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Antimicrobial Potential of Herbal Products Against *Clostridioides difficile*

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Abstract

Clostridioides difficile infection (CDI) is a major cause of hospital-acquired diarrhea, with significant morbidity and mortality. In the past decade, the incidence rates of community-acquired CDI are increasing worldwide. Antibiotic therapy which alters the normal colonic microbiota is the greatest risk factor for the development of these infections. The number of effective antibiotics for the treatment of CDI is limited, so it is necessary to explore alternative approaches in therapy. One of the approaches to solving this medical and public health problem is the use of plants and their products.

The aim of the present work is to explore the antimicrobial activity of herbal products against *C. difficile* in order to point out which herbal products are the most promising for the treatment and prevention of CDI.

We searched JSTOR, PubMed, ScienceDirect, Scopus, Springer Link and Web of Science databases for articles identifying herbal products targets or antimicrobial treatments for *C. difficile*. While searching certain keywords were used to find the publications that would be relevant in this review.

The study indicates that several essential oils, plant extracts, and their pure compounds exhibit a great antimicrobial potential against *C. difficile*, suggesting their possible use as an alternative to antibiotics or in combination with conventional antibiotics for the treatment of CDI.

Keywords: essential oils; plant extracts; pure component; *Clostridioides difficile*; antimicrobial activity

Abbreviations

CDI: *Clostridioides difficile* infection, AMR: antimicrobial resistance, MIC: minimal inhibitory concentration, PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses, EO: essential oil, SIC: sub-inhibitory concentration, IC50: half-maximal inhibitory concentration.

Introduction

Despite humanity's best efforts to contain bacterial infections inadequate use (most often overuse) of antibiotics and other therapeutics has put different bacterial species under immense pressure. As a consequence, more and more different resistant types are emerging, a phenomenon called antimicrobial resistance (AMR) [1]. There are many reasons for this occurrence. First of all, antibiotic overuse, both in medicine as well as agriculture, drives bacteria to quickly adapt. Inappropriate prescribing (using antibiotics to treat unsevere viral infections for example) as well as inappropriate usage (either shorter or longer than prescribed) also accelerate this event [2]. The ability of horizontal gene transfer enables bacteria to attain resistance in a very short period, thus, a large reservoir for infection may form [3, 4]. Hospitals and medical equipment (if not maintained properly) are optimal locations for such events to occur, not only because they are suitable for bacterial growth, but also because they are perfect places for bacteria to develop resistance - simply due to them being constantly exposed to major quantities of antibiotics. Another important factor is the patients themselves, who serve both as recipients and as conductors. Hospital personnel are exposed to the bacteria, and the patients are even more susceptible to infection because they are often immunodeficient – due to another illness or due to their age (very young or old). *Clostridioides difficile* (formerly: *Clostridium difficile*) [5] – a gram-positive, anaerobic bacteria that causes intestinal infections, diarrhea, and other clinical symptoms that may often lead to severe disease (CDI) [6] is considered one such threat because the number of its resistant types has increased over the last ten years. Even without the acquired resistance, *C. difficile* is well equipped to survive due to its ability to form spores that can be dormant in harsh environments and outside hosts. Once inside a host, the spores become active forms, causing infection. Even if the initial infection is suppressed a number of spores may survive, enabling the bacterium to cause a chronic or recurrent infection at a later stage. Another problem is the presence of toxins that are being produced, specifically toxin A and toxin B (TcdA, TcdB), that cause intestinal damage and also suppress commensal bacteria in the gut [7]. As an alternative to antibiotics, scientists all over the world are considering natural products such as plants and their compounds as possible novel therapeutics. Herbal medicine has been traditionally used to treat human diseases for centuries. Plants are an inexhaustible source of bioactive substances. As there are approximately 500,000 plant species exist on the planet, of which only 1% has been phytochemically investigated [8]. Among medicines prescribed in the USA, 25% is based on compounds isolated from plants [9]. Therefore, plants represent a great source of novel bioactive compounds. The first lines of treatment for CDI include antibiotics metronidazole and vancomycin [10]. The number of CDI cases is increasing globally due to the emergence of resistant strains to antibiotics used in clinical practice. Therefore, the investigation of alternative therapies for treating CDI such as herbal medicines is the focus of contemporary research. Certain plants show great promise in reducing or eliminating the pathogen through different mechanisms. Another seemingly viable approach is to use plant products together with antibiotics - such a therapy makes sense in the way that it puts the bacterium under even greater pressure in a way that it cannot evade all mechanisms of action that these antimicrobial substances provide. The aim of the present work is to explore the antimicrobial activity of herbal products against *C. difficile* in order to point out which herbal products are the most promising for the treatment and prevention of CDI.

Materials and Methods

Literature Search

The reporting of this systematic review was guided by the standards of the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) Statement [11, 12]. After the selection process of all publications had been done, it then had to be summarized. The summary of the selection process is presented in Figure 1. As was already mentioned, the methodological quality of the included systematic reviews is being assessed with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) [11].

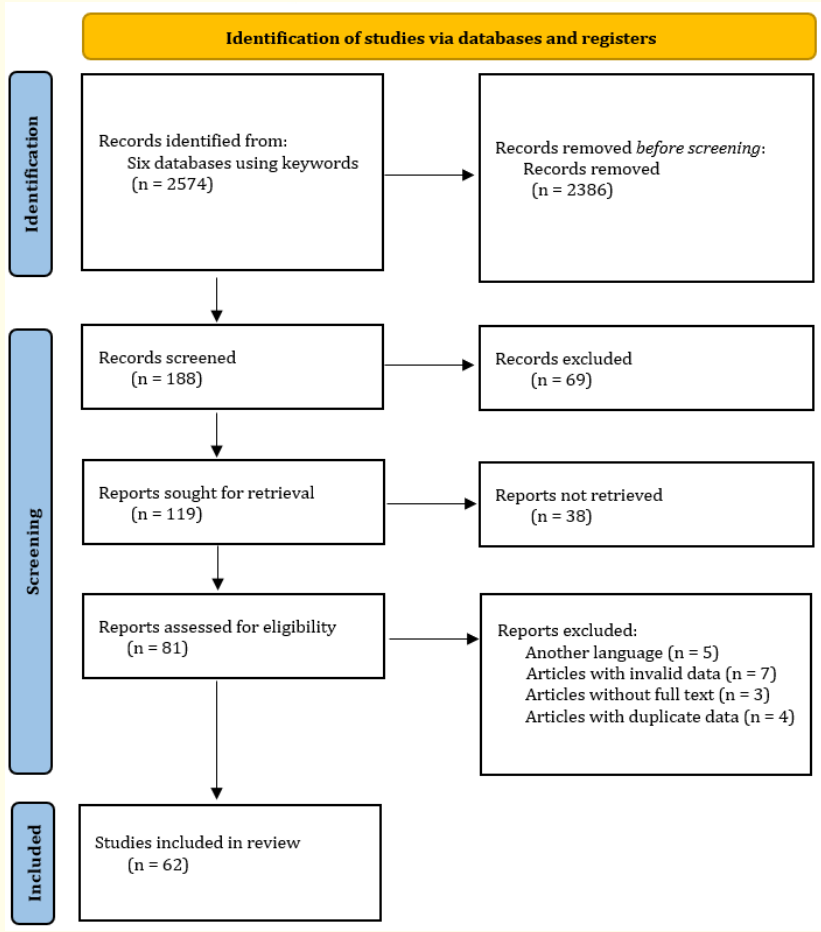

Figure 1: Flow diagram of the selection process.

Search strategy

Six bibliographic databases were searched (JSTOR, PubMed, Science Direct, Scopus, Springer Link and Web of Science) for relevant articles using the following keywords: "*Clostridium difficile*" or "*Clostridioides difficile*", "herbal" or "plant" or "natural products", "essential oils", "extracts", "antimicrobial" or "antimicrobial activity" or "antibacterial" or "antibacterial activity", "inhibition", "effects", "mechanisms of action" and "resistance". No limitations while searching the databases were applied. However, in order to consider a study, its abstract had to be written in English. After a detailed review and verifications of saved articles, duplicates were removed. Another round of searching for adequate articles was conducted and the missing published articles that were not identified in the first round were added.

Inclusion and exclusion criteria

Three reviewers independently screened article titles, abstracts, and, in some cases, the full text of the articles. All discrepancies were resolved by discussion between the three reviewers. The first step was to remove duplicate articles. In the next step the remaining articles were assessed for exclusion based on the next criteria:

1. no originality and relevance,
2. articles written in language that is not understandable,
3. duplicate articles,
4. invalid data,
5. articles without full text and only conference abstracts
6. articles without access.

After excluding a larger number of articles, the remaining ones were further proofread to see whether or not they fulfilled the set criteria.

Data extraction

In order to systemize the data, the following items were extracted from all included studies: 1) last name of the first author; 2) year of study; 3) year published; 4) country; 5) number of tested herbal extracts; 6) type of herbal extracts (e.g. essential oil, secondary metabolite, raw compound, etc.) 7) minimal inhibitory concentration (MIC) values; 8) zone of inhibition. All extracted data are visible in Table 1 and Table 2 in the Result section.

Results and Discussion

Different natural herbal raw materials as well as their processed products have demonstrated antimicrobial potential against *C. difficile*. Some of them are vegetable plants that people use in their diet, while others are used as health supplements. In this study, essential oils, plant extracts, and their bioactive compounds are being considered as possible anti-*C. difficile* agents.

The golden standard for investigating the antimicrobial activity of herbal products is the determination of MIC (Minimal Inhibitory Concentration) using the broth microdilution method. MIC represents the lowest concentration of an antimicrobial substance that inhibits the visible growth of a tested microorganism. Table 1 summarizes the MIC values for the investigated essential oils and plant extracts.

Family	***Plant species (Latin name)***	***Part used***	***Form of usage (unit)***	***MIC***	***Source***
Amaranthaceae	Epazote (*Dysphania ambrosioides*)	Leaves	Ethanol extract (mg/mL)	3.9	[13]
Amaryllidaceae	Garlic (*Allium sativum*)	Bulbs	Juice (% v/v)	0.4 - 0.8	[14]
			Powder (mg/mL)	4.7	[15]
		Clove	Powder (mg/mL)	9.4	[15]
		Bulbs	Tablet (mg/mL) *	37.5 - 75	[15]
		not specified	EO (mg/mL)	0.02 - 40	[16]
	Onion (*Allium cepa*)	Bulbs	Juice (% v/v)	>50	[15]
Apiaceae	Cuminum (*Cuminum cyminum*)	Aerial parts	EO (% v/v)	3.12-6.24	[17]
	Prickly parsnip (*Echinophora spinosa*)	Aerial parts	EO (% v/v)	0.25	[18]
		Ripe fruits	EO (% v/v)	0.13	[18]
	Garden angelica (*Angelica archangelica*)	Root	E0 (% v/v)	0.25	[19]

Arecaceae	Coconout (*Cocos nucifera*)	Fruit	Oil capsule (% v/v)*	>32	[15]
Aristolochiaceae	Barraztm (*Aristolochia paucinervis*)	Rhizome and leaves	Methanolic extract (µg/mL)	8-64	[20]
	Wild ginger (*Asarum heterotropoides*)	Root	Methanol extract (mg/mL)	14.70	[21]
Asphodelaceae	Aloe Vera (*Aloe vera*)	Clear gel	Gel (% v/v) *	16	[15]
Asteraceae	Artichoke (*Cynara cardunculus*)	not specified	Tablet (mg/mL) *	>150	[15]
	Ajenjo or warmwood (*Artemisia absinthium*)	Aerial	Ethanol extract (mg/mL)	0.48	[13]
	Estafiate or Louisiana wormwood (*Artemisia ludoviciana*)	Leaves	Ethanol extract (mg/mL)	0.48	[13]
Clusiaceae	African Mammee Apple (*Mammea africana*)	Bark	Crude methanol extract (µg/mL)		[22]
Cupressaceae	Cade juniper (*Juniperus oxycedrus* ssp. *macrocarpa*)	Aerial part	EO (% v/v)	>32	[23]
	Juniper (*Juniperus communis*)	Aerial part	EO (% v/v)	3.12 - 6.24	[17]
Fabaceae	Psoraleae Fructus (*Cullen corylifolium*)	Seeds	Phenolic extract (*µg*/mL)	8	[24]
Lamiaceae	Lavender (*Lavandula angustifolia*)	Aerial parts	EO (% v/v)	1.56 - 3.12	[17]
	Mint (*Mentha piperita*)	Leaves	Ethanol extract (mg/mL)	0.46	[13]
	Wild oregano (*Origanum minutiflorum*)	not specified	EO (mg/mL)	0.02 – 1.25	[16]
	Peppermint oil (*Mentha sp.*)	Aerial parts	EO (% v/v)	4-8	[14]
	Rosemary (*Rosmarinus officinalis*)	Leaves	Ethanol extract (mg/mL)	0.13	[13]
	Thyme (*Thymus vulgaris*)	Leaves	Ethanol extract (mg/mL)	0.49	[13]
Lauraceae	Cinnamon (*Cinnamomum zeylonicum*)	Bark	EO (% v/v)	0.39 - 1.56	[17]
		Bark	Powder (mg/mL)	75	[17]
		Root	Powder (mg/mL)	>150	[15]
Lythraceae	Pomegranate (*Punica granatum*)	Fruit	Juice (µg/mL)	390	[25]
		Fruit	Pomegranate extract (mg/mL)	12.5-25	[26]
Myristicaceae	Nutmeg (*Myristica fragrans*)	Fruit	EO (µL/mL)	2.5 - 10	[27]

Myrtaceae	Clove (*Syzygium aromaticum*)	Flower buds	EO (µL/mL)	1.25 – 2.5	[27]
	Tea tree (*Melaleuca alternifolia*)	Aerial parts	EO (% v/v)	0.39 - 1.56	[17]
Oleaceae	Jasmine (*Jasminum officinale*)	Aerial parts	EO (% v/v)	3.12 - 12.5	[17]
Piperaceae	Black pepper (*Piper nigrum*)	not specified	EO (mg/mL)	0.04 - 40	[16]
Rutaceae	BIOCITRO (*Citrus paradisi, C. reticulata blanco, C. aurantium* ssp. *bargamia, C. sinensis*)	Fruit	Extract powder (µg/mL)	16 - 32	[28]
	Lemon (*Citrus limonum*)	Aerial parts	EO (% v/v)	6.24	[17]
	Rue (*Ruta graveolens*)	Aerial parts	Ethanol extract (mg/mL)	0.5	[13]
Solanaceae	Toloache (*Datura ferox*)	Leaves	Ethanol extract (mg/mL)	0.85	[13]
Zingiberaceae	Ginger (*Zingiber officinale*)	Rhizome	EO (µL/mL)	2.5 - 10	[27]
			Juice (% v/v)	>50	[17]
			Powder (mg/mL)	>150	[17]
			Tablet (mg/mL) *	>150	[17]
	Turmeric (*Curcuma longa*)	Root	Powder (mg/mL)	>150	[15]
		not specified	Tablet (mg/mL) *	>150	[15]

* *Solvent* – 20% DMSO (dimethyl sulfoxide).

Table 1: MIC (Minimal Inhibitory Concentration) values of essential oils (EOs) and plant extracts against C. difficile.

Disc diffusion and agar well diffusion methods are commonly used for preliminary screening of the antibacterial activities of plant extracts, but these methods cannot be used to determine the MICs of plant extracts. Table 2 shows the zones of inhibition for those herbal products for which the MIC values were not determined.

Family	***Plant species (Latin name)***	***Part used***	***Form of usage (solvent)***	***Inhibition zone (mm)***	***Source***
Burseraxeae	African myrrh (*Commiphora myrrha*)	not specified	Water extract	≥15	[29]
Pinaceae	Cedarwood (*Cedrus atlantica*)	Sawdust or wood chips	EO	9.05 – 10.51	[17]
Ranunculaceae	Black seed (*Nigella sativa*)	Seed	2% oil (Methanol)	≥15	[29]
Zingiberaceae	Ginger (*Zingiber officinale*)	Rhizome	Juice (20% DMSO)	10.5 – 15.3	[15]
			Powder (20% DMSO)	9.65 – 10.9	

Table 2: The inhibition zone diameter of *C. difficile* obtained by the disc diffusion method for several natural products.

According to the literature data, one of the greatest contributions in the field of testing plant derived products against *C. difficile* was given by Roshan and associates [15, 30, 31, 14]. The antimicrobial activity of the different EOs depends on their chemical composition and the bacterial species or strain. The reason why bacteria fail to develop resistance to essential oils lies in the fact that essential oils are a mixture of substances with different mechanisms of action, unlike antibiotics, which contain only one active substance against which bacteria could develop resistance. Based on the results of the research, clove, ginger, and nutmeg essential oils exhibited the strongest activity against *C. difficile*, as shown in Tables 1 and 2 (Table 1, Table 2). Clove EO demonstrated the best antimicrobial activity against *C. difficile* (MICs = 1.25 – 2.5 µL/mL,) followed by ginger and nutmeg EOs with the same MICs range of 2.5-10 µL/mL [27]. The major bioactive compound of clove EO is eugenol (at least 50%), followed by eugenyl acetate, β-caryophyllene, and α-humulene [32]. Nutmeg EO's main bioactive substances are pinene derivates, elemicin, 4-terpineol, myristicin, eugenol, and linalool [33]. The main components of ginger EO are α-zingiberene, β-sesquiphellandrene, ar-curcumene, α-farnesene, β-bisabolene and geranial [34]. Lipophilic properties of essential oils have been suggested to play an important role in antimicrobial activity, which allows them to permeate through the cell membrane and in interaction with polysaccharides, fatty acids, and phospholipids causes loss of cellular membrane integrity, leakage of cellular contents, and interference with proton pump activity, leading to bacterial cell death [32]. Although the antimicrobial mechanism of essential oils has not been completely elucidated [35], it is considered that their constituents such as monoterpenoids, sesquiterpenoids, phenolic compounds, and their derivatives (aldehydes, ketones, alcohols, esters) are responsible for the marked antimicrobial activity of essential oils [36]. The synergistic action of all EO constituents is mainly responsible for their antimicrobial effects. Investigation of the potential application of EOs and pure EO compounds [37] found that essential oils of coriander, curcuma, clove, commercial blend of EO ('Agolin') and chestnut extract inhibited growth of the *C. difficile* at a half-maximal inhibitory concentration (IC_{50}) varying from 41 to 464 p.p.m., while nerolidol, thymol, geraniol, methylisoeugenol, geranylacetate, eugenol and linalool showed an IC_{50} in the ranging from 323 to 563 p.p.m. In a separate experiment [38], in which the fecal suspensions were amended by the addition of a pure culture of *C. difficile*. Numbers of *C. difficile* were suppressed by thymol and methyl isoeugenol at 500 p.p.m. and to a lesser extent with methylisoeugenol at 100 p.p.m. Thymol at 100 p.p.m. had no effect. Eugenol and geraniol gave rather similar suppression of *C. difficile* numbers at both 100 and 500 p.p.m., while nerolidol had no significant effect. Essential oils of wild oregano, black pepper and garlic reduced in vitro biofilm production, with the best activity of oregano oil [16]. Bearing in mind pure EO components, the best anti-clostridial activity was recorded for mammea A/AA and cannabidiol. Mammea A/AA (phenyl coumarin) isolated from *Mammea africana* showed the lowest MIC of 0.25 µg/mL, whose mechanism of action needs to be elucidated [22]. Cannabidiol, the main non-psychoactive ingredient of the cannabis plant, exhibited very strong activity against *C. difficile* (MIC 2 - 4 µg/mL), whose primary mechanism of action is membrane disruption, but also exhibited excellent activity against biofilms, little propensity to induce resistance, and topical *in vivo* efficacy [39]. Asiatic acid derived from *Centella asiatica*, which has been widely used in traditional medicine in Asia, displayed substantial inhibitory effects on *C. difficile* with MICs ranging from 10 to 20 µg/mL [40]. Curcuminoids, the major constituents of turmeric, were very effective with MIC values ranging from 4 to 32 µg/mL, and among them especially curcumin which inhibited 50% of the tested *C. difficile* isolates (MIC_{50}) at a concentration of 16 µg/mL and 90% (MIC_{90}) at 32 µg/mL [41]. Among tested purified hope constituents [42], the strongest anti-*C. difficile* effects were displayed with xanthohumol (MICs = 32 - 107 µg/mL). It is considered that hops-derived compounds affect bacterial cell membrane integrity, interfere with fatty acid metabolism, and lead to an accumulation of protons intracellularly and subsequent cells [43]. The major component of the cinnamon EO, cinnamaldehyde, in a pure state demonstrated a high anti-clostridial effect (MICs = 0.01 - 0.02% v/v) [15, 17]. Cinnamaldehyde, as well as some of its derivatives, have been shown to inhibit the cell division process by binding to FtsZ, lowering the uptake or use of glucose and altering bacterial cell membrane integrity and permeability [44]. Carvacrol and thymol are monoterpenoid phenols present in oregano and thyme oils. Studies [45, 30] demonstrated that the SIC (sub-inhibitory concentrations that do not inhibit bacterial growth) of carvacrol at 0.10 mg/mL and trans-cinnamaldehyde at 0.05 mg/mL were effective in significantly reducing *C. difficile* toxin production. The results of the germination experiments [46] revealed that the MIC of carvacrol (1.2 mM) completely inhibited the spore outgrowth. Also, the SIC of baicalin (700 µg/mL), the active ingredient of Chinese herbal medicine *Scutellaria baicalensis*, significantly reduced toxin synthesis, sporulation and spore outgrowth in *C. difficile* isolates [47]. The wild ginger root constituents, δ 3-carene and methyleugenol, showed the most potent growth inhibition of *C. difficile*, with equal MIC values of 0.70 mg/mL [21]. MICs for pure turpentine ranged from 0.78 - 6.24% v/v [17]. Zingerone and menthol (the active constituents of ginger and peppermint, re-

spectively) were responsible for the antidiarrheal effect [48, 49]. The MIC value of both compounds for *C. difficile* strains was 9.4 mg/mL [15]. Another investigation [30] established that zingerone at 0.3 mg/mL protected the Vero and HT 29 cell lines from damage from *C. difficile* toxins. A study [50] demonstrated the growth inhibition of *C. difficile* mediated by the fatty acids derived from virgin coconut oil, especially lauric acid. Lipolyzed coconut oil at a concentration of 1.2% v/v inhibited 99.9% of *C. difficile* growth and the transmission electron microscopy showed the disruption of both the cell membrane and the bacterial cytoplasm exposed to 2 mg/ml of lauric acid. Further research [51] revealed that lauric acid, exhibited an inhibitory effect on *C. difficile* vegetative cell growth (MIC = 0.08 - 0.16 mg/mL), spore outgrowth, and biofilm formation and that the cytotoxic effect of this fatty acid was mediated via production of reactive oxygen species and plasma membrane damage. In another study [52], lauric acid was shown to have high antimicrobial activity against pathogenic Bacteroides and Clostridium, but low inhibitory activity against commensal lactic acid bacteria, suggesting that lauric acid might modulate intestinal health. Berberine is an isoquinoline alkaloid present in many species from the genera Berberis, Mahonia and Coptis. In Asia, berberine hydrochloride tablets are sold for the treatment of intestinal infectious diseases and diarrhea [53]. The study [54] found that cell growth from *C. difficile* spores was completely inhibited at berberine concentrations of 640 mg/L. Susceptibility testing of 12 *C. difficile* strains [55] has shown that the MIC values of berberine ranged from 256 to 1024 mg/L (median ca. 491.3 mg/L). Mechanisms of berberine action include binding to nucleic acids as well as to the protein FtsZ and blocking cell division [56-58]. Regarding the crude herbal extracts, the phenolic extract of psoralen fructus [24] was the most active against *C. difficile* (MIC = 8 μg/mL), and their components, isobavachalcone and 4′-O methylbavachalcone were very potent compounds (MICs = 4 μg/mL). Another study revealed that rosemary, mint, wormwood, thyme, and methanol extracts demonstrated excellent anti-clostridial activity, with MICs of 0.13, 0.46, 0.48, 0.49, and 0.50% v/c, respectively [13]. Garlic juice at 0.4 - 0.8% v/v [14] was very effective against *C. difficile* strains. The main compound responsible for its antimicrobial activity is an organosulfur compound allicin which in a pure state demonstrated anti-*C. difficile* activity with MICs of 2.3 - 4.7 mg/mL [15]. Allicin reacts with thiol groups and can inactivate essential enzymes of bacteria [59] whose primary target is inhibition of RNA synthesis, whereas DNA and protein syntheses showed delayed and partial inhibition. Black seed 2% methanol extract and myrrh water extract also exhibited excellent anti-clostridial action [29]. Pomegranate extract with MICs of 12.5 - 25 mg/mL The MIC of pomegranate juice, rich in polyphenol punicalagin, was found to be 390 μg/mL for all four *C. difficile* hypervirulent strains [25]. The potential antibacterial mechanisms of puniglacin (ellagitannin) include iron chelation, suppression of cell wall synthesis, and damage to cell membranes [60]. Fresh onion bulb extract (12.5% v/v) significantly decreased toxin production and activity [30]. At the same concentration, onion extract significantly reduced spore production, as well as coconut oil (16% v/v) [31]. Vancomycin is a preferred antibiotic for treating *C. difficile* infection, but approximately 20% of treated patients develop recurrent disease [61]. Examination of the combination of herbal products and antibiotics indicated a possible synergy between trans-cinnamaldehyde and vancomycin and a partial synergy between trans-cinnamaldehyde and metronidazole against *C. difficile* [15]. Additive effects were found between cinnamon essential oil and vancomycin [17]. Studies in a mouse model [61] have suggested that a combination of berberine and vancomycin was more effective than vancomycin alone for treating CDI. Also, an in vitro study [55] observed the synergistic effect of the sub-MIC (1/2 MIC) berberine chloride with vancomycin. Bearing in mind the data presented, we can agree with the statement [62], that natural antimicrobial agents derived from plant sources are an innovative and sustainable option in controlling pathogens. However, it is necessary to investigate in vivo efficiency and toxicological safety of herbal products, as well as their effects on normal intestinal microbiota.

Conclusion

The study indicates that several essential oils, plant extracts, and their pure compounds exhibit a great antimicrobial potential against *C. difficile*, suggesting their possible use as an alternative to antibiotics or in combination with conventional antibiotics for the treatment of CDI. The antibacterial mechanism of plant-derived products is mainly attributed to the disturbing of the cell membrane integrity, which leads to the leakage of cellular contents and ultimately bacterial death. In addition, herbal products show inhibitory activity on fundamental processes: cell division, production and activity of toxins, biofilm formation and sporulation cycle of *C. difficile*, and by doing so reduce its durability. However, additional well-designed future studies are needed to determine the stability, toxicity and safe doses of herbal products.

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