih pacijenata sa dijarejom i potvrđenom *Clostridioides difficile* infekcijom (CDI)), kao i efekat ulja na formiranje biofilma i spora. Rezultati su pokazali različite stepene antimikrobne aktivnosti, u rasponu od jake do slabe, u zavisnosti od soja, sa koncentracijama u rasponu od 0.08 do 40 mg/ml. Etarsko ulje (EO) u koncentraciji 2xMIC i MIC značajno je smanjilo proizvodnju biofilma kod 89% i 84% testiranih sojeva, respektivno. Formiranje spora je takođe značajno smanjeno, kada je tretirano sa 0.5xMIC i MIC EO. Uzimajući u obzir antiklostridijsku aktivnost EO semena mirođije, inhibiciju proizvodnje biofilma i sporulacije, ovaj prirodni proizvod se klasifikuje kao odličan kandidat za dopunski tretman CDI.

Ključne reči:
mirođija, etarsko ulje, *Clostridioides difficile*, antibiofilmska aktivnost, antisporulaciona aktivnost

Introduction

Clostridioides difficile is an anaerobic spore-forming pathogen that is causative agent of serious nosocomial toxin-mediated enteric disease worldwide. The disease is manifested with a wide range of severity, from mild, self-limiting diarrhea to pseudomembranous colitis and even toxic megacolon

which can be life-threatening and often results in death (Awad et al., 2014). In a healthy individuals, human microbiota working in synergy and thus provides protection from pathogens. *Clostridioides difficile* establish itself in the host when the gut microbiota is disturbed, usually after antibiotic treatment administered for other conditions. The bacterial species produces spores under stress conditions, which are a dormant form of the bacterium. Spores are the infectious particle critical for *C. difficile*-mediated infection and transmission (Awad et al., 2014). After entering the host in the spore form, *C. difficile* passes through the stomach into the small intestine, where it begins the germination process, transitioning into the metabolically active vegetative cell form. Toxin production then occurs, leading to host tissue damage and disease (Awad et al., 2014; Smits et al., 2016; Schäffler & Breitrück, 2018). In addition to antimicrobial therapy, which disrupts the intestinal microbiota and enables the unhindered germination of spores and release of toxins, it has been confirmed that recurrent infections can be unequivocally linked to the production of biofilms in certain isolates of C. *difficile*. Fact that recurrence of CDI is attributed to the strain which initially caused disease (Figueroa et al., 2012), indicates that the pathogen somehow avoids treatment with antibiotic therapy. Biofilm and spore formation may be a key mechanisms of *C. difficile* persistence in the gut as well as transmission and relapse (Frost et al., 2021; Normington et al., 2021). Antimicrobial resistance in biofilm can increase from 10 to 1000 times more compared to planktonic bacteria (Wei et al., 2018). Recent research suggests that *C. difficile* biofilms can serve as a niche for generating modified spores, which favor maintenance of a dormant cells, aiding bacterial persistence and disease recurrence (Frost et al., 2021). Antimicrobial drugs can lead to sporulation and biofilm formation due to unfavorable environment, so it seems that preventing development of these structures in the host intestine could be promising approach in the control of pathogen survival.

To successfully control CDI, in addition to antibiotic treatment, it may be beneficial to consider new strategies and approaches aimed at countering C. difficile's ability to develop resistance to conventional treatments and its subsequent proliferation. Natural products could serve as good source of anticlostridial agents (Phanchana et al., 2021). Bioactive compounds derived from essential oils could provide an opportunity to discover novel potentially effective molecules to combat this problem. The diverse of chemical composition allows essential oils to exhibit multiple mechanisms of action in reducing bacterial cell viability including effect on pH homeostasis and equilibrium of inorganic ions, inhibition of NADH oxidation, and/or structural and functional damage of the cell membrane (Tortajada-Girbés et al., 2021). Therapeutic agents that are capable of reducing *C. difficile* spore production (Mooyottu et al., 2017) and biofilm formation (Normington et al., 2021) could significantly minimize relapse of CDI and consequent transmission.

Anetum graveolens L. (commonly known as dill, family *Apiaceae*), is an aromatic annual plant originated from Mediterranean and West Asia widely used as spice and medicinal herb. Dill is commonly utilized in food and pharmaceutical industries and frequently used to treat various health problems, among other gastrointestinal disorders, flatulence and gastro-intestinal spasms (Chahal et al., 2017; Ozliman et al., 2021). Essential oil can be extracted from various parts of plant such as seeds, leaf and flower. Studies on the chemical composition of dill seed essential oil reported that major compounds were carvone and limonene whereas dill apiole, trans-dihydrocarvone and α-phellandrene were present in appreciable amounts (Chahal et al., 2017). Based on the reviewed published studies on the antimicrobial activity of *A. graveolens* seed essential oil, significant to moderate antibacterial activity was found against both Gram-positive and Gram-negative bacteria, as well as against fungi (Elgayyar et al., 2001). To date, limited literature is available on the use of natural products against *C. difficile* (Hammond & Donkor, 2013; Finegold et al., 2014; Justin & Antony, 2016; Aljarallah, 2016; Cermak et al., 2017; Roshan et al., 2017; Piotrowski et al., 2017; Harnvoravongchai et al., 2018; Wultańska et al., 2020; Aleksić et al., 2022).

The aim of this study was to evaluate whether natural antimicrobial components of the essential oils of well-known and widely used herb *Anetum graveolens* L. (eng. Dill) could influence growth, biofilm formation and sporulation in selected *C. difficile* strains isolated from patients with confirmed CDI.

Materials and Methods

Test microorganisms

In total 42 clinical strains (designated as CD_1 – CD_{42}) of *C. difficile* were isolated from stool specimens of hospitalized patients with diarrhea and CDI on the territory of Serbia. Reference strains, *C. difficile* ATCC 9689 ($A^+B^+CDT^-$), *C. difficile* ATCC 43593 ($A^-B^-CDT^-$) and *C. difficile* ATCC-BAA 1870 ($A^+B^+CDT^+$) were used as control strains. The presence of genes that encode toxins was confirmed by multiplex PCR. Based on capillary

gel-based electrophoresis, 6 different ribotypes were determined. The most prevalent ribotypes were RT 001 (20/42; 48%) and RT 027 (18/42; 43%), while RTs 012, 015, 020 and 205 were represented by only one isolate (2.4%). All the tested isolates are sensitive to metronidazole, vancomycin and tigecycline with MIC_{50} values of 0.25, 0.38, and 0.016 µg/mL, respectively. Among 42 strains tested, 36 showed resistance to moxifloxacin. Two strains (CD4 and CD13) are resistant to rifampicin (with MICs 4 and 32 µg/mL, respectively) in addition to moxifloxacin resistance (Aleksić et al., 2022).

Essential oils

Dill seeds essential oil (*Anetum graveolens* L.) (AlekPharm, Serbia) obtained by steam distillation method was purchased at a local health food market. As stated in the manufacturer's specification, the main components of dill seed essential oil are carvone, limonene and (Z)-dihydro carvone.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentrations (MICs) of essential oil were determined using the broth microdilution method in a 96-well plate according to described methodology (Wultańska et al., 2020), with slight modifications. Suspensions were made from the 24 h old *C. difficile* culture on Columbia 5% Blood Agar and adjusted to 3 McFarland turbidity by using a densitometer (DEN-1, BioSan). An initial stock solution was prepared by dissolving essential oil in 50% dimethyl sulfoxide (DMSO) in concentration of 200 mg/mL. Serial dilutions were made in Brain Heart Infusion (BHI) broth (HiMedia, Mumbai, India), with starting concentration of 40 mg/mL, which was serially diluted (dilution factor 2) to the final concentration of 0.02 mg/mL. Then, BHI broth (180 µL) containing the decreasing concentrations of essential oils was inoculated with suspension of the tested *C. difficile* strains (20 µL, 3 McFarland turbidity, 10-fold dilution of the 3 McFarland in each well) and incubated anaerobically at 37 °C for 48 h. Inoculated BHI broth without essential oil was used as a positive control, while the wells containing sterile BHI broth were used as negative control. The DMSO was tested previously and showed no effect toward the tested strains in used concentrations (10% DMSO and lower concentrations). After incubation, the MICs were determined visually as the lowest concentrations without visible growth in the medium. The minimum bactericidal concentration (MBC) was determined by transferring the suspension from the wells with no visible growth (containing essential oils in MIC and higher concentrations) to 5% Columbia agar plates. Plates inoculated in this way were incubated at 37 °C for 48 h under anaerobic conditions, and after this period the plates with the lowest concentration of oil with no grown colonies were determined as MBCs.

Anti-biofilm production assay

To investigate the potential of the isolated strains to form biofilm, the tested isolates were first subjected to biofilm forming ability crystal violet (CV) assay as previously described (Aleksić et al., 2022). The wells of the microtiter plates containing 180 µl of the BHI broth containing 0.5% yeast extract (Torlak, Serbia) and 1% glucose (Centrohem, Serbia) were inoculated with 20 µl of suspension (3 McFarland) prepared as described for microdilution method, to achieve the final concentration of ~10^6 CFU/ml. The plates were incubated at 37 °C for 72 h under anaerobic conditions afterwards the well content was aspirated, and the wells were washed twice with phosphate saline buffer (pH 7.4). Wells with BHI broth without the inoculums were used as the controls. Then the plates were dried, stained with 1% (w/v) solution of CV for 15 min, washed and destained by addition of 250 µl of ethanol (96%, v/v). Following 30 min of destaining procedure, the obtained solutions were transferred into a new microtiter plate and the absorbance of each well content was measured at 595 nm using an ELISA reader (Multiscan Ascent, Labsystems, Finland). According to their biofilm producing ability, the strains were classified into the following groups: none, weak, moderate, and strong biofilm producers (Stepanović et al., 2007; Tijerina-Rodríguez et al., 2019).

The potential of dill seeds essential oils to inhibit biofilm production of the investigated *C. difficile* strains was done by using the same procedure. The only difference was that the wells contained three different inhibitory concentrations of the essential oil (0.5 x MIC, MIC and 2 x MIC) together with inoculum and cultivation media. The experiment was performed in triplicate and the inhibition was detected as a decreased absorbance at 595 nm compared to positive controls (wells without essential oils). Differences in the biofilm formation were calculated using two-way ANOVA and Tukey post-hoc analysis (GraphPad PRISM v.6.0.) and considered as statistically significant for p values < 0.05.

Spore formation assay

The effect of the dill seed EO on *C. difficile* spore formation was performed using a combination of the previously described protocols (Garneau et al., 2014; Frost et al., 2021) with slight modifications. For the

sporulation test, the reference strains of the most abundant RTs in the tested sample were selected (*C. difficile* ATCC-BAA 1870/A+B+CDT+/RT 027 and *C. difficile* ATCC 9689/A+B+CDT−/RT001). Briefly, BHI with (sub)inhibitory concentration of EO (0.5xMIC and MIC) was inoculated with 3 McFarland suspension that is made from 24 h old culture of *C. difficile* on Columbia 5% Blood Agar (final concentration of ~10^6 CFU/ml) and incubated anaerobically at 37 °C for 10 days. Inoculated wells without addition of EO were used as the controls. A sample (15 µL) from each well of liquid culture was placed to microscope slide, dried at room temperature, fixed by heat and stained according to Schaeffer-Fulton method (Hussey & Zayaits, 2016). For the presence of endospores and their quantification, slides were examined using a Leica DM 1000 light microscope under the oil immersion lens (1,000X). Images from 3 different fields were taken on each slide and were processed with ImageJ program (Schneider et al., 2012). Vegetative cells and spores were counted in 3 fields for each dilution. The percentage of spores in each image was calculated as follows: $[N_{spores} / (N_{spores} + N_{vegetative\ cells})] *100$. An experiment was performed in triplicate. The data were analyzed using one-way ANOVA. Differences between means were considered to be significant at $p<0.05$.

Results and discussion

Clostridioides difficile infection (CDI) is one of the biggest concerns in current world medical practice. The infections caused by these bacteria are treated primarily with vancomycin, metronidazole, or both, and these drugs have been available for the past 30 years. However, treatment options have significant limitations. Importantly, following the treatment, *C. difficile* infections (CDIs) recur in approximately 25% of individuals treated with either vancomycin or metronidazole, and some patients experience multiple recurrences (Babakhani et al., 2012). Given the limited antimicrobials available for treating CDI and the increase of resistance to these drugs, an antimicrobial agent with strong inhibitory effects on both *C. difficile* vegetative cells and sporulation seems like a promising approach. This type of antimicrobial agent would be capable to not only treat CDI but also prevent its recurrence (Sholeh et al., 2020; Chiu et al., 2021). Alternative therapeutic agents that can attenuate *C. difficile* virulence without disrupting the normal gut flora represent a viable control approach against the pathogen. As reported by Semenyuk et al. (2014), biofilm cells possess 100-fold greater resistance to the antibiotic metronidazole than do planktonic cells cultured in liquid media. This report suggest that *C. difficile* cells and spores in biofilms have specialized properties that may facilitate infection (Semenyuk et al., 2014). In this context, the bioactive components of essential oils could serve as beneficial and potentially effective alternatives to combat this problem. In recent years, there has been a growing interest in the use of natural products as an alternative in the treatment of numerous infections, primarily due to the belief that they are safe and widely available at low prices. Humans have been using natural products as traditional medicine for treating illnesses for centuries. Several plant extracts and plant-derived compounds possess antibacterial activity against *C. difficile* and their action has been investigated (Phanchana et al., 2021). Processed products, aloe vera gel, peppermint oil, artichoke capsules, and garlic tablets demonstrated antimicrobial activity against *C. difficile* (Roshan et al., 2017). Plant-derived compounds such as allicin (derived from garlic), cinnamon powder, zingerone (derived from ginger), menthol (derived from peppermint), trans-cinnamaldehyde (cinnamon bark), δ-3-Carene (monoterpene derived from the root of *Asarum heterotropoides*) and some essential oils exhibited a certain anticlostridial potential (Phanchana et al., 2021).

Numerous essential oils (EOs) possess antimicrobial activity, but despite their multipurpose and widespread use, only a small fraction is used commercially. The antimicrobial activity of EOs can be attributed to their composition, volume and interactions with pathogens. The essential oils affect one or more targets within the pathogen, which is related to different active compounds present in their composition. In most cases, two to three primary constituents of EO account for ≤85% of the biological activity of the oil (El-Tarabily et al., 2021), but minor compounds can also be important due to synergistic interaction with the dominant ones (Pejčić et al., 2021).

Based on the data presented in **Tab. 1**, it can be concluded that the tested oil exhibited varying degrees of antimicrobial activity, ranging from strong to weak, depending on the strain, with concentrations ranging from 0.08 to 40 mg/ml. Bactericidal activity spanned from 0.08 to complete inactivity. Notably, considering the characteristics of the tested strains, as previously described (Aleksić et al., 2022), it appears that strong biofilm producers exhibited greater resistance to the tested agent.

The antimicrobial tolerance of biofilms has emerged as a significant challenge to medical scientists in various healthcare sectors. Most recurrent episodes of CDI are attributed to the original strain/ribotype (Figueroa et al., 2012), suggesting

Table 1. MICs and MBCs of the dill EO

Strain	MIC	MBC	Strain	MIC	MBC
[1]**CD+**	5.00	5.00	**CD21**	0.63	0.63
[2]**CD-**	2.50	2.50	**CD22**	5.00	5.00
[3]**CD027**	1.25	2.50	**CD23**	5.00	10.00
CD1	40.00	>40.00	**CD24**	0.63	1.25
CD2	40.00	>40.00	**CD25**	5.00	5.00
CD3	40.00	>40.00	**CD26**	5.00	10.00
CD4	40.00	>40.00	**CD27**	2.50	2.50
CD5	20.00	20.00	**CD28**	1.25	1.25
CD6	20.00	20.00	**CD29**	1.25	2.50
CD7	40.00	>40.00	**CD30**	2.50	2.50
CD8	10.00	10.00	**CD31**	0.08	0.08
CD9	40.00	>40.00	**CD32**	1.25	1.25
CD10	40.00	>40.00	**CD33**	0.63	2.50
CD11	20.00	20.00	**CD34**	0.32	0.32
CD12	10.00	10.00	**CD35**	2.50	5.00
CD13	20.00	20.00	**CD36**	0.63	1.25
CD14	5.00	10.00	**CD37**	2.50	5.00
CD15	5.00	10.00	**CD38**	1.25	5.00
CD16	2.50	5.00	**CD39**	2.50	2.50
CD17	20.00	20.00	**CD40**	2.50	5.00
CD18	10.00	10.00	**CD41**	1.25	2.50
CD19	5.00	5.00	**CD42**	2.50	5.00
CD20	2.50	5.00			

that *C. difficile* can evade antibiotic treatment and the host's defense system, possibly by occupying a protective niche in the gut where antibiotic therapy is ineffective (Normington et al., 2021).

Within the biofilm, *C. difficile* cells undergo metabolic remodeling compared to planktonic cells and have a different array of proteins/organelles on the cell surface (Poquet et al., 2018). These facts indicate that production of an increased, dense biofilm could have an important clinical relevance in the treatment failure and recurrence of CDI (Vuotto et al., 2016).

The formation and development of biofilms is a complicated process that involves different stages which can be the target of natural antibiofilm agents for the prevention of biofilm development (Mishra et al., 2020). Effects of the tested EO on biofilm formation of *C. difficile* strains are presented in **Fig. 1**. Among the thirty-seven tested strains, thirty-four exhibited reduced biofilm production when treated with 2xMIC EO. The minimum inhibitory concentration (MIC) of EO effectively inhibited biofilm formation in thirty-two out of the thirty-seven tested isolates. Subinhibitory (0.5xMIC) concentrations enhanced biofilm formation in 7 tested strains. It is not an unexpected phenomenon, considering that antibiotic resistance in biofilms can increase from 10 to 1000 times compared to planktonic bacteria (Wei et al., 2018). In the study of Vuotto et al. (2016), when sub-inhibitory concentrations of metronidazole were applied, an increased biofilm production among metronidazole-susceptible *C. difficile* isolates has been reported (Vuotto et al., 2015). This situation could be clinically relevant when there is exposure to low doses of antibiotics, as is the case at the beginning or end of antibiotic therapy, which could possibly explain ineffective treatment (Petrof et al., 2013; Normington et al., 2021). Strains CD3 and CD11 (both strong biofilm producers), showed enhanced biofilm production in all three tested EO concentration.

Considering significance of spore formation in occurrence, course and prognosis of the CDI, effect of EO on sporulation in two reference strains were

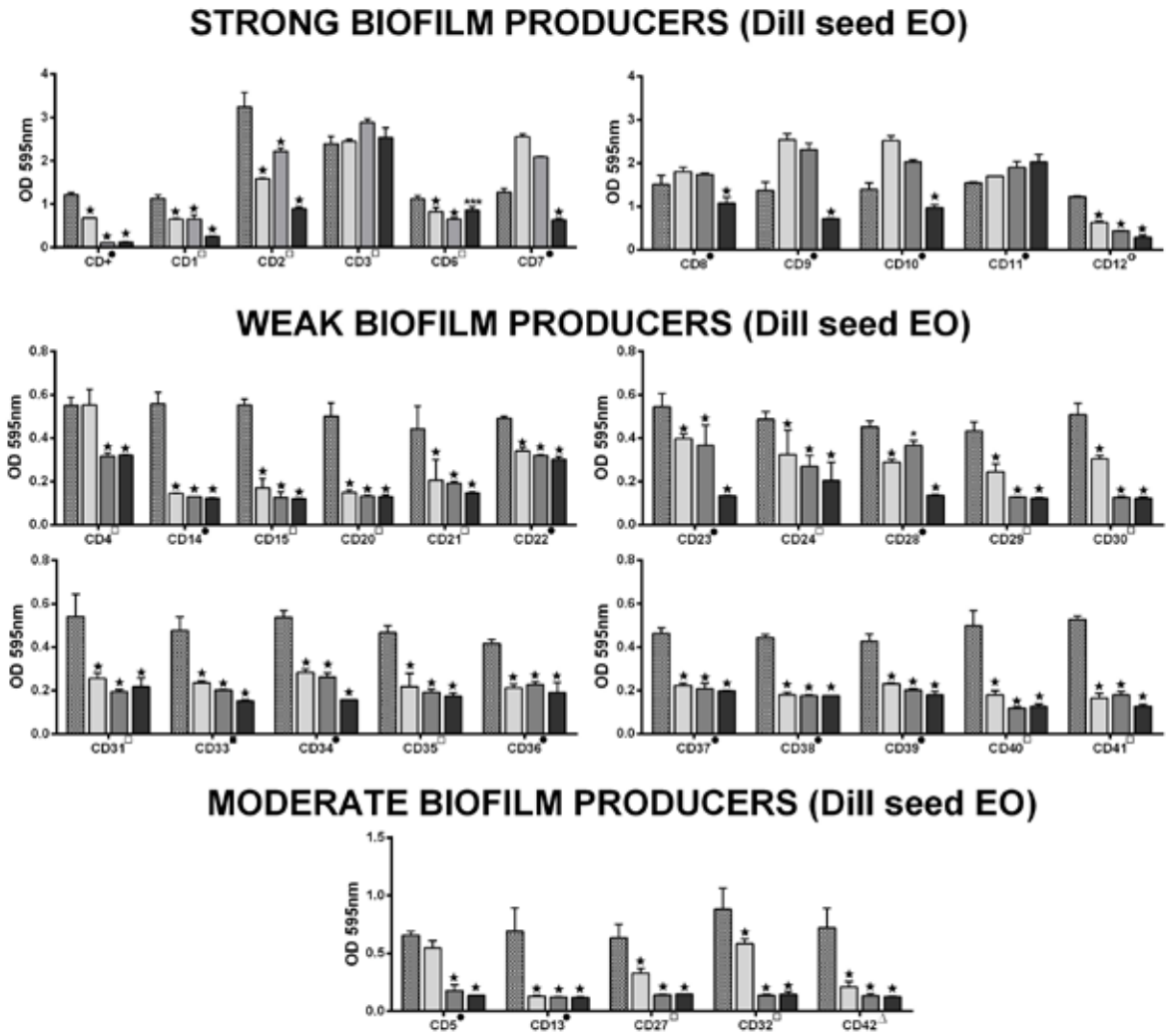

Fig. 1. Biofilm formation by the tested *C. difficile* strains treated with 0.5xMIC, MIC and 2xMIC of dill seed essential oil for the three defined categories of biofilm formed (strong, weak and moderate). The bars show the average values from the three measurements. The error bars show the standard deviations. An asterisk shows a statistically significant differences (**p*< 0.05; ***p*< 0.01; ****p*< 0.001; *p*< 0.0001) in the average OD_{595} values when the same strain was grown in presence or absence of the essential oils; *p*<005)

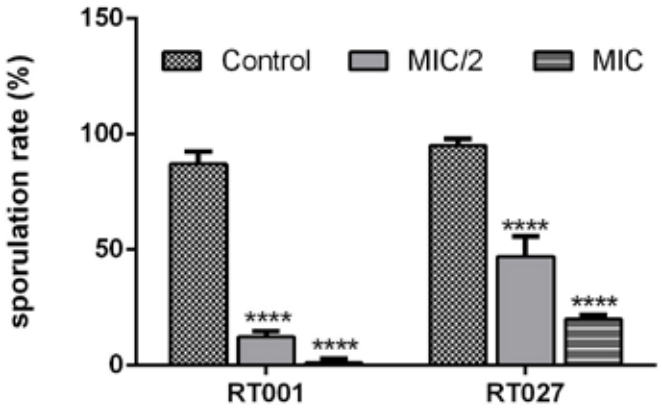

Fig. 2. Effect of dill seed EO on spore production

investigated. The test included two concentrations, reference strains belonging to RT001 and RT027 and prolonged incubation (10 days).

In both strains, spore formation was significantly reduced, when treated with 0.5xMIC and MIC, as well (**Fig. 2**). Better activity is exhibited against RT001 strain with more than 70% of reduction when treated with 0.5xMIC, while MIC concentration reduced the spore number to only 1% of the spore production exhibited by untreated control. Up to now, only one study reported the effects of natural products on sporulation of *C. difficile* (Roshan et al., 2018). This research tested 22 natural products and among them, only three (coconut oil, fresh onion bulb extract and fresh ginger rhizome extract) demonstrated inhibitory effects in concentrations of 6.3% (v/v), 8% and 25%, respectively. The inhibition was up to 86% for the fresh onion bulb extract, which is close to our reduction here obtained for 0.5xMIC of the tested oil.

Conclusion

Clostridioides difficile infection is one of the biggest concerns in current world's medical practice. Along with the appearance of antibiotic resistance, the choice of treatment is complicated by the increase in the disease recurrence rate. The emergence of recurrent CDI could be a consequence of properties of the infectious agent related with biofilm and spores formation, together with antimicrobial resistance. Considering exhibited anticlostridial activity and especially observed antibiofilm action and sporulation-reducing potential of the dill seed EO, it can be regarded as an excellent candidate for supplementary treatment of CDI. Furthermore, natural extracts and natural product-based agents possess fewer side effects due to their low toxicity levels and to date, no bacterial resistance to this type of antimicrobial agents has been recorded. This is an additional reason to expand research on this subject, pinpoint the active compounds, investigate their efficacy in combination with antibiotics and then, as the final step, examine their effectiveness *in vivo.*

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