Research Article

Centella asiatica Essential Oil (CEO) Intercalated Montmorillonite Nanoclay; Development, Characterization, and Bioactivity Assessment

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Abstract

Centella asiatica Essential Oil (CEO) is used in traditional ayurvedic medicine in China, India, and Sri Lanka. It has health benefits such as anticancer, antioxidant, antiulcer, and antimicrobial activities. But its low water solubility, low thermal, chemo and photostability, have limited its commercial applications. Intercalation of EO in nanomaterials is a feasible and successful solution to increase oil stability. Currently, there is no information available on the CEO nanoclay intercalation. Bright yellow coloured CEO was extracted using Clevenger apparatus. The oil yield was 0.04% (v/w), and the density was 830 kg m-3. Oil was subjected to GC-MS and identified 43 compounds including terpenes and chromenes accounting for 95.056% of the total. The most abundant compounds were α-humulene (23.74%), caryophyllene (18.64%), and β-farnesene (11.16%). Intercalated oil (MMT+CEO) was obtained by liquid assisted grinding. XRD results validated the successful CEO intercalation by detecting an increment of montmorillonite interlayer space from 11.9 Å to 14.2 Å. A comparison of FTIR spectra showed additional peaks after intercalation. The plate-like appearance of MMT was marginally interrupted in intercalation. The in vitro antioxidant activity of CEO was assessed by DPPH assay and obtained 508.6 ± 0.5 µg/ml as the IC_{50} value. α-humulene and Caryophyllene were identified as the major antioxidants. The IC_{50} value for the positive control; ascorbic acid, was 4.4 ± 2.3 µg/ml. According to the Kirby Bauer assay, a moderate antimicrobial activity was observed against Staphylococcus aureus. Low and negligible activities were demonstrated against Candida tropicalis and Escherichia coli. The minimum inhibitory concentration of CEO and MMT+CEO against S. aureus was 25 and 100 (µl/ml) respectively. The Colony counts of poison food technique decreased in MMT+CEO with time. Although in vitro antimicrobial activity

doesn't change qualitatively with intercalation, the quantitative activity was higher in non-intercalated form suggesting that nanoclay can act as a physical barrier.

Keywords: *Antimicrobial activity, Centella asiatica, essential oil, montmorillonite, nano intercalation*

1. Introduction

Centella asiatica L. (Local name; *Gotukola*) is a perennial creeper belonging to the family Apiaceae. The plant and its essential oil (EO) are rich in many secondary metabolites including sesquiterpene hydrocarbons, flavonoids, tannins, phytosterols, amino acids, and terpenoids (Francis and Thomas, 2016). It has been reported that the compounds in *C. asiatica* possess numerous bioactivities such as anti-inflammatory, antiulcer, radioprotective, antidepressant, immunomodulating, anticancer, anxiolytic, antioxidant, anticonvulsant, anti-psoriatic, cardioprotective, skin maintenance, wound healing, hepatoprotective, memory enhancing, burn healing, antimicrobial, larvicidal, anti-hyperglycemic, and neuroprotective activities (Chong *et* al., 2011; Goldstein and Goldstein, 2012; Roy *et al*., 2013). *C. asiatica* essential oil (CEO) is used in medicinal, food and cosmetic industries due to its antimicrobial and other therapeutic properties. Although CEO has an encouraging potential to maintain and promote health, while preventing diseases; their low water solubility, low thermal stability, high volatility, side effects, and sensitivity to moisture, light, and oxygen have limited their applications (Mwaniki and Kamau, 2003).

EO nanoencapsulation is a feasible approach to modulate drug release by increasing shelf life, bioavailability, pH stability, thermal stability, photo stability, and bioactivity. Additionally, nanoencapsulation decreases toxicity and reduces the evaporation of active compounds. Furthermore, it maintains the controlled release, site specific action, and therapeutic rate (Singh *et al*., 2009; Gavini *et al.*, 2005). The encapsulation techniques are widely used to transform EOs into long-lasting powders or capsules with wall materials which can protect the active compounds from environmental factors by decreasing the oil volatility (Bilia *et al*., 2014). This can improve the stability of EOs against enzymatic degradation and can achieve the desired therapeutic levels in targeted tissues within a short time using small doses. In addition, nanocarriers ensure the easier and safer handling of liquid substances by changing them into solid powders. They can mask the poor taste of EOs, deliver multiple active ingredients consecutively, improve the water solubility of hydrophobic ingredients, and enhance bioavailability and efficacy (Bilia *et al.*, 2014). Intercalation may increase the effectiveness of bioactive compound

concentration in areas of food systems, where targeted microorganisms are preferentially located (Weiss *et al*., 2009).

Up to now, very little systematic research has been conducted to evaluate the use of nanomaterials in EO intercalation (Weiss *et al*., 2009). Therefore, the present work is targeted to intercalate CEO in montmorillonite nano clay (MMT). Moreover, the *in vitro* antimicrobial and antioxidant activities of nanomaterial were compared with that of pure CEO to find the suitability of using this nanomaterial in the food, drug and packaging industries.

2. Material and Methods

The whole plants of *C. asiatica* were purchased from a local vendor at Wijerama, Sri Lanka. The manufacturer of MMT (Sodium salt, Cloisite–Na+) was Southern Clay, USA. All chemicals used were reagent grade and used without further purification. Distilled water (DW) was used for all solution preparations. Pure cultures of the test organisms *Candida tropicalis* (ATTC 13803), *Escherichia coli* (ATTC 25922), *Staphylococcus aureus* (ATTC 25923) and *Bacillus cereus* (ATTC 11778) were used for the experiments.

2.1. Extraction and characterization of CEO

The whole plant of *C. asiatica* was washed with running tap water for 10 minutes and cut into small pieces. A mass of 300 g of plant materials in 250 ml of DW was subjected to hydrodistillation for 6 hrs in a Clevenger- type apparatus (Francis and Thomas, 2016). The extracted oil was carefully separated from water using a micropipette and dried over anhydrous Na_2SO_4 and stored in sealed Eppendorf tubes at -20 °C until further use. The weight and volume of the CEO were recorded.

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the CEO

The GC-MS analysis was performed using Agilent Technologies Gas Chromatograph model 7890A coupled to Agilent 19091S-433HP-5MS 5% Phenyl Methyl Silox column. The split ratio was 10:1, the run time was 42 min, and the oven temperature was 60 - 250 °C with a 5 °C /min rate. The flow rate was 14 ml/ min (Francis and Thomas, 2016). Compounds were identified by calculating Retention Indices (RI) and comparing mass spectra with NIST MS Search Library Software version 2.0.

2.3. Preparation of MMT+CEO by nanoencapsulation

The MMT platelets were not regular in shape and the width was 15- 150 nm. It was oven dried to remove water. The CEO was mixed with MMT in a 1:2 (v/w) ratio and ground for 30 min using an agate mortar and pestle to facilitate the movement, diffusion, and stabilization of CEO in-between MMT clay layers.

2.4. Characterization of CEO intercalated MMT (MMT+CEO)

X-ray diffractogram (XRD) of MMT+CEO was obtained by Rigaku Ultima IV X-ray diffractometer operating in reflection mode with Cu-Kα radiation (40 kV, 30 mA) and diffracted beam monochromator, using a step scan mode. The 2θ value changed from 3^o – 60^o with a step size of 0.02^0 (Madusanka *et al*., 2015). The same procedure was performed for pure MMT. Powder XRD patterns were then compared with each other and analyzed.

Thermo Scientific Nicolet S10 Fourier Transform (FTIR) Spectrometer was used to obtain FTIR spectra of MMT+CEO and pure MMT and compared the results. OMNIC Spectra was used as the analysis program.

The morphology and topography of MMT+CEO were studied using a HITACHISU 6600 Scanning Electron Microscope (SEM). Samples were loaded separately on either side of the stub containing carbon tapes. Excess samples and bulky particles were removed by spraying air. Gold sputtering was performed to ionize samples and obtained the images.

2.5. *In vitro* antioxidant activity of CEO.

2.5.1. DPPH- radical scavenging assay test.

The *in vitro* antioxidant activity of CEO was determined as a measure of radical scavenging using DPPH according to Patil *et al.,* (2010) with some modifications. The initial absorbance of 25 mg/l DPPH solution was measured at 517 nm and adjusted to a value of around 0.7 by diluting in methanol. Then, 2 ml of DPPH was mixed with 50 µl of different concentrations (400 µg/ml, 450 µg/ml, 500 µg/ml, 550 µg/ml, 600 µg/ml and 650 µg/ml) of CEO in 1 cm path- length cuvettes and vortexed well. Prepared samples were kept in the dark for 15 min before measuring the absorbance at 517 nm. The radical scavenging activities were calculated for each concentration using the following equation and the absorbance measurements.

Radical scavenging activity = [(OD control – OD sample) / (OD control)] × 100%

IC_{50} value of CEO was acquired by plotting a graph via Microsoft Excel 2010. Ascorbic acid was used as the standard and methanol was the negative control.

2.5.2. Identification of antioxidant compound/s in CEO

A TLC bio-autograph was developed to identify the antioxidant constituent/s of CEO. Exactly 1 µL of CEO was placed on a 20 cm × 1.5 cm TLC Silica gel 60 F_{254} Merck KGaA (Darmstadt, Germany) plate. The chromatographic chamber was saturated with hexane and chloroform – 12:8 (total volume 20 mL) solvent system. The TLC plate was developed and air dried for 5-10 min before observing under the UV light to identify UV sensitive compounds. Further, the plates were sprayed with 0.2% DPPH to identify the antioxidant compound/s. The regions containing antioxidant compound/s were scraped and eluted in hexane. It was vortexed and centrifuged at 5000 rpm for 5 min and the supernatant was subjected to GC-MS for further identification.

2.6. Screening of *in vitro* antimicrobial activity

2.6.1. Inocula preparation

Bacteria and *Candida sp.* were cultured in Nutrient Agar (NA) and Yeast Extract Peptone Dextrose Agar (YPDA) respectively. A loop full of overnight grown pathogens was inoculated in separate McCartney bottles containing 5 ml of sterile distilled water (SDW). The optical densities (OD) were recorded at 600 nm using a UV spectrophotometer (Crom Tech CT-8200, China) and OD 600 was adjusted to 0.5 McFarland (approximately 5×10^6 CFU/ml) standard.

2.6.2. Disc diffusion / standard Kirby-Bauer test

An aliquot of 100 µl of fresh bacteria (0.5 McFarland) and *C. tropicalis* were spread on Muller Hinton Agar (MHA) and Glucose Muller Hinton Agar (GMHA) respectively. A sterile filter paper disc was impregnated in 5 µl CEO + 5 µl of 10% (v/v) dimethyl sulfoxide (DMSO). Similarly, discs with MMT+CEO (10 mg), and pure MMT (10 mg) in DMSO were prepared (Kouame *et al*., 2018). As positive controls, chloramphenicol (for *S. aureus* and *B. cereus*), gentamicin (for *E. coli*), and ketoconazole (for *C. tropicalis*) were used. Plates were incubated at 37 °C for 24 hrs and the zones of inhibition (ZOI) were measured.

2.6.3. *In vitro* Minimum Inhibitory Concentration (MIC) against *S. aureus* using micro broth dilution method.

The Minimum Inhibitory Concentration (MIC) was determined according to the micro-broth dilution method (Kouame *et al*., 2018). Microplates (96 wells) were filled with 100 µl of Muller Hinton Broth (MHB). An aliquot of 100 µl of CEO (100 µl/ml) in Tween 80% was added to the first well. From this first dilution, 100 µl was transferred to the second well to half the concentration (50 µl/ml). This was repeated to make a series of two-fold dilutions (25 µl/ml, 12.5 µl/ml, 6.025 µl/ml, 3.125 µl/ml, 1.56 µl/ml, 0.78 µl/ml, 0.39 µl/ml, 0.19 µl/ml, 0.09 µl/ml, 0.04 µl/ml). From the final wells, 100 µl was discarded to maintain a fixed volume. The same procedure was repeated to obtain two-fold dilutions for MMT+CEO. Aliquots of 100 µl *S. aureus* suspension (0.5 McFarland) were added to each well except for blanks. Blanks were treated with 100 µl SDW. The positive control was chloramphenicol antibiotic (1 mg/ml). Negative control was made without CEO. After the incubation at 37 °C for 24 h, 40 µl of 1% (w/v) triphenyl tetrazolium chloride (TTC) was added and incubated for another 30 min. The lowest concentration which gave no colour change was taken as the MIC.

2.6.4. Assessment of *in vitro* antimicrobial activity by poison food technique.

A working solution was prepared using 50 µl of CEO, 50 µl of Tween 80% and 2 ml of SDW. Then, 200 µl of this working solution, 50 µl of fresh *S. aureus* (0.5 McFarland), and 15 ml of molten MHA were poured into Petri dishes (Kim *et al*., 2011). Similarly, 100 mg of MMT+CEO was used instead of CEO to prepare another working solution. The negative control was SDW instead of oil. Pure MMT dissolved in SDW, was another control. Plates were incubated at 37 °C for 24 hrs. The tests were repeated every 2 hrs using the same working solutions, but freshly prepared inocula for a total of 6 hrs. Colony counts were obtained by using AutoCAD, 2016 software.

3. Results and Discussion

3.1. Extraction and characterization of CEO

The yield of extracted CEO from shoots was 0.04% and the density was 830 kg/m^3. It was bright yellow and had a powerful odour. Francis and Thomas, 2016 reported the shoot CEO yield as 0.05% (v/w). A colourless mild scented CEO with a 0.06%

(v/w) yield was recorded in Oyedeji and Afolayan (2005). Different levels of secondary metabolites may result in slight differences in colour and scent (Florczak 2014). CEO yield data in past literature favors the data obtained from this study. According to Florczak (2014), the yield of EO depends upon the number of oil sacs present in the plant material. It was recorded that the lavender leaf includes a large number of oil sacs since it is an aromatic plant while *C. asiatica* has only a few EO sacs per leaf (Florczak, 2014). This could be a reason for the very low yield of the CEO. steam distillation was the most efficient method to obtain the greatest yield of CEO out of different extraction methods such as steam distillation, water distillation, solvent extraction and, soxhlet method (Mwaniki *et al*., 2003). The collected CEO was stored in dark at -80 °C as they can decompose at room temperature.

The GC-MS profile contained 43 compounds accounting for 95.056% of the total. Each compound had a matching percentage of ≥80% with the compounds in the library database. The molecular weights of each compound were lower than 300 g/mol. The most abundant compound was α-humulene (23.743%), having a known antimicrobial activity (Oyedeji and Afolayan, 2005). The second and the third abundant compounds were caryophyllenes (18.64%) and β-farnesene (11.155%). Oyedeji and Afolayan (2005) determined the composition and antibacterial activity of the CEO in South Africa. They reported 40 compounds and the major constituents were α-humulene (21.06%), β-caryophyllene (19.08%), bicyclogermacrene (11.22%), myrcene (6.55%) and germacrene B (6.29%). According to the literature, CEO composition varies significantly. It can be concluded that the EO chemical composition is affected by geo-climatic location, soil nature, plant age, harvesting time, and the leaf size used for the extraction (Florczak, 2014). According to Asakawa *et al.*, (1982) CEO extracted from the whole plant was dominated by sesquiterpenes, which is in agreement with our results. But the compositional constituents and their abundance were different. Monoterpenes, sesquiterpenes, esters, amino acids and chromenes were identified during our study. Table 1 shows the chemical profile of CEO with the content percentages. The CEO composition is exceptionally complex because they contain a broad chemical spectrum of different chemical species (Rehman *et al.*, 2016).

Table 1: Chemical constituents and their proportions of CEO analysed by GC-MS.

No	Retention time	Compound	Content %
1	4.617	beta-Thujene (monoterpene)	0.168
2	4.748	alpha-pinene (monoterpene)	0.720
3	5.070	Camphene (monoterpene)	0.022
4	5.696	beta-pinene (monoterpene)	0.496
5	6.090	beta-Thujene (monoterpene)	0.765
6	6.474	alpha-phellandrene (cyclic monoterpene)	0.100
7	6.819	2-Carene (monoterpene)	0.192
8	7.063	O-Cymene (monoterpene)	0.139
9	7.166	Limonene(monoterpene)	0.155
10	8.020	γ-Terpinene (terpene)	2.077
11	8.946	Terpinolene(terpene)	0.097
12	14.459	Bornyl Acetate (ester)	0.083
13	15.856	delta-elemene (sesquiterpene)	0.045
14	16.164	Alpha-Cubebene (sesquiterpene)	0.473
15	16.391	Clovene (sesquiterpene)	0.037
16	16.606	Cyclosativene (sesquiterpene)	0.075
17	16.945	Copaene (sesquiterpene)	9.059
18	17.251	Beta-Cubebene (sesquiterpenoid)	2.989
19	17.671	Caryophyllene (sesquiterpene)	0.126
20	17.845	Benzyloxycarbonyl amino	0.254
21	**18.153**	**Caryophyllene (sesquiterpene)**	**18.640**
22	18.253	Beta-Cubebene (sesquiterpenoid)	0.241
23	**19.012**	**α-humulene (sesquiterpene)**	**23.743**
24	**19.092**	**β-Farnesene (sesquiterpenoid)**	**11.155**

25	19.429	Delta-cadinene (sesquiterpene)	0.791
26	19.651	Beta-Cubebene (sesquiterpene)	5.435
27	19.739	beta-Selinene (sesquiterpene)	0.183
28	19.858	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene	0.974
29	20.009	alpha-Muurolene (sesquiterpene)	0.376
30	20.150	beta-Elemene (sesquiterpenoid)	0.372
31	20.330	gamma-Muurolene	0.407
32	20.575	Beta-Cadinene (sesquiterpene)	2.567
33	20.769	1,4-Cadinadiene (sesquiterpenoid)	0.312
34	20.888	Beta-Cadinene (sesquiterpene)	0.080
35	21.344	Gamma-Elemene (sesquiterpene)	0.082
36	21.501	Nerolidol (sesquiterpene)	1.888
37	21.958	Caryophyllene oxide (sesquiterpene)	0.734
38	22.174	beta-Gurjunene (sesquiterpenoid)	0.115
39	22.322	1,2-Dihydropyridine, 1-(1-oxobutyl)	0.090
40	22.429	Ledol (sesquiterpene)	0.323
41	23.378	Copaene (sesquiterpene)	0.192
42	23.573	Alpha-Cadinol (sesquiterpenoid alcohol)	0.470
43	23.773	Precocene II (chromenes)	7.814
		Total	95.056

3.2. Nanoclay intercalation and characterization of CEO+MMT

Initially 1:1 (v/m) ratio of MMT: CEO was used for the encapsulation. But, after 30 min of grinding the composite was sticky and semi-solid in nature. As the resulting composite should have a proper solid texture, the ratio was adjusted to 2:1 (MMT: CEO). The ratio 3:1 was also tested but not selected since it had no significant texture difference and further lowering of EO concentration has the potential to reduce the bioactivity. However, XRD data (Figure 1a) confirmed that all three ratios are successful in intercalation but have no significant difference in d values. When EO molecules move to the gallery between MMT layers, intercalation occurs. MMT provides room for the CEO molecules to sit within the interlayers as well as on the outside surfaces. It can retard the degradation of oil components and give a slow releasing behavior. This could enhance the shelf life of the CEO when incorporated into industrial products such as foods.

Sodium montmorillonite (Na+ MMT-) is a naturally occurring, non toxic, layered material with a large specific surface area. The cation exchange capacity, drug-carrying capability, and cost effectivity make it suitable for the synthesis of bio nanocomposites (Mahmood *et al*., 2017; Uddin, 2008).

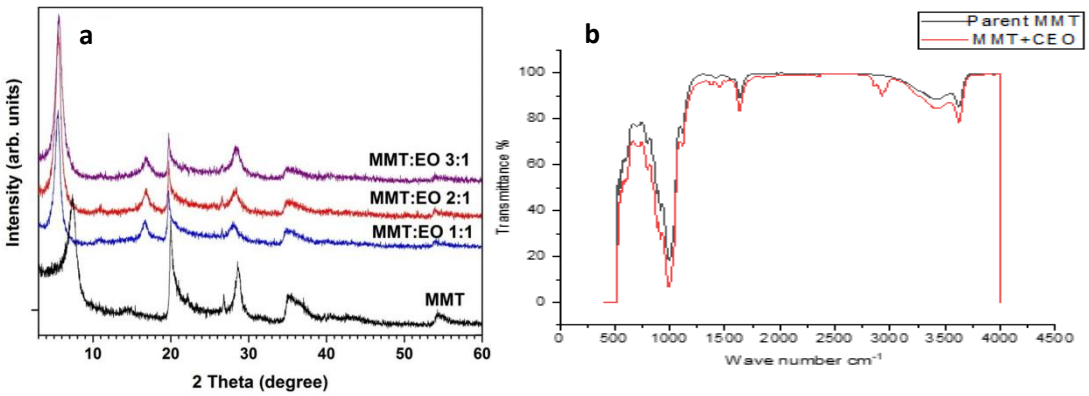

Figure 1: (a) XRD traces of Parent MMT (d_{001} = 11.9 Å), MMT+CEO in different v/w ratios (d_{002} = 14.2 Å), (b) FTIR spectrum of Parent MMT and MMT+CEO (1:2)

3.2.1. X-ray Diffraction (XRD)

The MMT layers move apart providing sufficient space for CEO molecules to intercalate. This eventually increases the d value in XRD as depicted in figure 1a. The initial interlayer spacing (d_{001}) of oven-dried MMT was 11.9 Å. During CEO

intercalation, the movement of MMT layers elevated the interlayer distance (d_{002}) to 14.2 Å (Figure 1a) and the increment is independent of the oil to clay ratio. According to Bragg's Law d and 2σ are inversely proportional variables [$2d \sin \sigma = n\lambda$]. This expansion of layers is an indication of CEO interaction.

3.2.2. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR spectrum of MMT+CEO demonstrated some extra peaks compared to MMT. These peaks may indicate the chemical varieties in CEO. Peaks around 2900 cm^{-1} due to C-H stretching, peaks around 1500 cm^{-1} due to stretching vibrations of C=C of benzene rings, peaks at 1400-1300 cm^{-1} due to symmetrical deformations of CH_2 and C-OH groups, and peaks at 2800 cm^{-1} due to carboxylic acid derivatives have only appeared in MMT+CEO (Meng *et al*., 2019). The major constituent α-humulene ($C_{15}H_{24}$) contains an 11-membered ring and consists of three isoprene units with three non-conjugated C=C double bonds (Alwakil *et al*., 2022). The additional peaks that appeared in intercalated MMT suggest the presence of CEO majoring α-humulene. We can conclude that the nature of bonding has changed after nanoencapsulation and this can be used to validate the adsorption of oil into nanoclay material (Figure 1b).

3.2.3. Scanning Electron Microscopic (SEM) analysis

Parent MMT displayed typical plate-like morphology in SEM images (Figure 2). This plate-like appearance is slightly maintained even after the intercalation of the CEO. In addition to that, some agglomerations on MMT surfaces were observed due to the adsorption of CEO particles (Figures 2 b and c).

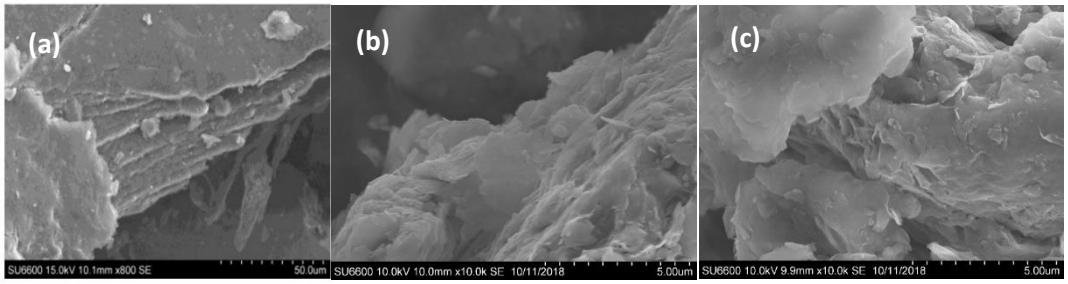

Figure 2: SEM image of (a) parent MMT (Madusanka *et al*., 2015); (b) and (c) MMT+CEO

3.3. *In vitro* antioxidant activity of CEO and MMT+CEO

3.3.1. DPPH- radical scavenging assay test

The mean IC_{50} values of CEO and ascorbic acid standard were 508.6 ± 0.5 μg/ml and 4.4 ± 2.3 μg/ml respectively. According to the results, CEO exhibited comparatively moderate antioxidant activity as assessed by DPPH free radical scavenging assay.

3.3.2. TLC-bio autography

Two antioxidant zones were identified based on the TLC-DPPH assay results of the CEO. The two antioxidant spots at Rf = 0.55 and 0.48 were detected as caryophyllene (retention time – 12.785 min, matching percentage – 95%) and α-humulene (retention time – 13.401 min, matching percentage – 90%) respectively. The antioxidant activity of caryophyllene was previously reported by Espinosa-Ahedo *et al*., (2022). Notably, this study recorded the antioxidant potential of α-humulene extracted from CEO for the first time.

3.4. *In vitro* antimicrobial activity of CEO and MMT+CEO

3.4.1. *In vitro* assessment of antimicrobial activity by disc diffusion test (standard Kirby-Bauer test)

Pure CEO and its intercalated forms exhibited antimicrobial activity against Gram (+) bacteria (*S. aureus* and *B. cereus*) and *C. tropicalis*. But none of them showed antimicrobial activity against Gram (-) *E. coli* bacteria. According to ZOI in figure 3, the activity was higher against *S. aureus* than *B. cereus* and negligible against *C. tropicalis*. Although the presence of ZOIs indicated the antimicrobial activity of CEO, their diameters were very small (< 8 mm). Therefore, the disc diffusion assay was preferably used as a qualitative test to find the most susceptible pathogen. The same trend was observed in both CEO and MMT+CEO showing that the antimicrobial potential does not qualitatively change due to intercalation. Higher activity of the CEO against *S. aureus* than *B. cereus* was also reported by Oyedeji and Afolayan, 2005. Based on these data *S. aureus* was selected for further studies. The small ZOI could be probably due to the slow diffusion of active compounds through agar. Furthermore, the hydrophobicity and limited solubility of CEO can compromise the diffusion rate (Ganjali *et al*., 2016).

The great variety of sesquiterpenes in CEO is accountable for its antimicrobial activity. Probably the components such as α and β pinene, β caryophyllene, phellandrene, cymene, clovene, cyclosativene, α-humulene, and nerolidol detected in this study could be responsible for the activity (Vieira *et al*., 2009). The effectivity

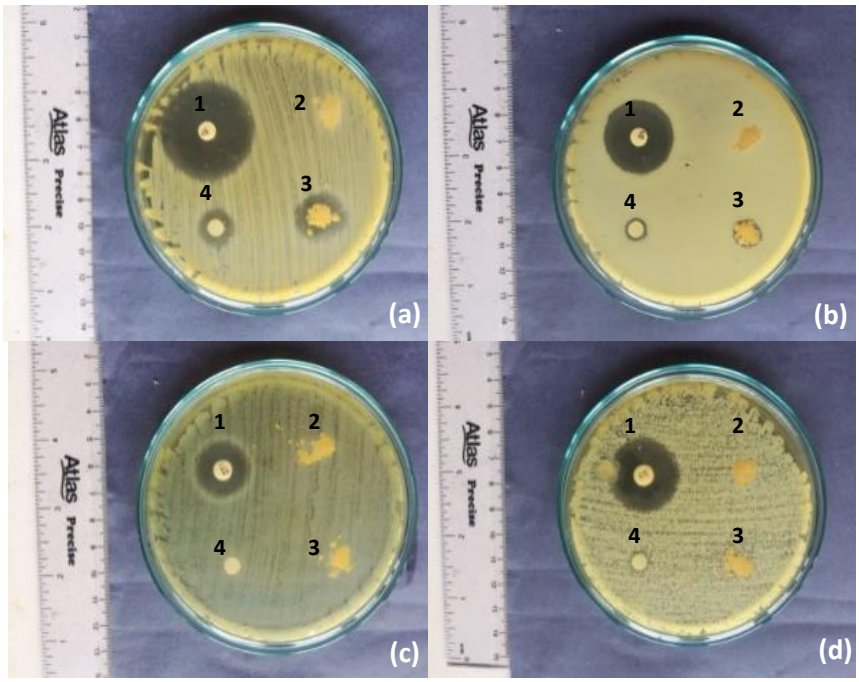

Figure 3: In vitro antibacterial activity of CEO 1) positive control 2) MMT 3) MMT+CEO 4) CEO (a) Against *S. aureus* (positive control - Chloramphenicol, 30 µg). (b) *B. cereus* (positive control - Chloramphenicol, 30 µg). (c) *E. coli* (positive control - Gentamicin, 10 µg). (d) *C. tropicalis* (positive control – Ketoconazole ,25 µg)

of β caryophyllene against *S. aureus* was previously reported by Kim *et al*., 2008. These data confirm that the use of CEO in traditional medicine is very effective. Since pure oil can be negatively get affected by various environmental factors such as temperature, pH, and radiation, nanoencapsulation might be a good solution to increase the shelf life of CEO. Nevertheless, due to the low yield of CEO, it is very important to protect bioactive compounds from evaporation.

3.4.2. *In vitro* minimum inhibitory concentration (MIC) of CEO against *S. aureus*

The CEO had a lower MIC value (25µl/ml<mic) and a higher antimicrobial efficacy than MMT+CEO (100 µl/ml<mic). This may be due to the slow release of active compound/s from MMT+CEO to the matrix because of the presence of its nanoclay barrier. In the meantime, this barrier protects the active compounds from degradation.

3.4.3. *In vitro* antimicrobial activity of CEO by colony counting using the pour plate method

The initial set of plates had the highest number of colonies in the poison food technique assay (SDW: 3034.67 ± 95, CEO: 2818 ± 52, MMT+CEO: 2932 ± 74). This may be due to the low availability of bioactive compounds. The set of plates prepared after 6 hrs, had the lowest number of colonies (SDW-3077 ± 67, CEO-505 ± 83, MMT+CEO-2095 ± 127). When allowed to stand for 6 h; bioactive compounds had more time to diffuse to the surrounding media. The release of oil from MMT+CEO increased with time; thus, the suppression of pathogens increased accordingly. Conferring to the colony counts and MIC values, the antimicrobial activity is comparatively high in the non-intercalated form. With time the release of CEO from nanocomposite must be increased. Therefore, the suppression of pathogens must be increased with time. This trend was observed in our study. The reason for this observation may be the presence of the nanoclay barrier around the intercalated form. Although the intercalated form displayed reduced activity, the antimicrobial quality was preserved even after the encapsulation. Therefore, we can conclude that the antimicrobial effect was not eliminated by encapsulation.

Maryan *et al*., 2013 reported the antibacterial activity of MMT and its effect on cotton fiber suggesting the potential use of MMT with other antimicrobial agents in the textile industry to control the growth of bacteria in cellulose fibers. Another research reported that methylcellulose (MC), carvacrol (CRV), and MMT nanocomposite films have potential use as active antimicrobial packaging materials. The nanocomposite films on sausage reduced *E. coli* and *S. aureus* counts by 0.9 and 0.7 log CFU/mL compared to the control (Tunç *et al.*, 2011).

The reduced activity due to the slow release of active compounds after the nano clay intercalation has previously been reported in the literature (São Pedro *et al.*, 2013). Future research should be focused on exploring the potential use of intercalation to

extend the shelf life of EOs. Additionally, studies must be conducted to ensure the reduced activity is due to a controlled release. This will lead to the successful use of nanoclay intercalation in the food and pharmaceutical industries to gain more profitable and useful results.

4. Conclusion

The importance of CEO in traditional medicine has been previously reported in the literature. The present study was carried out to evaluate the effect of nanoencapsulation on *in vitro* antimicrobial activity of essential oil extracted from *C. asiatica* medicinal herb. Additionally, the chemical composition of CEO grown in Sri Lanka was also investigated. This research has encompassed the study of the antimicrobial activity of intercalated CEO (MMT+CEO) for the first time.

The CEO was dominated by α-humulene (23.743%), caryophyllene (18.640%), and β-farnesene (11.155%), thus the oil was abundant with sesquiterpenes and chromenes. CEO showed *in vitro* antioxidant activity, indicating the potential use as an alternative natural antioxidant. Caryophyllene and α-humulene gave rise to the antioxidant activity of CEO. I*n vitro* antimicrobial tests showed a moderate antimicrobial activity of CEO against *S. aureus*. But it was less effective against *Candida* sp. and *E. coli*. Enhancement of *in vitro* antimicrobial activity of nanoencapsulated form was not reported but the general qualitative activity was retained even after the encapsulation. Both forms were active against similar types of pathogens, but the quantitative activity was higher in the nonencapsulated form.

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