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The evaluation of *Tagetes minuta* as antimicrobial agent against antibiotic resistant *Clostridium difficile* associated with diarrhoea in children under the age of five

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Abstract: Diarrhea infections is a global health problem and regarded amongst the most prevalent cause of mortality in children under five-years, due to their still developing immune system, more especial children from developing countries. These infections spread through contaminated-food, lack of access to safe-drinking-water, poor-hygiene by mothers handling pediatric, bottle instead of breastfeeding, and low-socio-economic status. *Clostridium difficile* is a foodborne pathogen linked to numerous outbreaks in children, causing diarrhea. Antibiotics have traditionally been used to treat such bacterial infections. However, overreliance and misuse of antibiotics led to bacteria developing resistance. Consequently, researchers begun exploring alternative treatments, such as essential oils (EOs), to mitigate the impact of antibiotic-resistant bacteria. The purpose of the study was to investigate the use of *Tagetes minuta* EOs as an alternative antimicrobial against *C.difficile*. *Tagetes minuta* EOs was characterized using, gas-chromatography-mass-spectrometry (GC-MS). Antimicrobial activities of *T. minuta* against *C. difficile* was done using Bioassay and Minimal Inhibitory Concentration (MIC) methods. To analyse structural damages on the *C. difficile* cells after treatment, Scanning Electron Microscopy (SEM) was used. GC.MS results indicated that α-Thujene (71.59%) and 1,8-cineole 3.30c%), were identified as major compounds. Furthermore, these components are known to have antibacterial activities. Bioassay results indicated an inhibition zone of 29 mm, while MIC results of *T. minuta* against *C.difficile* was 12.5 (µg/ml), indicating high inhibition zone. *Tagetes minuta*, was effectives against *C. difficile,* This indicates that *T. minuta* can be used as potential alternative antibacterial agent against *C. difficile* responsible for diarrhoea in children. Furthermore, *T. minuta* is considered safe for humans and animals and are biodegradable compared to the antibiotics.

Keywords: antimicrobial agent, diarrhoea, children, biodegradable

Introduction

Diarrhoea occurs when there is an infection in the gut or intestinal tract, normal as three or more loose stools a day that may last for up to fourteen days. It may be caused by a bacteria, virus, or parasites (Bhattacharya, 2017). Children under the age of five are the most vulnerable groups due to their still developing immune system, more especial children from developing countries (World Health Organization, 2005). as such Diarrhoea is rated as the second leading cause of death after pneumonia in these group, and it is considered a significant health issue globally (World Health Organization, 2005; Liu et al., 2016). It is also known to cause high annual mortality rates among young children worldwide (World Health Organization, 2005; Najim et al., 2019).

Various studies have investigated the prevalence of diarrhoea in these vulnerable group, and it seems highly likely that it spreads through contaminated food, lack of access to clean/healthy drinking water, poor hygiene practices by mothers, bottle instead of breastfeeding, and low socio-economic status (Akinyemi et al., 2018; Cohen et al., 2019; Cohen et al., 2019). Other sources include polluted soil, poor food preparation, and inadequate food and meal storage practices (Kuhn, 2019).

The symptoms can vary from light to severe and can manifest as abdominal colic, nausea, vomiting, dehydration, diarrhoea, and fever (Cohen et al., 2019). Despite many advances in the field of food safety and environmental health, billions of people worldwide are at risk of unsafe food leading to diarrhoea infections (Fung et al., 2018).

According to a 2016/2017 World Health Organisation (2017) survey, unsafe food products that contained harmful bacteria, viruses, parasites, or chemical substances caused over two hundred diseases ranging from diarrhoea to cancers. Diarrhoeal diseases are the most common illnesses resulting from the consumption of contaminated food (World Health Organization, 2017). A data synthesis that was conducted by Kirk et al. (2017) on the global and regional disease burden of 22 foodborne diseases estimated that 580 million such illnesses occurred in 2010, and mostly were related to diarrhoeal. *Clostridium difficile* Bactrial pathogens has been reported to be amongst the leading coause of diarrhoeal illness (Franz et al., 2019).

Clostridium difficile is a member of the *Clostridium* genus which is a large prokaryotic genus encompassing several clinically relevant species (Ramos & Rodloff, 2018). It is gram-positive spore-forming anaerobic bacteria that causes disease in humans and animals (Primavilla et al., 2019). This bacterium has for years been considered a nosocomial human pathogen that usually causes infectious diarrhoea in patients after hospitalisation and antibiotic treatment (Primavilla et al., 2019). *C. difficile* is a common human enteric pathogen (Cimolai, 2019). The pathogens symptoms may vary from mild diarrheal symptoms to fulminant panniculitis, while many associated systemic manifestations - including death - have been reported (Cimolai, 2019). In addition, *C. difficile* is also a major diarrhoea-causing pathogen in people of all ages and poses a significant threat to vulnerable and immunocompromised populations (Plants-Paris et al., 2019).

In 2007, *C. difficile*-causing pseudomembranous colitis contributed to the death of patients five times more often than MRSA (Ramos & Rodloff, 2018). Moreover, patients with *C. difficile* infection who were treated in intensive care units experienced a longer hospital stay and a higher mortality (Ramos & Rodloff, 2018).

The diarrhoea infections are normal treated using antibiotics, their discovery and development in the pharmaceutical industry in the twentieth century have been a most influential medical advancement (Uddin et al., 2021). Antibiotics have been of great benefaction to humans as they combat infections against bacteria and have saved millions of lives. However, with the advent of the twenty-first century, infections caused by multidrug-resistant (MDR) bacteria have been increasing around the globe (Uddin et al., 2021).

Nowadays antibiotic resistance is a universal public health concern as it has implications for the successful treatment of human and animal infections (Mbanga et al., 2021). Due to the escalation of resistance against most categories of antibiotics and increasing challenges to treat infections, the need for new strategies and antimicrobials has intensified (Iseppi et al., 2021). For this reason, the pharmaceutical industry and research communities have shown interest in exploring the biodiversity of the world of plants and their derivatives as they contain important sources of molecules with a potentially broad spectrum of action. For example, essential oils (EOs) extracted from aromatic plants have shown a wide range of antimicrobial activity against pathogens in various studies (Arena et al., 2018; Iseppi et al., 2021).

Essential oils (EOs) are reported to be effective in treating infections caused by antibiotic resistant pathogens. EOs, has been considered because they are safe and limit risk to humans, animals, and the environment (Yulia et al., 2019). Furthermore, EOs are well known as antimicrobial agents that can be used to control food spoilage and foodborne pathogenic bacteria because they have a wide range of active chemical components such as Terpene hydrocarbons (monoterpenes and sesquiterpenes) and oxygenated compounds (phenols, alcohols, ketones, aldehydes, esters, lactones, oxides, ethers, and coumarins), furthermore, (Chen et al., 2019).

Bacteria are less likely to develop resistance to them than to antibiotics due to their multiple target (site (Chandra et al., 2017). The current study investigated the inhibitory effects of *Tagetes minuta* essential oil against *C. difficile* (causative agent of diarrhoea) for use as alternative antibacterial agent.

Materials and methods

Gas Chromatography coupled with Mass Spectrometry (GC-MS)

Tagetes minuta was characterised using gas chromatography that was equipped with an AB-1MS (30 Mx0.25 mm id 0.25 μm) capillary column (Kirbaslar et al., 2009; Adams, 2012; Igwaran et al., 2017). To prepare samples for analysis, the EO was dissolved in hexane (10%) followed by an injection at a split ratio of 50:1 in a Finnegan Focus Gas Chromatograph that was set at 230˚C. At an injection temperature of 25°C, Helium was used as a carrier gas at a constant flow of 1 $mLmin^{-1}$ (at a split ratio of 50:1). The chromatograph oven was optimized at an initial temperature

of 40˚C for 4 minutes and then raised to 5˚Cmin^{-1} to 200˚C. This was held at 200˚C for 1 minute and then raised by 5˚C to 220˚C where it was held for 10 min. Mass analysis of the oils was done using a Finnegan Focus DSQ mass spectrometer. The ion source temperature was set at 250˚C with an ionization voltage of 70 eV and mass scan range of 50-650 amu. The identification of the compounds followed the procedure as proposed by Kirbaslar et al. (2009). Individual GC peaks and mass spectra were determined by searching commercial libraries followed by expert matching of MS data (Kirbaşlar et al., 2009).

Strain used

The one strain of *Clostridium difficile* used in the study was obtained from Thermofischer Scientific: Microbiology division in Randburg. The microorganism was cultured on plate count agar (PCA) at 37°C according to (Setlhare, 2017; Mostafa et al., 2018). The cultures were grown for 24 hours at 37°C on PCA.

Bio-Assay preparation

The antimicrobial activity of the *Tagetes minuta* essential oil and ethanol (96%) used as a negative control and was determined by bioassay (Ghabraie, 2014) based on the agar diffusion method for infection-causing bacteria (*Clostridium difficile*). After 24 hours of cultivating the organism, a final bacterial inoculum of 5x10^5 CFU.ml^{-1} was prepared using Mueller-Hinton Broth (MHB). Then 200 µl of the organism was taken from the bottles and transferred into the prepared petri dishes containing a plate count ager (0.5% m.v-1 agar) and spread over the plate using a 'hockey stick' glass rod. A well (0.5 cm in diameter and depth) was then constructed at the centre of the plate containing an organism, and 46 µl of essential oil was added into the well. Ethanol (96%) was used as control and was added separately to the wells of the prepared plates. All the plates were incubated at 37°C for 24 h. To prevent evaporation of the essential oil from the plate, the oil was permitted to disperse in the agar before incubation.

Minimal Inhibitory Concentration (MIC)

To determine the inhibitory effect of the *Tagetes minuta* essential oil on the selected *Clostridium difficile*), a broth microdilution method (Shawish et al., 2014) was carried out using a 96-well disinfected microtiter plate. A final bacterial inoculum of 5x10^5 CFU.ml^{-1} was developed using Mueller-Hinton Broth (MHB) and reciprocated into a 96-well sterile microtiter plate (Becton, Dickinson and Company, Sparks, USA). The analysed essential oil samples were added to the first row of wells and serial dilutions were performed to achieve final concentrations of 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8 mg.ml^{-1}. To indicate growth after 24 hours of incubation, 40 µl of p-iodonitrotetrazolium violet (INT) [Sigma] solution (showing a pink to violet colour) was added to each well After infusion, the plates were incubated at 37°C for 24 hours. This analysis process was run twice in parallel for each concentration. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism in a broth dilution susceptibility test. The minimum inhibitory concentration was characterised in the first well without visible growth of bacteria.

Scanning Electron Microscopy (SEM)

To assess if structural changes occurred in the bacteria due to the function of the essential oils, scanning electron microscopy (SEM) was performed as proposed by Van Wyk and Wingfield (1991). Briefly, throughout the bio-assay preparatory process, cells susceptible to *Tagetes minuta* and a control specimen were fixed using 3% v/v of a sodium phosphate buffered glutaraldehyde solution at pH 7.0 and a similarly buffered solution (1% m.v-1) of osmium tetroxide for 1 h. Subsequently, the material was dehydrated in a graded series of the ethanol solution (30%, 50%, 70%, 90%, and 100%) for 30 min per solution). Thereafter, ethanol-dehydrated material was critical-point dried, mounted, and coated with gold to make it electrically conductive. This preparation for each sample was examined using a scanning electron microscope (SEM).

Positive Control

Study done by sethlare (2017) has proven that both gram negative and gram positive bacteria have develop resistant mechanism against the current use antibiotics such as penicill in, ampicillin, mezlocillin, peperacillin, cefazolin, cefotaxime, ceftazidime, aztreonam, imipenem, Aminoglycosides gentamicin, tobramycin, amikacin, netilmicin, streptomycin, kanamycin, therefore no positive control was done in the study.

Results and Discussion

Gas Chromatography coupled with Mass Spectrometry (GC-MS)

According to Prakash et al. (2015) and Bakkali et al. (2008) the antimicrobial activities of essential oils depend mainly on their compositions, it was therefore crucial to characterise the *Tagetes minuta* and elucidate its mode of action against *Clostridium difficile.* The chemical composition was thus analysed using *gas* chromatography mass spectrometry and the results are shown on Table 1 in terms of the identity and the percentage content of the individual components that were identified. In the study 15 compounds were identify representing 93.11% of the tested compound. *Tagetes minuta* most consisted of terpenes as major compounds which are known to possess antimicrobial activities (Omayma et al., 2020). These included *α*-Thujene (71.59%) and trans-Thujone (*β*-thujone) (14.45%), as major compounds, while they were other minor compounds detected ranges between 0.13-3.30 %. The minor compounds identified included *p*-Cymene 1,8-cineole (eucalyptol) (3.30%), 2-Bornanone (camphor) (1.15%) and other compounds reflected on Table 1. The results from the current study, differ from the study conducted in Djibout which indicated that as the *Tagetes minuta* oil consists of major compound such as 3-Methyl-2-butenoic acid, cyclobutyl ester (20.8%), artemisia (17.9%), p-Ment- 6-en-2-one (12.4%), (-)-spathulenol (11%) and estragole (9.5%) (Abdoul-Latif et al., 2022). Moreover, another study done in Zimbabwe, has indicated different major composition from the current study as it indicated high amount of dihydrotagetone (2.2–43.3%), (Z)-p-ocirnene (20.7–41.4%) and (Z)-tagetenone (0.3–31.4% (Chagonda et al., 1999). However, such results are not surprising as studies have proven that the yield and composition of the essential oil may differ depending on the plant and the area where it is grown, plant organ, stage of growth, latitude and methods of extraction all affect the composition of oil (Aparco et al., 2022).

Table 1: Chemical composition of *Tagetes minuta* obtain using Gas Chromatography coupled with Mass Spectrometry (GC-MS).

Compounds	*Tagetes minuta* Essential Oils (%)
Camphene	0.35
p-Cymene	0.78
1,8-cineole (eucalyptol)	3.30
2-Bornanone (camphor)	1.15
ar-Curcumene	0.13
Borneol	0.28
Isopentyl 2-methyl butanoate	0.22
Sabinene	0.23
trans-Thujone (β-thujone)	14.45
Z-β-Ocimene	0.17
α-Pinene	0.06
α-Thujene	71.59
β-Myrcene	0.14
β-Pinene	0.11
β-Selinene	0.15

Bioassay

After analysing the chemical composition of the selected essential oil, an antimicrobial activity test was conducted using a bioassay method. The bioassay method was performed to investigate whether *Tagetes minuta* inhibits the growth of *Clostridium difficile*. According to the results obtained, in the bioassay method, when *Tagetes minuta* was applied in the bioassay method against *C. difficile*, a zone of inhibition (I) was observed (Figure 1A and B) except for ethanol where only maximum growth was observed (figure 1 C). Undiluted *Tagetes minuta* showed the largest inhibition zone of 29 mm in diameter as observed in figure 2A, while the diluted *Tagetes minuta* produced an inhibition zone of 14 mm against the tested organism as indicated in figure 2B. when ethanol was tested as a negative control no inhibition zone was observe against the tested organism as indicated in figure 1C. The results obtain in the current study are in line with the study done by Gakuubi et al. (2016) were three bacteria indicated high inhibition zone when tested against undiluted *Tagetes minuta*. The highest antibacterial activity of undiluted *Tagetes minuta* EOs was observed in *P. savastanoi pv. phaseolicola* with inhibition zone of 41.8 mm while, for *X. axonopodis* pv. *phaseoli* and *X. axonopodis pv. manihotis*, the oils produced inhibition zones of 26.8 mm after 24 hours of incubation (Gakuubi et al., 2016). Antibacterial activity of *T. minuta* EOs has been reported in literature with most studies

reported that Gram-positive bacteria are more susceptible to the effects of EOs in comparison to Gram negative bacteria (Gakuubi et al., 2016). However, In the current study and the study done by Gakuubi et al. (2016) the results reveal the opposite as oils was more effective against Gram-negative bacteria than the gram positive. All the bacteria from Gakuubi et al. (2016) were gram negative, which has inhibition zone ranging from 41.8 mm to 26.8 mm, while the current study, the bacteria tested was gram positive bacteria with and inhibition zone of 25 mm for the undiluted oil, was the inhibition zone for the diluted oil was 14 mm. However, De Olanda et al. (2019) had different results of antimicrobial activity using *Salmonella typhimurium*, as it was resistant to *Tagetes minuta.*

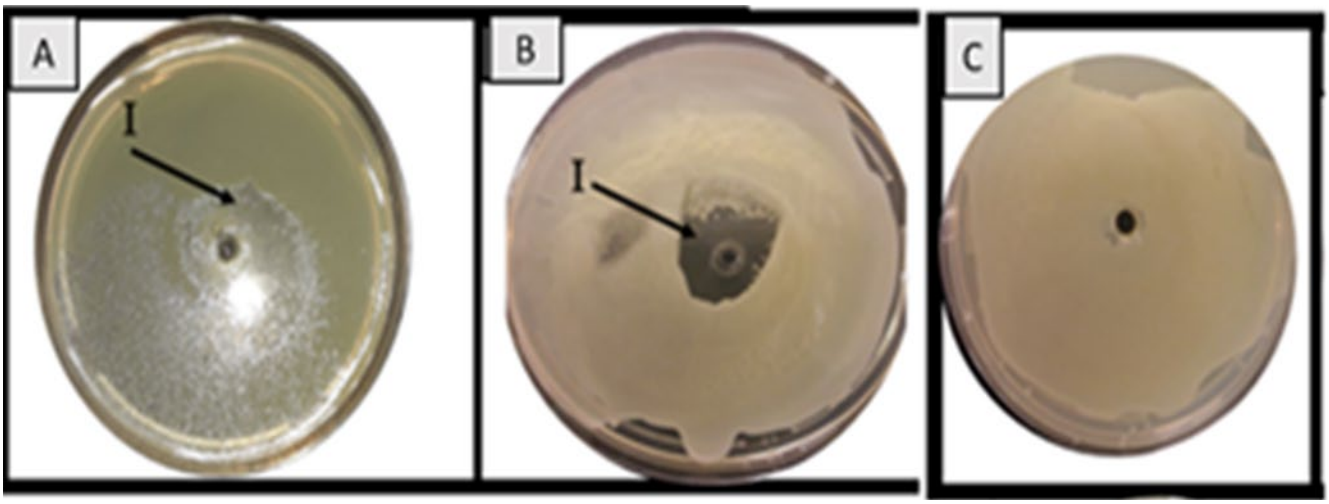


Figure 1: A bioassay showing cells of *C. difficile* cells treated with T*agetes minuta* (A=Undiluted oil, B=Diluted oil), (C) Untreated (control) cells with no antimicrobial activity. I – denotes the zone of inhibition.

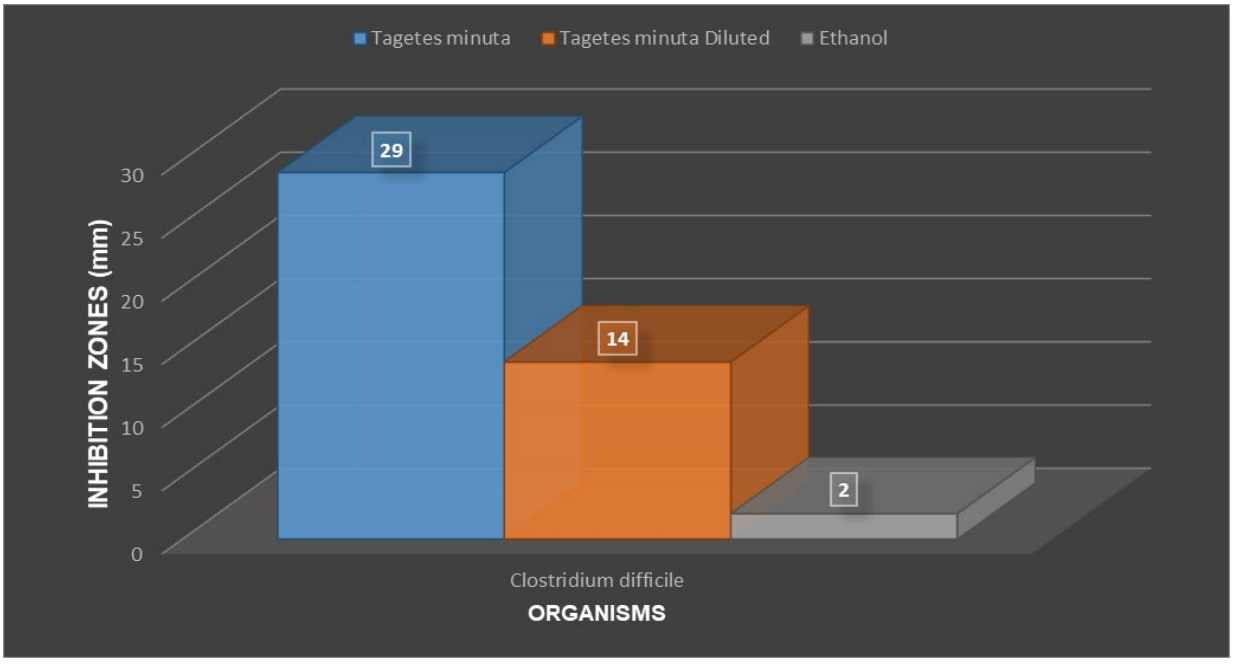


Figure 2: Antimicrobial activity of *Tagetes minuta* against *Clostridium difficile* using bioassay measured in mm.

Minimal Inhibitory Concentration (MIC)

As the inhibitory effect of the essential oil was evaluated, MICs were observed for the studied essential oil on *Clostridium difficile* (Table 2). From the results, For inhibitory effect of *tagetes minuta* on *C. difficile* isolates, the undiluted *tagetes minuta* essential oil completely inhibited bacterial growth due to its high antimicrobial activity (Table 2) indicated by the no growth (-) and the diluted essential oil showed an MIC of 12.5 mcg/ml (Table 2) for treated *Clostridium difficile*cells (Table 2). Moreover ethanol alone was not effective against the tested organism indicated by the strong growth (+++).

Table 2: Minimum Inhibitory concentration of *Tagetes Minuta* on *Clostridium difficile*

Key: **(-)** indicates no growth of bacteria, **(+)** indicates minor growth, **(++)** indicates medium growth, **(+++)** indicates strong growth. Colour GREEN indicates where the essential oil started to completely kill the organism, and the colour BLUE indicates where the organism's growth started to decrease

Dilution of EOs and ethanol(µg/ml)	**≥100**	**≥50**	**≥25**	**≥12.5**	**≥6.3**	**≥3.1**	**≥1.6**	**≥0.8**
Ethanol	+++	+++	+++	+++	+++	+++	+++	+++
Diluted Tagetes	-	-	-	-	+	+	++	++
Undiluted Tagetes	-	-	-	-	-			

Scanning Electron Microscopy (SEM).

To assess the morphological changes on the bacterial cells of organisms (*clostridium difficile* causing diarrhoea treated by *Tagetes Minuta*, an analysis was performed using a scanning electron microscopy from the MIC results. The results showed that at different concentration levels, *Tagetes minuta* was able to cause structural changes on the bacterial cells of *C. difficile* as shown in **figure** 3. During MIC observation, the results showed that *Tagetes minuta* began to inhibit the growth of *C. difficile* at a concentration of 12.5µg/ml. **Figure 3** reveal the changes of the *C. difficile* cells that began in **figure 3.B** (EO with 12.5 µg/ml concentration) to **figure 3.C** (with 100µg/ml concentration of oil). From the concentration of 12.5µg/ml (**figure 3.B**) Up to 100µg/ml concentration (**figure 3.C**), *Tagetes minuta* managed to cause holes on the surface of the cell wall (**figure 3. B.i** & **figure 3.C.iv**), incomplete cell division (**figure 3.B.ii & figure 3.C.v**), abnormal cell breaking and cell lysis (**figure 3.A.iii**). This shows that the *Tagetes minuta* oil indeed has the antimicrobial effect on the *C. difficile* bacterium. These changes are visible as holes on the cell wall, and this must have compromised the cell integrity by creating an imbalance of electrolytes that triggered swelling of the cell, incomplete cell division, and eventually cell death. Das et al. (2022) discussed similar findings, arguing that essential oils can disturb the motion of normal microbial growth by reducing the ATP production and modifying protein synthesis. Moreover, these structural modifications suggest energy limitations and therefore the inhibition of the toxin production by the bacterial cells, as proposed by Dashamiri et al. (2019) and Zhu et al. (2022). It is possible that the secretion of toxins was prevented by these modifications in the bacterial membrane due to the attachment of the essential oil that disturbed the phospholipid bilayer and led to the trans-membrane transport process, as proposed by Zhu et al. (2022). This may therefore have limited the release of toxins into the immediate environment.

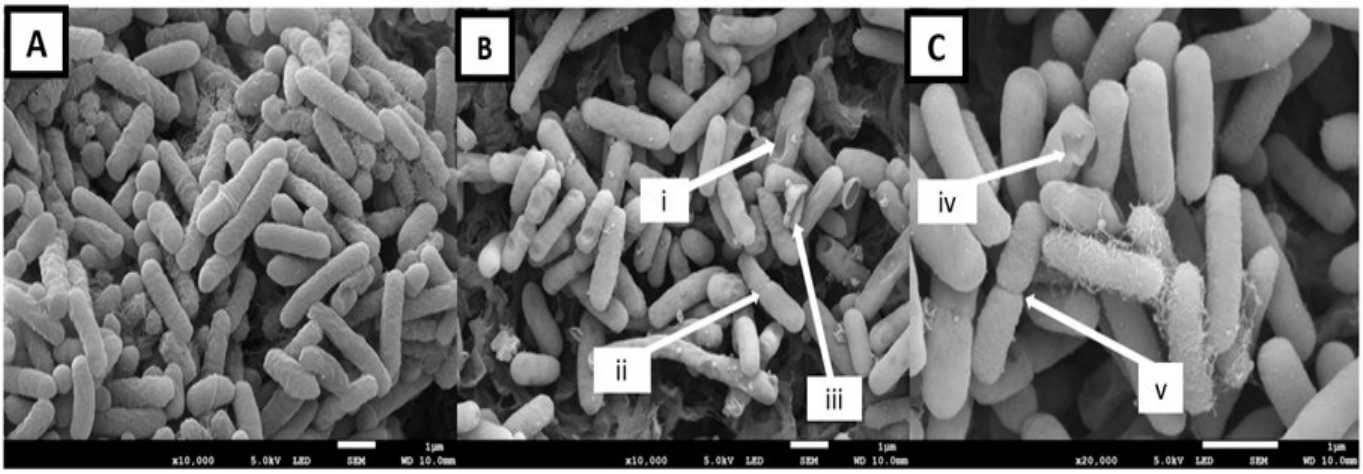


Figure 3: Scanning Electron Microscopy analysis of *Clostridium difficile*. A= Untreated bacterial cells, B= Treated cells at a concentration of 12.5µg/ml with *Tagetes minuta*, C= Cells treated with *Tagetes minuta* at a concentration of at 100µg/ml. i & iv = Holes on the surface of the cell wall, ii & v= Incomplete cell division, iii=cell lysis

Conclusions and Remarks

Infectious diseases and food-borne illnesses triggered by *Clostridium difficile* can cause severe health effects and can even lead to death among the residing population, especially in the developing regions of the world. The continual emergence of antibiotic-resistant microorganisms has prompted researchers in this study to search for new antimicrobial agents that are more effective against the resistant microbial pathogens. Structural modification of the antimicrobials (against which microbial resistance has been developed) is reported to improve the effectiveness of antimicrobial agents against bacteria, fungi, and viruses. Such alternative treatment methods included essential oils as potential antimicrobial agents for the fight against infectious diseases and food-borne illnesses that cause severe health effects and even death especially in patients with weakened immune systems. Therefore, it is advised that plants with these potential therapeutic or medicinal values can be successfully utilised for preventing and treating various ailments and food-borne illnesses.

From the results obtained in this study, it can be concluded that essential oils and their play a significant role in combating bacterial antibiotic resistance and can be used in traditional medicine to cure some of the common disorders and degenerative diseases in humans. The effectiveness of these procedures has been attributed mainly to the presence of active phytochemicals or bioactive compounds in essential oils observed in this study. Given the scope of searching new antimicrobial agents, antimicrobials derived from Tagetes minuta plant materials are regarded as natural and safe compared t. Moreover, plant-based medicine is highly recommended due to the increasing concern of consumers reported in the literature (Djilani and Dicko, 2012) regarding the use of antibiotics.

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