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CHEMICAL PROFILING, BIOLOGICAL ACTIVITIES, AND NANOFORMULATION OF ESSENTIAL OILS FROM JUNIPERUS EXCELSA M. BIEB. AND TAGETES MINUTA L.

Maryam[*1], Syed Abdul Khaliq Jan[2], Salahud Din[3], Qadar Khan[4], Shahab Alam[5], Imtiaz Ali Shah[6], Tamanna Alam[7], Shabana Irum[8], Rizwan Malik[9], Sohail Ahmad[10], Tara Malik[11], Habiba Bibi[12], Shazia Dilbar[13]

*[*1,2]Department of Chemistry, Shaheed Benazir Bhutto University, Sheringal Dir (U), Khyber Pakhtunkhwa, Pakistan*

[3,4,910,13]Department of Botany, university of Swat, Pakistan

[5,11]Swat College of Nursing, Qambar, Swat, KPK, Pakistan

[6]Swat College of Pharmaceutical Sciences, Gogdara, Swat, KPK, Pakistan

[7]Iqra National University, Odigram, Swat Campus, Swat, KPK, Pakistan

[8]Department of Botany, Hazara University, Mansehra, KPK, Pakistan

[12]Department of Nursing, Zubair College of Nursing and Allied Health Sciences, Swat, Pakistan

[*1]qadarkhan1131@gmail.com/zarachemist33@gmail.com, [2]abdukhaliq@gmail.com, [3]taxuscontorta@gmail.com, [4]qadarkhan1131@gmail.com, [5]adrianblake2222@gmail.com, [6]imtiazkhanusa@gmail.com, [7]zohan490ef@gmail.com, [8]shabanairum911@gmail.com, [9]malik0720741@gmail.com, [10]suhailahmud@gmail.com, [11]taraijaz0741@gmail.com, [12]habibanasarkhan.swat@gmail.com, [13]shazia.dilbar@uswat.edu.pk,

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Maryam

Abstract

Juniperus excelsa (Cupressaceae) and Tagetes minuta (Asteraceae) are aromatic, essential oil-bearing plant species reported from northern Pakistan. This study aimed to characterize their essential oils (EOs), formulate and evaluate EO-based nanoemulsions and microcapsules, and investigate their biological activities. Plant materials were collected from Sheringal, Upper Dir, Pakistan, and essential oils were extracted by steam distillation. Gas chromatography-mass spectrometry (GC-MS) analysis identified α-pinene and D-limonene as the major constituents of J. excelsa EO, whereas dihydrotagetone and (E)-ocimene predominated in T. minuta EO. The formulated nanoemulsions exhibited particle sizes of up to 50 nm and relatively uniform distributions, with T. minuta EO nanoemulsion showing smaller particle sizes than that of J. excelsa. Microencapsulation yields were 49.3% and 52.0% for T. minuta and J. excelsa, respectively, while the corresponding moisture contents were 4.28% and 4.01%. The resulting microcapsules exhibited comparable spherical morphologies and three distinct particle-size populations. Both essential oils demonstrated inhibitory

activity against the Gram-positive bacterial strains Bacillus atrophaeus and B. subtilis, although their inhibition zones were smaller than those produced by the reference antibiotics. Similarly, antifungal activity was observed against Aspergillus parasiticus and A. niger, but remained lower than that of the reference antifungal agents. Cytotoxicity assessments across concentrations of 50-1000 μg/mL indicated that the 50 μg/mL concentration of both EOs was non-toxic to normal fibroblast cells, suggesting favourable biocompatibility at this concentration. Overall, the findings demonstrate the potential of J. excelsa and T. minuta essential oils as sources of bioactive compounds and provide a basis for further investigation of their nanoformulations and possible pharmaceutical applications.

INTRODUCTION

Essential oils (EOs) are complex mixtures of volatile, hydrophobic secondary metabolites produced by different plant species. They are commonly obtained from leaves, flowers, fruits, seeds, bark, wood, and roots, primarily through distillation. These aromatic compounds have attracted considerable scientific interest because of their diverse chemical compositions and potential pharmacological, antimicrobial, and industrial applications (Baser & Buchbauer, 2010; Dima & Dima, 2015). Among essential oil-bearing plants, Juniperus excelsa M. Bieb. (Cupressaceae) and Tagetes minuta L. (Asteraceae) are important aromatic species with considerable phytochemical and biological significance.

The genus Juniperus comprises approximately 70 species worldwide, with five species reported from Pakistan (Adams, 2004; Kangshan et al., 2010). Junipers exhibit considerable ecological adaptability across different geographical regions and altitudinal ranges. They are particularly valued for their aromatic wood, essential oils, ornamental properties, and reported biological activities (Adams, 2004; Harrington & Fisher, 1999). Juniperus excelsa, commonly known as Greek juniper and locally referred to as Obashta or Sanober in Pakistan, is an evergreen tree or shrub distributed across parts of the Mediterranean, Middle East, and western Asia (Adams, 2004; Shanjani et al., 2010). The species has considerable medicinal and phytochemical importance. Previous investigations have identified several volatile constituents in its essential oil, including α-pinene, limonene, sabinene, cedrol, α-cedrene, terpinene-4-ol, camphene, myrcene, borneol, and germacrene D (Adams, 1990; Shanjani et al., 2010; Thappa et al., 1987; Unlu et al., 2008). Furthermore, extracts and essential oils of J. excelsa have demonstrated antioxidant, antimicrobial, antitumour, and other pharmacological activities (Khanavi et al., 2019; Nabi et al., 2012; Ozturk et al., 2011; Unlu et al., 2008).

Similarly, Tagetes minuta, commonly known as wild marigold, is an aromatic member of the family Asteraceae, native to South America but now widely distributed across different continents (Cornelius & Wycliffe, 2016). The species has diverse applications in food, perfumery, cosmetics, and traditional medicine (Amat, 1983; Cornelius & Wycliffe, 2016). Its essential oil has received considerable attention because of its complex chemical composition and biological properties. Previous investigations have reported diverse volatile constituents, including tagetones and other oxygenated and hydrocarbon compounds, with variations according to geographical origin, harvesting season, developmental stage, and plant part (Chalchat et al., 1995; El-Deeb et al., 2004; Wanzala & Ogoma, 2013). The species has also demonstrated antimicrobial, antioxidant, insect-repellent, and cytotoxic potential, supporting its importance as a source of naturally derived bioactive compounds (Ali et al., 2014; Dos Santos et al., 2017; Karimian et al., 2014; Salehi et al., 2018).

Despite their promising chemical and biological properties, the practical application of essential oils is often limited by their volatility, hydrophobicity, environmental sensitivity, and susceptibility to degradation. These limitations can affect their stability, solubility, and biological performance (Baser & Buchbauer, 2010; Dima & Dima, 2015). Nanoemulsion technology offers a potential approach to overcoming some of these challenges by dispersing oil droplets into an aqueous phase at nanoscale dimensions. Nanoemulsions generally exhibit small droplet sizes and useful physicochemical properties,

making them attractive for pharmaceutical, food, and cosmetic applications (Ee et al., 2008; Gupta et al., 2016). Their properties can be adjusted through formulation composition and processing conditions, while their applications include different routes of drug delivery and the incorporation of poorly water-soluble bioactive compounds (Calligaris et al., 2016; Kumar et al., 2019; Wilson et al., 2022).
Considering the phytochemical diversity and reported biological properties of J. excelsa and T. minuta, their essential oils represent promising candidates for nanoemulsion-based formulations. However, investigating the chemical composition of oils obtained from specific plant organs and evaluating their biological activities in formulated systems can provide further insight into their potential applications. Therefore, the present study focuses on the extraction and GC–MS characterization of essential oils from J. excelsa leaves and T. minuta seed cones, followed by the formulation and physicochemical characterization of their respective nanoemulsions. The antioxidant, antimicrobial, and cytotoxic activities of the essential oil-based nanoemulsions are subsequently evaluated to assess their biological potential and suitability for possible pharmaceutical applications.

2. MATERIALS AND METHODS

2.1. Preparation of Essential Oils

Fresh leaves and seed cones of Juniperus excelsa and aerial parts of Tagetes minuta were collected, cleaned, and chopped into small pieces before extraction. Essential oils (EOs) were extracted separately by hydrodistillation using a Clevenger-type apparatus. Briefly, 300 g of each plant material was subjected to hydrodistillation for 4 h with a sufficient volume of distilled water. The essential oils were separated from the aqueous phase and collected from the upper oil layer of the apparatus. The extraction procedure was repeated until sufficient quantities of essential oils were obtained. The extracted oils were weighed, and their yields were calculated relative to the dry weight of the plant material. Their physical characteristics, including colour, odour, density, and viscosity, were also recorded. Finally, the essential oils were transferred into airtight containers and stored at 4 °C until further analysis. Hydrodistillation is a widely used method for extracting volatile constituents from aromatic plants, including Juniperus species (Semerdjieva et al., 2019; Shanjani et al., 2010; Baser & Buchbauer, 2010).

2.2. Chemical Composition of Essential Oils

The chemical composition of the essential oils was determined using gas chromatography-mass spectrometry (GC-MS). The analysis was performed using a gas chromatograph equipped with an HP-5MS capillary column (5% phenyl methyl siloxane; 30 m × 0.25 mm internal diameter × 0.25 μm film thickness) coupled to a mass-selective detector operating in electron-impact (EI) ionisation mode at 70 eV. A flame ionisation detector (FID) was also used for chromatographic detection. Helium was employed as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was programmed to increase gradually at rates ranging from 1 to 6 °C/min according to the analytical programme. Samples were diluted in pentane and injected using a split ratio of 1:1000. Mass spectra were recorded over a scanning range of m/z 50–800. The retention times and mass spectral profiles of the individual constituents were recorded for subsequent identification and characterisation. GC-MS is a well-established analytical technique for characterising volatile constituents and determining the chemical profiles of essential oils (Rawat et al., 2007; Adams, 1990; Zheljazkov et al., 2018).

2.3. Identification of Chemical Compounds

The volatile constituents of the essential oils were identified by comparing their mass spectra and calculated retention indices (RIs). Retention indices were calculated using a homologous series of n-alkanes analysed under the same chromatographic conditions. The obtained mass spectra were compared with reference spectra available in commercial spectral libraries, including the National Institute of Standards and Technology (NIST) and Wiley mass spectral libraries. Compound identification was further supported by comparing the calculated retention indices with published literature values and, where available, those of authentic reference compounds. The combined use of mass spectral matching and retention-index comparisons provides a reliable approach for identifying volatile constituents in essential oils (Adams, 2000; Chalchat et al., 1995; El-Deeb et al., 2004; Zheljazkov et al., 2018).

2.4. Preparation of Nanoemulsions

Nanoemulsions were prepared using the essential oils of J. excelsa and T. minuta as the oil phases, Tween 20 and Tween 80 as surfactants, ethylene glycol as a cosolvent, and deionised water as the aqueous phase. Tween 20 and Tween 80, with hydrophilic–lipophilic balance (HLB) values of approximately 16.7 and 15, respectively, were selected because of their hydrophilic characteristics and suitability for formulating oil-in-water (O/W) emulsions. Different surfactant-to-oil ratios (S/O) ranging from 0.2 to 1 were evaluated to obtain stable nanoemulsion formulations. The mixtures were homogenised using an ultrasonic processor operating at 20 kHz for 15 min, with a maximum power output of 200 W. The resulting formulations were evaluated for their suitability and stability as essential oil-based nanoemulsions. The formulation and stabilisation of nanoemulsions depend on the appropriate selection of surfactants, oil-to-surfactant ratios, and emulsification conditions (Gupta et al., 2016; Calligaris et al., 2016; Kumar et al., 2019; Wilson et al., 2022).

2.5. Morphological Characterisation by Scanning Electron Microscopy (SEM)

The morphology and structural characteristics of the essential oil-based nanoemulsions were examined using scanning electron microscopy (SEM). Before analysis, the nanoemulsion samples were appropriately prepared to obtain representative images of their morphological characteristics. The samples were diluted in ethanol, where necessary, to facilitate the separation and visualisation of dispersed droplets while minimising interference from the surrounding emulsion matrix. The prepared samples were subsequently subjected to microscopic examination to assess their surface morphology, particle distribution, and structural characteristics.

2.6. Biological Activities

2.6.1. Antioxidant Activity of Essential Oil-Based Nanoemulsions

The antioxidant potential of the essential oils and their corresponding nanoemulsions was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free-radical scavenging assay. The assay was performed following established procedures with slight modifications. All chemicals and solvents used were of analytical grade. Stock solutions of the essential oil samples were prepared by dissolving 500 μg of each sample in 1 mL of methanol. A 0.075 mM DPPH solution was prepared separately in methanol.

For the assay, 0.1 mL of each sample solution was mixed with 3.9 mL of DPPH solution. The mixture was homogenised at 2500 rpm for 2 min to ensure uniform mixing. The initial absorbance was recorded at 517 nm using a double-beam spectrophotometer. Subsequently, the reaction mixtures were incubated at room temperature for 30 min in the dark, after which their absorbance was measured again at 517 nm. The control consisted of 0.1 mL of methanol mixed with 3.9 mL of DPPH solution. The concentration required to scavenge 50% of the DPPH radicals (IC_{50}) was determined through serial dilutions and corresponding absorbance measurements. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ DPPH free radical scavenging} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad \text{(Eq. 1)}$$

The DPPH assay is widely employed to evaluate the free-radical scavenging potential of plant extracts and essential oils (Ozturk et al., 2011; Orhan et al., 2011; Karimian et al., 2014; Bakkour et al., 2013).

2.6.2. Antimicrobial Activities

The antimicrobial potential of the essential oils and their nanoemulsions was evaluated against selected pathogenic bacterial and fungal strains. The bacterial panel included species belonging to the genera Escherichia, Haemophilus, Shigella, and Staphylococcus, whereas the fungal panel included representatives of Aspergillus and Candida. The microbial strains were obtained from the National Institute of Health (NIH), Islamabad, Pakistan, with their corresponding American Type Culture Collection (ATCC) identification codes. Standardised microbiological procedures were employed to evaluate the antibacterial and antifungal activities of the prepared formulations. The antimicrobial potential of essential oils from Juniperus and Tagetes species has previously been documented in several studies (Unlu et al., 2008; Tereschuk et al., 1997; Hamouda et al., 2001; Semerdjieva et al., 2020; Dos Santos et al., 2017).

2.6.3. Cytotoxic Activity and Statistical Analysis

The cytotoxic potential of the essential oil-based nanoemulsions was evaluated using the Alamar Blue assay against human hepatocellular carcinoma (HepG2) cells. The experiments were conducted at the Research Centre for Interdisciplinary Biomedical Research (RCBIR), COMSATS University Islamabad, Lahore Campus. HepG2 cell lines were obtained from the American Type Culture Collection (ATCC). Normal mouse fibroblast NIH/3T3 cells were also used to evaluate the potential cytotoxic effects of the formulations on non-cancerous cells and to identify suitable non-toxic concentrations. HepG2 cells were cultured in T25 tissue culture flasks under standard cell culture conditions until confluence was achieved. The cells were subsequently subcultured to obtain sufficient numbers for experimental analysis. Approximately 50,000 cells were seeded into each well of 24-well tissue culture plates and exposed to different concentrations of the prepared samples. The plates were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

Following incubation, the culture medium was removed, and Alamar Blue reagent was added to the wells at the specified working concentration. The plates were further incubated for 4 h at 37 °C under 5% CO_2 in the dark. The resulting dye solution was transferred into 96-well plates, with nine replicates prepared for each experimental treatment. Absorbance was recorded at 570 nm using a microplate reader. Untreated cells served as the negative control. The absorbance readings were subsequently converted into percentages relative to the control to determine the effects of the treatments on cell viability. The use of Alamar Blue assays for evaluating the cytotoxic potential of plant-derived compounds and natural products has been reported in previous studies (Nabi et al., 2012; Khanavi et al., 2019; Ali et al., 2014; Pant et al., 2021).

All experimental data were subjected to statistical analysis using appropriate statistical tests. Student's t-test was applied to assess differences between experimental treatments and their corresponding controls, where applicable. Results were expressed as arithmetic means ± standard error of the mean (SEM). Differences were considered statistically significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. RESULTS

3.1 Chemical composition of J. excelsa and T. minuta essential oil

Fifty compounds were identified by GC-MS analysis of the essential oil of Juniperus excelsa. The major constituents of the JEO are, α-pinene (53.44%) and D-limonene (30.28%), β-Linalool (4.99%), β-Myrcene (3.08%), Cedrol(2.85), β-Pinene(1.71%) and o-Cymene(1.71%). Several differences were observed with respect to previous studies, as Khanavi et al., [89] reported α-pinene as 73.27%, 79% and 67.71% respectively in JEO. Bakkour et al. [90] respectively D-limonene as 3.49 %, 15% and 4.52% in JEO. Our results show that the oil constituents were dominated by α-pinene (53.44%) and D-limonene (30.28%). It was found that D-limonene was comparatively greater compared to other components of J.excelsa Essential oil.

The analysis of TMO using GC-MS revealed that its primary constituents were dihydrotagetone, making up about 34.1% of the composition, followed by E-ocimene at 20.0 %, tagetone at 17.2%, cis-β-ocimene at 8.1%, Z-ocimene at 5.4%, limonene at 3.2%, and epoxyocimene at 2.1%. Several differences were observed with respect to previous studies, as EL-Deeb et al., [91] identified the main components of TMO using Mass Spectrometry (GC-MS) techniques. The primary components identified were tagetone at 11.52% concentration, 5-octyn-4-one,2,7-dimethyl at 11.52%, propanedinitrile, dicyclohexyl- at 10.45%, and 2-pinen-4-one at 8.03%. Additionally, smaller amounts of 1-acetoxy-p-menth-3-one at 0.17% and 9-octacenamide (Z) at 0.48% were also detected as part of the sample composition (Table 1).

Table 1. Comparative chemical composition and relative abundance of essential oil constituents of Juniperus excelsa and Tagetes minuta as determined by gas chromatography–mass spectrometry (GC–MS).

S. No.	Constituents	J. excelsa R. time (s)	Area	Conc. (%)	T. minuta R. time (s)	Conc. (%)
1	α-Thujene	7.943	24,758	0.09	–	–
2	α-Pinene	8.258	14,257,747	53.44	9.44	0.33

3	Camphene	8.847	164,692	0.62	–	–
4	Thujene	9.837	251,751	0.94	–	–
5	β-Pinene	10.008	455,192	1.71	–	–
6	β-Myrcene	10.624	821,434	2.08	–	–
7	α-Phellandrene	11.389	2,562	0.01	–	–
8	o-Cymene	12.193	456,354	0.71	–	–
9	D-Limonene / Limonene	12.422	8,079,583	30.28	10.3	3.1
10	Eucalyptol	12.525	13,149	0.05	–	–
11	Sabinene	–	–	–	9.8	0.4
12	cis-3-Hexenyl acetate	–	–	–	10.06	0.15
13	p-Cymene	–	–	–	10.35	0.93
14	cis-β-Ocimene	–	–	–	10.38	7.9
15	Dihydrotagetone	–	–	–	10.62	33.8
16	allo-Ocimene	–	–	–	11.36	0.36
17	E,Z-Epoxyocimene	–	–	–	11.5	2
18	Tagetone	–	–	–	11.61	16.1
19	cis-Tagetone	–	–	–	11.8	0.2
20	p-Mentha-1,8-dien-3-one	–	–	–	12.09	0.18
21	Z-Ocimene	–	–	–	12.4	5.3
22	E-Ocimene	–	–	–	12.51	19.9
23	β-Linalool	15.92	1,331,341	3.99	–	–
24	L-Pinocarveol	17.631	32,747	0.12	–	–
25	Borneol	18.771	36,782	0.14	–	–
26	p-Menth-1-en-4-ol	19.022	109,869	0.41	–	–
27	p-Cymen-8-ol	19.307	99,413	0.37	–	–
28	p-Menth-1-en-8-ol	19.494	71,606	0.27	–	–
29	Thymol	–	–	–	12.83	0.48
30	Carvacrol	–	–	–	12.97	0.47
31	cis-Isoeugenol	–	–	–	13.99	0.86
32	L-Carvone	20.751	90,355	0.34	–	–
33	p-Menthan-3-one, 1,2-epoxy-	21.417	5,902	0.02	–	–
34	Caryophyllene	24.053	166,950	0.63	–	–
35	trans-α-Bergamotene	23.875	1,668	0.01	–	–
36	Alloaromadendrene	24.202	48,439	0.18	–	–
37	α-Caryophyllene	24.682	3,138	0.01	–	–
38	Humulen-(v1)	24.833	7,359	0.03	–	–
39	Germacrene D	25.525	16,400	0.06	–	–
40	β-Bisabolene	25.399	19,921	0.07	–	–
41	Germacrene D	–	–	–	14.98	0.43
42	E-Caryophyllene	–	–	–	14.2	0.32
43	α-Humulene	–	–	–	14.54	0.15
44	Epiglobulol	26.288	2,940	0.01	–	–
45	Ledol	26.468	19,600	0.07	–	–
46	Spathulenol	26.468	19,600	0.07	15.83	0.42

47	Globulol	26.468	20,416	0.08	–	–
48	Viridiflorol	26.553	17,069	0.06	–	–
49	Cedrol	26.991	783,262	2.85	–	–
50	(+)-Selin-7(11)-en-4,α-ol	27.49	16,731	0.06	–	–
51	α-Bisabolol	27.838	14,988	0.06	–	–

3.2 Nanoemuulsion

The nano-emulsion produced with EO extracted from 2 different species different plants demonstrate different particle size and volume frequency distribution. As shown in (Figure 1) all of the emulsions present two peaks in droplet curve frequency. The lowest particle size was observed around 17 to 20 nm size for all of the emulsions, however; the frequency distribution was recorded too low. Another peak was observed around 25 to 40 nm particle size with maximum frequency distribution (16 %). These results clearly indicate that emulsion was composed of particle size range up to 50 nm in two different categories. Overall, the emulsions present fair distribution but it was interesting to observed a significant decrease in particle size for different plant species i.e., EO from T. minuta present lowest particle size in emulsion with lower frequency rate. The lower rate was possibly because the emulsion slightly demolishing and separation of interface, while the highest rate was observed for J.Excelsa EO emulsion.

The similar trend was also observed in emulsion microscopy. The freshly prepared nano-emulsion was used to capture the microscopic pictures of oil droplets. The results were evident that no flocculation was observed among droplet at zero time with fair distribution in aqueous phase. A think dark interface was observed around the oil droplet which was possibly the combination of EO, olive oil and standard protein. Generally speaking, as EO have volatile are in nature and a fast moving of droplet was observed at microscopic slide. The moving of droplet indicates the evaporation of EO from emulsion interface and resulting the rapture of emulsion interface after a while. However, the microphotograph of the emulsions present similar sizes and distribution as earlier noted in frequency distribution results.

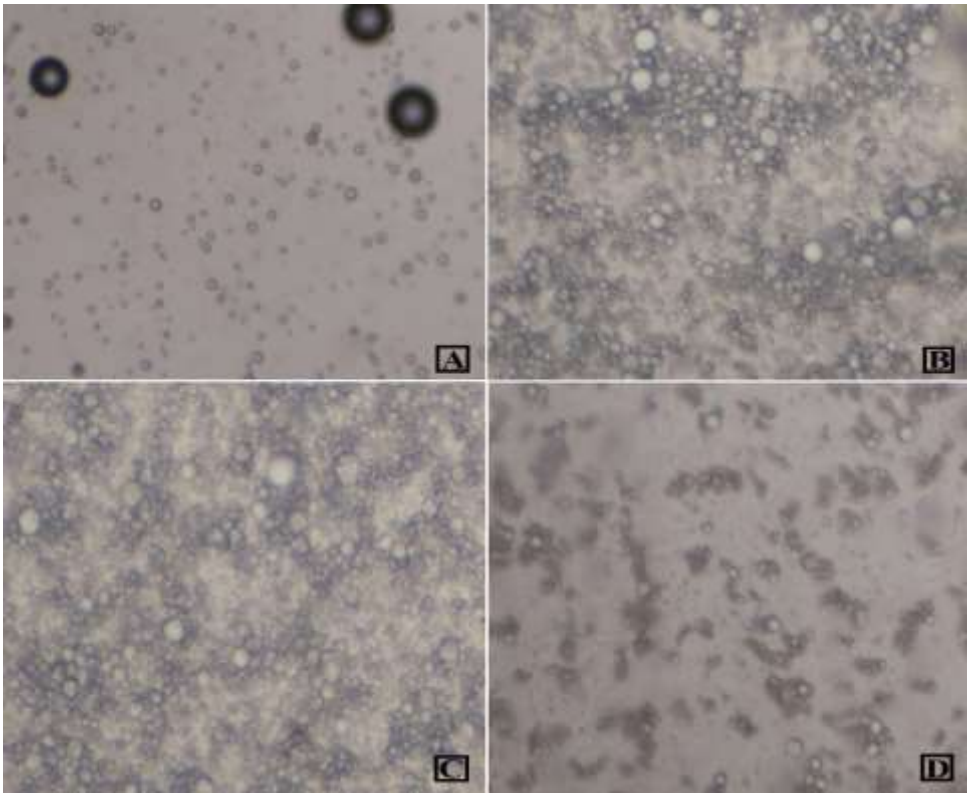

Figure 1. Microscopic characterisation of essential oil-based nanoemulsions of Tagetes minuta (A, B) and Juniperus excelsa (C, D).

3.3. SEM T. minuta and J. excelsa EOs

The microencapsulation yields were 49.3% for T. minuta and 52% for j excelsa essential oils, while the moisture content results were 4.28 % and 4.01% for T. minuta and J. excelsa Eos, respectively (Figure 2). The observed morphologies for both microcapsules (MC) were similar, exhibiting spherical particles with smooth surfaces and occasional concave depressions. Both types of MC displayed three main particle size populations, constituting 96.08% and 99.02% of the measured particles for T. minuta and J. excelsa Eos, respectively. T. minuta featured smaller particles with mean sizes of 6.12, 118.18, and 665.10 µm for each population and the entire sample (D10 = 5.5o µm; D50 = 6.50 µm; D90 = 375.14 µm). In contrast, J. excelsa showed mean sizes of 7.0, 111.8, and 3751.09 µm for separate distributions (D10 = 7.04 µm; D50 = 3511.44 µm; D90 = 4227.36 µm).

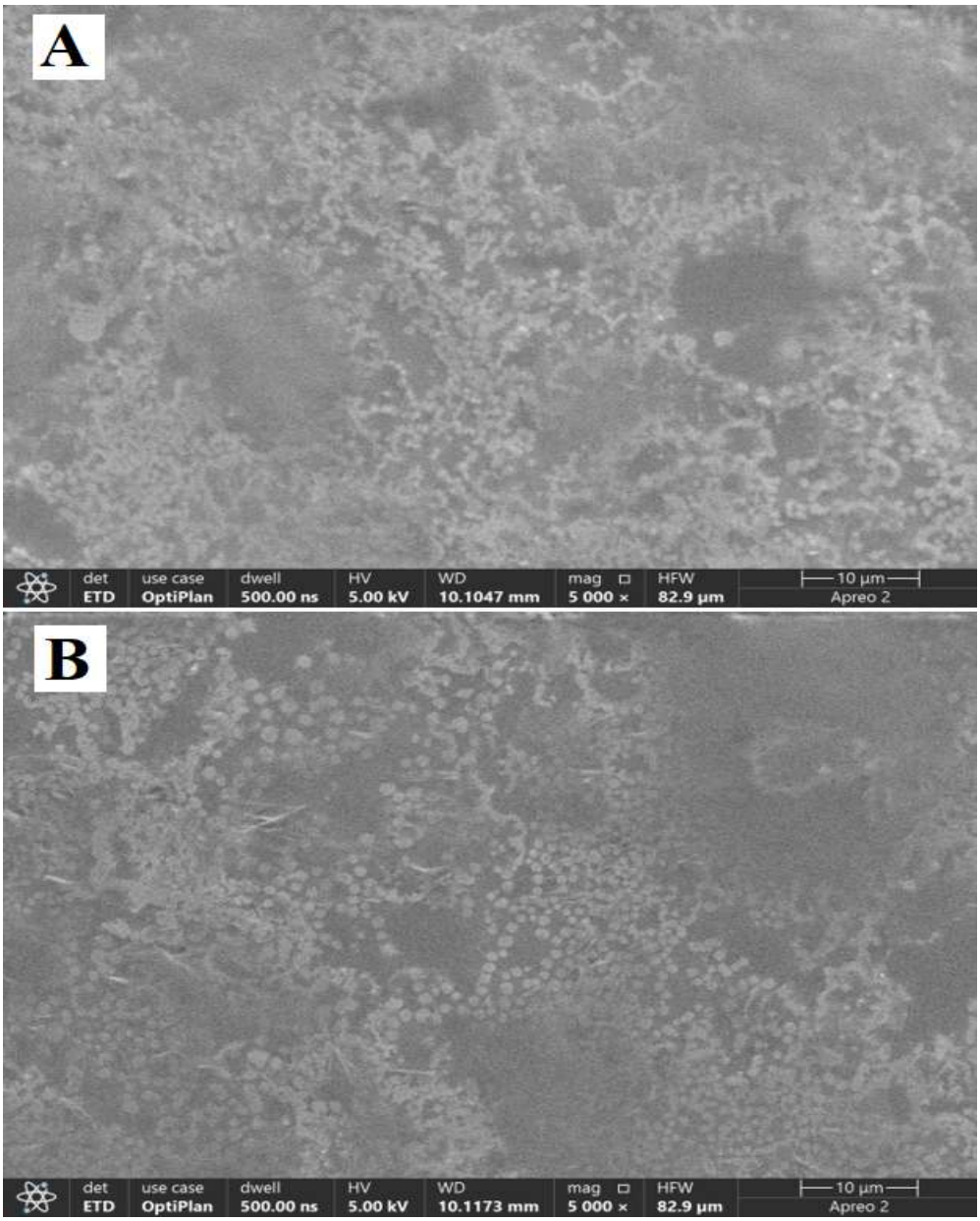

Figure 2. Scanning electron microscopy (SEM) images showing the microcapsule morphology of essential oils of Tagetes minuta (A) and Juniperus excelsa (B).

3.4. Antimicrobial Activities

The results of an antimicrobial screening test for T.minuta essential oil (EO) against different strains of bacteria and fungi. The values represent the zones of inhibition in millimeters (mm) at two different concentrations (50 µg/ml and 100 µg/ml) of the essential oil. Additionally, reference antibiotics (Levofloxacin, Erythromycin, and Fluconazole) and DMSO (a control) were used for comparison. For Gram-Negative Bacterial Strains (S. typhi and K. pneumonia), at both concentrations of the essential oil (50 µg/ml and 100 µg/ml), there was a zone of inhibition. However, Levofloxacin (an antibiotic) showed larger zones of inhibition. For Gram-Positive Bacterial Strains (B. atrophaeus and B. subtilis), the essential oil also exhibited zones of inhibition at both concentrations, although Erythromycin (an antibiotic) had larger inhibitory zones. In the case of Fungal Strains (A. parasiticus and A. niger), the essential oil displayed zones of inhibition at both concentrations, but Fluconazole (an antifungal medication) showed larger inhibitory zones (Table 2). The results indicate the antimicrobial potential of T.minuta essential oil, but it appears to be less effective than the antibiotics and antifungal agents used as references in this study.

Table 2. Zones of inhibition in the antimicrobial screening of Tagetes minuta essential oil.

Microbial group	Test microorganism	Sample	50 µg/mL	100 µg/mL
Gram-negative bacteria	S. typhi	EO	13.6 ± 0.06*	16.7 ± 0.01*
		Levofloxacin	16.9 ± 0.04	20.8 ± 0.02
		DMSO	–	–
	K. pneumoniae	EO	12.8 ± 0.04*	16.6 ± 0.05*
		Levofloxacin	16.4 ± 0.02	20.2 ± 0.03
		DMSO	–	–
Gram-positive bacteria	B. atrophaeus	EO	14.6 ± 0.03*	17.7 ± 0.04*
		Erythromycin	17.4 ± 0.02	21.8 ± 0.05
		DMSO	–	–
	B. subtilis	EO	15.2 ± 0.04*	18.8 ± 0.02*
		Erythromycin	16.8 ± 0.04	21.5 ± 0.03
		DMSO	–	–
Fungal strains	A. parasiticus	EO	14.04 ± 0.04*	19.07 ± 0.02*
		Fluconazole	15.2 ± 0.04	19.3 ± 0.02
		DMSO	–	–
	A. niger	EO	14.04 ± 0.02*	18.2 ± 0.04*
		Fluconazole	16.4 ± 0.02	20.5 ± 0.06
		DMSO	–	–

The reference antibiotics and antifungal agent generally produced larger inhibition zones than the EO, indicating that the essential oil showed promising but comparatively lower antimicrobial activity under the tested conditions. No inhibition was recorded for the DMSO negative control, supporting the conclusion that the observed effects were associated with the tested antimicrobial preparations rather than the solvent. Overall, T. minuta essential oil demonstrated broad-spectrum in vitro antimicrobial potential, particularly against B. subtilis and the tested fungal strains (Figure 3). Further investigations, including MIC and MBC/MFC assays, are required to establish its potency and determine its potential applications.

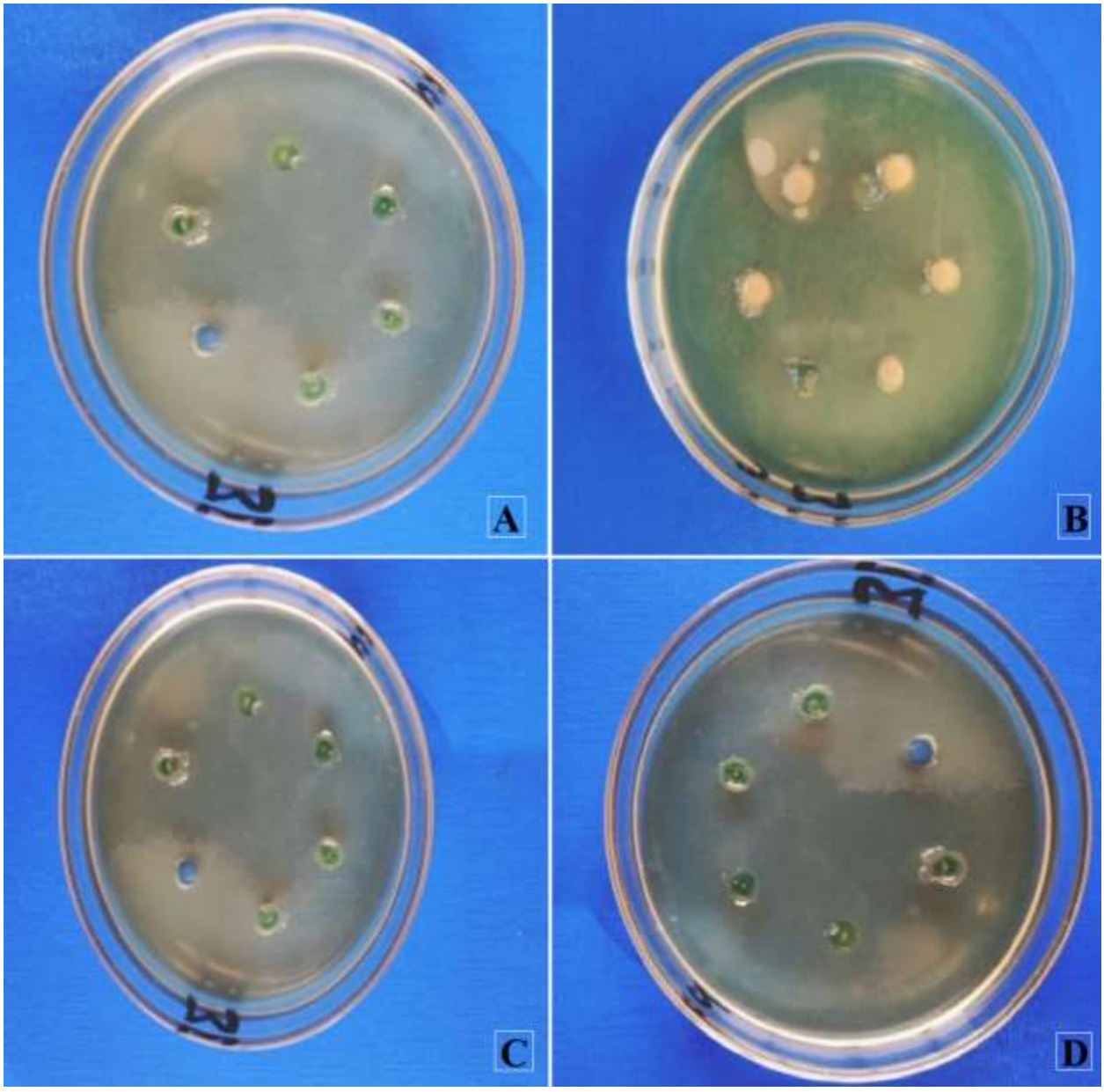

Figure 3. Zones of inhibition demonstrating the antimicrobial activity of Tagetes minuta essential oil against selected bacterial and fungal strains.

3.5 Normal and cancer Cell Line assays

Standard procedures were carried out in determining the anticancer effect [92]. The cancer cell lines $HepG_2$ were purchased from ATCC. The essential oils of J. excelsa and T. minuta were selected in a dose range of 50-1000 µg/mL of the cell culture media (3 replicate each). The dose of 50 µg/mL was selected based on a study of effectiveness done using normal mouse fibroblast NIH_3T_3 cell line. The normal fibroblast cell line showed high biocompatibility at 50 µg/mL exposure for 24 h which means this dose was non-toxic to the normal non-cancerous cells. The cancer cell lines $HepG_2$ were cultured in T_{25} tissue culture flasks till confluence. The cells were passaged and cultured till appropriate number was obtained. At least 50000 cells were pre-seeded in 24 well Tissue culture plates with 50 µg/mL of both oils. The cells were incubated for 24 h at 37 °C with 5% CO_2. After 24 h, the media was removed from the wells containing cells and 0.02 M Alamar Blue dye was added for 4 h and incubated at 37 °C with 5 % CO_2 in dark. Then the Alamar blue was pipetted in 96 well plates and 3 replicates each of the variants were plated. The absorbance readings were taken in an absorbance plate reader (make model) at 570 nm wavelength for each sample as well as of the control (cell only on tissue culture plastic without any extract). The readings

were converted in percentage to evaluate the difference with that of control samples. For nano emulsions 50 μg/mL samples were applied (having 100 ppm oil concentration).

Juniper excelsa oil dominated by α-pinene and D-limonene; Tagetes minuta oil primarily contained dihydrotagetone. Emulsions showed varying particle size distributions; Tagetes minuta had smaller particles. Significant differences observed in antimicrobial effects compared to reference antibiotics. Variations noted in microencapsulation and nanoemulsion characteristics between the two plant oils. Tagetes minuta and Juniper excelsa oils exhibited differing yields and moisture contents. Microcapsules displayed distinct size populations for each oil. Tagetes minuta oil tested against bacteria/fungi at 50 μg/mL and 100 μg/mL. Zones of inhibition measured; compared with Levofloxacin, Erythromycin, and Fluconazole. Juniper excelsa and Tagetes minuta oils at 50-1000 μg/mL tested on HepG2 cells; 50 μg/mL non-toxic to normal cells.

4. Discussion

4.1. Chemical composition of essential oils

The GC–MS analysis revealed distinct chemical profiles for the essential oils of Juniperus excelsa and Tagetes minuta, reflecting differences in their major volatile constituents. The essential oil of J. excelsa was predominantly composed of α-pinene (53.44%) and D-limonene (30.28%), which together accounted for approximately 83.72% of the reported composition. Other notable constituents included β-linalool (3.99%), β-myrcene (2.08%), cedrol (2.85%), and β-pinene (1.71%). This profile indicates that the oil was principally characterised by monoterpene hydrocarbons, particularly α-pinene and D-limonene, accompanied by smaller proportions of oxygenated terpenoids and sesquiterpenes. The predominance of α-pinene in the present study is consistent with previous investigations reporting this compound as a major constituent of J. excelsa essential oil. Adams (1990) documented the chemical composition of J. excelsa leaf oil, while Shanjani et al. (2010) demonstrated that essential-oil composition can vary according to the plant part, drying conditions, and harvesting season. Similarly, Unlu et al. (2008) reported α-pinene as an important constituent of J. excelsa essential oil. Khanavi et al. (2019) also identified α-pinene as a major component of the leaf oil. However, the relative abundance of α-pinene in the present study (53.44%) differs from the higher proportions reported in some previous investigations. Such differences may be associated with geographical origin, environmental conditions, plant developmental stage, plant material, and extraction procedures.

An important feature of the present J. excelsa oil was its relatively high D-limonene content (30.28%). This proportion is notably higher than the values reported by Bakkour et al. (2013) for essential oils obtained from green and ripe J. excelsa berries. The variation suggests that D-limonene abundance may be influenced by differences in plant material and growing conditions. Previous studies have also demonstrated considerable chemical variation among Juniperus species and populations, supporting the existence of geographical and ecological influences on essential-oil composition (Cantrell et al., 2013; Zheljazkov et al., 2018, 2021). Furthermore, Shanjani et al. (2010) showed that harvesting season and post-harvest drying can influence the relative proportions of volatile compounds. The comparatively high D-limonene concentration observed here therefore represents a notable characteristic of the analysed J. excelsa oil, although additional seasonal and population-level sampling would be required to determine whether this profile is consistent across the species. The chemical composition of T. minuta essential oil was markedly different from that of J. excelsa. Its major constituents were dihydrotagetone (33.8%), E-ocimene (19.9%), tagetone (16.1%), cis-β-ocimene (7.9%), Z-ocimene (5.3%), limonene (3.1%), and E,Z-epoxyocimene (2.0%). These compounds indicate that the oil was primarily characterised by oxygenated monoterpenes and ocimene-related compounds. The predominance of dihydrotagetone, together with tagetone and ocimene derivatives, is consistent with the characteristic chemical profile frequently reported for T. minuta essential oil.

Nevertheless, considerable variation has been documented in the chemical composition of T. minuta oils from different geographical regions. Chalchat et al. (1995) demonstrated that the essential-oil composition of T. minuta varies according to harvesting period, geographical location, growth stage, and the plant part extracted. Similarly, El-Deeb et al. (2004) reported a different chemical profile in material collected in Saudi Arabia, in which tagetone was a major constituent, accompanied by several other

compounds not detected in the present analysis. Differences between the present findings and those of El-Deeb et al. (2004) may reflect geographical and environmental variation, differences in plant material, and analytical or extraction conditions. Other investigations have also reported variation in the composition and biological properties of T. minuta essential oil (Ali et al., 2014; Salehi et al., 2018; Wanzala & Ogoma, 2013). Taken together, the contrasting profiles of the two oils demonstrate that J. excelsa was characterised mainly by α-pinene and D-limonene, whereas T. minuta was dominated by dihydrotagetone, E-ocimene, and tagetone. These differences are important when interpreting their biological activities and selecting suitable formulations for further investigation. Essential-oil composition is closely associated with biological properties, and differences in the abundance and combination of volatile constituents may contribute to variation in antimicrobial and other bioactivities (Baser & Buchbauer, 2010; Salehi et al., 2018).

4.2. Nanoemulsion characteristics

The nanoemulsions prepared using the essential oils of J. excelsa and T. minuta exhibited differences in their droplet-size distributions. Both formulations showed two principal frequency peaks: a less prominent population at approximately 17–20 nm and a more abundant population within the 25–40 nm range. The occurrence of two distinct populations suggests a heterogeneous droplet-size distribution rather than a completely uniform nanoemulsion system. Nevertheless, the observed droplet sizes, which extended to approximately 50 nm, indicate the formation of finely dispersed oil droplets under the applied preparation conditions. The T. minuta formulation showed a shift towards smaller droplet sizes, although this population had a relatively low frequency, whereas the J. excelsa formulation exhibited a higher frequency in the predominant droplet-size range. These differences may be related to the distinct chemical compositions of the oils, particularly their relative proportions of monoterpene hydrocarbons and oxygenated monoterpenes. Differences in the physicochemical properties of oil phases can influence interfacial behaviour and droplet formation during emulsification. However, the present observations alone do not establish a direct relationship between individual chemical constituents and droplet size.

Nanoemulsion formation and stability are influenced by several formulation parameters, including oil composition, surfactant concentration, interfacial tension, homogenisation conditions, and droplet-size distribution (Gupta et al., 2016; Wilson et al., 2022). Previous research has demonstrated that droplet size can be affected by the preparation technique and processing conditions, including high-pressure homogenisation and ultrasound-assisted emulsification (Calligaris et al., 2016; Ee et al., 2008). Moreover, droplet size is an important factor influencing the physical and oxidative stability of oil-in-water emulsions (Nakaya et al., 2005). Microscopic observations further supported the formation of dispersed oil droplets in both formulations. At the initial observation, the droplets appeared reasonably distributed throughout the aqueous phase, with no evident flocculation. The dark interfacial appearance surrounding individual droplets may represent the oil–water interface and associated formulation components. The apparent movement and subsequent disappearance or disruption of droplets during microscopic observation may indicate limited physical stability or possible volatilisation of essential-oil constituents. However, these observations should be interpreted cautiously because droplet movement, evaporation, and interfacial disruption cannot be conclusively distinguished through microscopy alone. Consequently, additional measurements, including changes in droplet size, polydispersity index, zeta potential, and phase separation during storage, would be necessary to establish the long-term stability of the formulations.

4.3. Microencapsulation yield, moisture content, and morphology

The microencapsulation process produced yields of 49.3% for T. minuta and 52.0% for J. excelsa. The slightly higher yield obtained for J. excelsa indicates a modest difference in encapsulation performance between the two oils under the applied experimental conditions. The moisture contents were relatively similar, measuring 4.28% for T. minuta and 4.01% for J. excelsa. These results suggest that both microencapsulated preparations had comparable residual moisture contents, although their storage stability would also depend on encapsulating-wall composition, environmental humidity, and packaging conditions. SEM observations revealed broadly similar morphological characteristics in the microcapsules of both oils. The particles were generally spherical, with relatively smooth surfaces and occasional concave

depressions. Such morphological features are relevant to the physical characteristics of encapsulated materials because particle structure and surface properties may influence the protection and release of entrapped compounds. The formation of spherical microcapsules is also consistent with the use of emulsion-based encapsulation approaches for essential oils and other hydrophobic materials. Li et al. (2022), for example, demonstrated the formation of essential-oil-containing composite microcapsules using a Pickering-emulsion-based approach.

Despite their broadly similar morphology, the two formulations exhibited different particle-size distributions. The reported mean particle sizes for T. minuta were 6.12, 118.18, and 665.10 µm, whereas those for J. excelsa were 7.0, 111.8, and 3751.09 µm across the three reported populations. The larger third population in the J. excelsa formulation suggests the presence of substantially larger particles or aggregates compared with the corresponding T. minuta population. This difference may be relevant to formulation uniformity and subsequent release behaviour. However, particle-size measurements should be interpreted alongside the distribution percentiles and the analytical method used, as the presence of a small number of large particles can substantially influence the reported mean. Overall, the results indicate that the two essential oils could be incorporated into microcapsules with broadly comparable yields, moisture contents, and surface morphologies, but with differences in their particle-size distributions. These findings highlight the importance of evaluating both encapsulation efficiency and particle characteristics when developing essential-oil-based formulations.

4.4. Antimicrobial activity of Tagetes minuta essential oil

The antimicrobial screening demonstrated that T. minuta essential oil inhibited all six tested microorganisms, including two Gram-negative bacteria, two Gram-positive bacteria, and two fungal species. Inhibition zones increased with increasing EO concentration from 50 to 100 µg/mL, indicating a concentration-related response under the experimental conditions. Among the Gram-negative bacteria, Salmonella typhi showed an inhibition zone of 16.7 ± 0.01 mm at 100 µg/mL, while Klebsiella pneumoniae showed 16.6 ± 0.05 mm. These results indicate that the oil was active against both tested Gram-negative organisms, although the reference antibiotic, levofloxacin, produced larger inhibition zones. Among the Gram-positive bacteria, Bacillus subtilis exhibited the largest inhibition zone produced by the essential oil (18.8 ± 0.02 mm), followed by B. atrophaeus (17.7 ± 0.04 mm) at 100 µg/mL. The comparatively higher inhibition against B. subtilis suggests that susceptibility differed among the bacterial species tested. A similar variation was observed in the fungal assays: Aspergillus parasiticus showed an inhibition zone of 19.07 ± 0.02 mm, whereas A. niger showed 18.2 ± 0.04 mm at the higher EO concentration. Nevertheless, fluconazole produced larger inhibition zones than the essential oil against both fungal strains.

The observed antimicrobial activity is consistent with previous investigations demonstrating the biological potential of T. minuta and its essential-oil constituents. Tereschuk et al. (1997) reported antimicrobial activity in flavonoids isolated from T. minuta leaves, while Ali et al. (2014) investigated the chemical composition and antimicrobial properties of its essential oil. Dos Santos et al. (2017), in their systematic review, also highlighted the antimicrobial potential of T. minuta and identified opportunities for further investigation. Similarly, Salehi et al. (2018) discussed the chemical diversity and biological activities of essential oils and extracts from Tagetes species. The antimicrobial effects observed in the present study may be associated with the combined activity of the major constituents, particularly dihydrotagetone, tagetone, E-ocimene, and other volatile compounds. Essential oils are complex mixtures, and their biological effects may involve interactions among multiple constituents rather than the activity of a single compound. However, the present study did not independently evaluate the antimicrobial activity of individual constituents; therefore, their specific contributions cannot be established. The differences between bacterial and fungal responses may also reflect differences in microbial cell-envelope structure and susceptibility to hydrophobic compounds.

The reference treatments produced larger inhibition zones than the EO in most comparisons, indicating that the oil was less inhibitory than the standard antimicrobial agents under the tested conditions. The absence of inhibition with DMSO supports its suitability as a negative solvent control. Overall, the findings demonstrate in vitro antibacterial and antifungal activity of T. minuta essential oil, with

comparatively greater inhibition against B. subtilis and A. parasiticus at 100 µg/mL. Nevertheless, inhibition-zone measurements alone do not establish the minimum concentration required to inhibit or kill microorganisms. Further evaluation through minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) assays would provide a more comprehensive assessment of antimicrobial potency.

4.5. Cytotoxicity and potential anticancer activity

The cytotoxicity experiment was designed to investigate the effects of J. excelsa and T. minuta essential oils on the human liver cancer cell line HepG2, while using the normal mouse fibroblast cell line NIH/3T3 to assess cellular compatibility. The preliminary selection of 50 µg/mL based on the reported response of NIH/3T3 cells provides a useful starting point for evaluating the effects of the oils on cancer cells. However, the results of the HepG2 assay, including cell viability percentages, concentration-dependent responses, and statistical comparisons, have not been provided. Therefore, no conclusion can yet be drawn regarding the anticancer activity or selectivity of either essential oil.

Previous studies provide a basis for investigating the cytotoxic potential of these species. Khanavi et al. (2019) examined the cytotoxic activity of J. excelsa leaf essential oil against breast cancer cell lines, while Nabi et al. (2012) investigated antitumour activity in extracts and fractions obtained from J. excelsa berries. For T. minuta, Ali et al. (2014) reported cytotoxic activity alongside its chemical and antimicrobial characterisation. These studies support further investigation of the biological properties of both species, but their findings cannot be directly extrapolated to the present HepG2 model because the plant materials, preparations, cell lines, and experimental conditions differ. To establish whether either oil has selective anticancer potential, the HepG2 results should be interpreted in relation to the response of normal NIH/3T3 cells across the same concentration range. A comparison of cancer-cell and normal-cell viability, together with calculation of the half-maximal inhibitory concentration (IC_{50}) and selectivity index, would provide stronger evidence of potential selectivity. The use of dose–response analysis and appropriate positive and vehicle controls would further strengthen the interpretation.

The comparative findings demonstrate that J. excelsa and T. minuta produce essential oils with substantially different chemical compositions and formulation characteristics. The dominance of α-pinene and D-limonene in J. excelsa, in contrast to the dihydrotagetone-, ocimene-, and tagetone-rich profile of T. minuta, provides a chemical basis for investigating their different biological and physicochemical properties. Both oils were successfully incorporated into nanoemulsion and microencapsulation systems, although differences were observed in droplet-size distributions, encapsulation yields, and particle-size populations. Furthermore, T. minuta essential oil demonstrated in vitro antimicrobial activity against the tested bacterial and fungal strains, although its inhibition zones were generally smaller than those produced by the reference drugs. These findings support the potential of both species as sources of bioactive essential oils and demonstrate the importance of formulation strategies for their further investigation. Nevertheless, additional studies are required to establish formulation stability, antimicrobial potency, and cytotoxic selectivity. In particular, quantitative cytotoxicity results, MIC/MBC/MFC determinations, and stability assessments would be necessary before drawing conclusions about their pharmaceutical or other practical applications.

CONCLUSION

The present study provides a comparative assessment of the chemical composition, formulation characteristics, microencapsulation properties, and biological activities of essential oils (EOs) obtained from Juniperus excelsa and Tagetes minuta collected from Sheringal, Upper Dir, Pakistan. GC–MS analysis revealed distinct chemical profiles between the two species. The essential oil of J. excelsa was dominated by α-pinene and D-limonene, whereas T. minuta was characterised primarily by dihydrotagetone, E-ocimene, and tagetone. These differences highlight the distinct chemical compositions and potential applications of the two essential oils. Both oils were successfully incorporated into nanoemulsion and microencapsulation systems, demonstrating their suitability for further formulation development. The microencapsulated oils exhibited spherical particle morphology, with relatively smooth surfaces, and showed comparable encapsulation yields and moisture contents.

Furthermore, T. minuta essential oil demonstrated concentration-dependent antimicrobial activity against the tested Gram-positive and Gram-negative bacteria and fungal strains, although its inhibitory effects were generally lower than those of the corresponding standard antimicrobial agents. Its comparatively strong activity against Bacillus subtilis and Aspergillus parasiticus indicates promising in vitro antimicrobial potential. Collectively, these findings establish J. excelsa and T. minuta as valuable sources of chemically distinct essential oils and provide a foundation for their potential development in pharmaceutical, food-preservation, and other applied formulations. However, further investigations are required to evaluate the chemical stability and long-term performance of the nanoemulsions and microcapsules, determine minimum inhibitory and fungicidal concentrations, and assess the cytotoxicity and safety of the formulations. Such studies will be essential to validate their practical applications and support the development of effective, plant-based products.

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