



Anti-Biofilm Efficacy of a Salep Mucilage–Based Pad Containing Nanoethosomal *Allium Jesdianum* Essential Oil Boiss. for Controlling Healthcare-Associated Infections

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Abstract

In this study, nanoethosomes (NE_{th}) solution was made with a mixture of lecithin and ethanol at specific methods and conditions. Then essential oil of *Allium jesdianum* (AJ_{EO}) was obtained and combined with the NE_{th} solution to prepare NE_{th}-AJ_{EO} suspension containing lecithin. Next particle size, zeta potential, encapsulation properties, scanning electron microscope, and thermodynamic stability of resulting NE_{th}-AJ_{EO} were determined. The NE_{th}-AJ_{EO} suspension showed remarkable thermodynamic stability when its NE_{th} solution had enough lecithin (5% w/v) and AJ_{EO} (500 µg/mL) in diluted ethanol (35% v/v). Later electrospinning apparatus was employed to make anti-biofilm pad. In this process, the NE_{th}-AJ_{EO} suspension was coated with a mixture of Salep mucilage and polyvinylalcohol (SA_{MU}-PVA) as a shell. The resulting pad was tested against both reference and clinical strains of *Acinetobacter baumannii*. This prevalent pathogenic is responsible for community-acquired and healthcare-associated infections (HAIs). AJ_{EO} solution at a concentration of 50 µg/mL reduced the viability of A375 melanoma cells and HSF normal fibroblast cells to approximately 70%. At 500 µg/mL, a more pronounced cell-line reduction was observed, indicating a dose-dependent cytotoxic effect. Structural evaluation based on Fourier-transform infrared spectroscopy (FTIR) confirmed the attachment of NE_{th}-AJ_{EO} suspension in the anti-biofilm pad structure. The release-behavior rate of AJ_{EO} (attached with the anti-biofilm pad) demonstrated that it could be controlled without temperature dependence. The final pad (containing 10%wt. NE_{th}-AJ_{EO}) showed significant mechanical properties and exhibited robust anti-biofilm activity, effectively impacting both the growth and metabolic viability of *Acinetobacter baumannii* strains. Air–liquid interface biofilm formation revealed a notable decrease in both film thickness and surface coverage following treatment with the anti-biofilm pad. While the NE_{th}-AJ_{EO} showed high potential to be used as the cytotoxic agent on skin cancer cell lines, the prepared pad had anti-biofilm properties and underscored its therapeutic potential, which is against HAIs.

Keywords Anti-biofilm · *Acinetobacter baumannii* · Salep mucilage · Electrospun pad · Nanoethosome · *Allium jesdianum* Boiss

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Abbreviations

AFM	Atomic force microscopy
Anti-biofilm pad	Salep mucilage and poly (vinyl alcohol) nanofibers containing nanoethosome containing <i>Allium jesdianum</i> Boiss-essential oil
AJ _{EO}	<i>Allium jesdianum</i> Boiss-essential oil
ATR	Attenuated total reflection
DMSO	Dimetilsulfoxido
EE	Encapsulation efficiency
ELISA	Enzyme-linked immunosorbent assays
FBS	Fetal bovine serum
FESEM	Field emission scanning electron microscopy
FTIR	Fourier-transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
HAIs	Healthcare-associated infections
HSF	Human skin fibroblast
LC	Loading capacity
MDR	Multidrug-resistant
MHA	Mueller-Hinton Agar
MIC	Minimum inhibitory concentration
Nanoethosomes	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Drug delivery system, which has different composition of phospholipids, ethanol, and water to enhance skin penetration of active pharmaceutical ingredients
NE _{th}	Nanoethosome
NE _{th} -AJ _{EO}	Nanoethosome containing <i>Allium jesdianum</i> Boiss-essential oil
OD	Optical density
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PVA	Polyvinyl alcohol
RPMI 1640	Roswell Park Memorial Institute
SA _{MU}	Salep mucilage
SEM	Scanning electron microscopy
TSB	Tryptic Soy Broth
MC	Moisture content
WS	Water solubility
EAB	Elongation at break
YM	Young's modulus
ΔE	Total color difference
WI	Whitish index
YI	Yellowness index
WVP	Water vapor permeability
TPC	Total phenolic content
TFC	Total flavonoid content
GAE	Gallic acid equivalent
QUE	Quercetin equivalent

TRE	Trolox equivalent
ABTS	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
TPTZ	2,4,6-Tripyridyl-s-triazine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power

1 Introduction

Acinetobacter baumannii is a prevalent opportunistic pathogen associated with a wide range of healthcare-associated infections (HAIs), posing a serious global health threat due to its increasing resistance to multiple antibiotic classes, including β -lactams, carbapenems, fluoroquinolones, and aminoglycosides [1]. This bacterium employs various virulence and resistance mechanisms such as biofilm formation, efflux pumps, secretion systems, and membrane modifications [2]. Among these, biofilm formation is particularly critical, as it enhances antibiotic resistance and promotes persistent colonization on medical devices and host tissues [3]. Biofilms are structured microbial communities embedded in a protective extracellular matrix that significantly limits antibiotic penetration and immune clearance [4]. Therefore, disrupting *A. baumannii* biofilms represents a promising strategy to overcome chronic infections and combat antimicrobial resistance [5, 6]. Anti-biofilm agents work by reducing the adherence and virulence factors of pathogens without inhibiting their growth, thereby minimizing the risk of developing resistance.

Moreover, these therapies can improve bacterial susceptibility to antibiotics and enhance the effectiveness of the host immune response [7]. This underscores the critical need for anti-biofilm therapies and highlights the significance of discovering new anti-biofilm agents [8]. A variety of extracts and essential oils as natural compounds have been investigated for their potential to combat biofilms [9–12]. Within the *Allium* genus (belongs to the *Amaryllidaceae* family) *Allium jesdianum* Boiss. (known as Bon-e-Sorkh in Persian) stands out as a significant species. This plant is endemic and thriving in the northern, western, and southwestern regions of the Iran [13]. This genus is recognized for its potential health benefits due to its antibacterial, anticancer properties, and therapeutic components [14]. While various research efforts are needed to isolate its functional components, the impact of *Allium* essential oil in the form of nanostructures (utilized as herbal controlled-release systems) remains largely unexplored. To address microbial resistance, nanostructure-based targeting strategies have been proposed, particularly given the rapid degradation, biological variability, and limited biological persistence of essential oils [15]. Lipid-based carrier systems, such as nanoethosomes, are being investigated for their ability to deliver biochemical, pharmaceutical, and agricultural

agents. Nanoethosomes are flexible carriers and have efficacy in enhancing the dermal and transdermal delivery of different (hydrophilic and lipophilic) compounds in various studies [16]. For the successful commercialization of nanoethosomes, it is crucial to optimize their formulation by focusing on their size reduction, flocculation prevention, and coalescence minimization [17]. Nanoethosomes are phospholipid-based nanovesicles commonly used for dermal and transdermal delivery of molecules, particularly drugs. The double encapsulation technique—embedding nanoethosomes within electrospun nanofibers—is a novel strategy to enhance the controlled release and stability of bioactive compounds. In this system, lipid-based nanovesicles are incorporated into a polymeric solution and electrospun to form bioactive nanofibers. This approach offers promising potential for improving the efficiency of delivery and expanding the application of bioactives in pharmaceutical and biomedical fields.

Electrospun nanofibers are distinguished by their significant loading capacity, favorable surface-to-volume ratio, and are particularly effective for hybrid controlled-release systems [18]. A wide variety of both natural and synthetic polymers can be processed into fine nanofibers via electrospinning. Among natural polymers, biopolymers such as nucleic acids, proteins, and polysaccharides are commonly used due to their biodegradability and biocompatibility [19]. Salep, a flour produced from ground orchid tubers, contains a considerable proportion of glucomannan (with range of 16–55%). Salep is recognized for its polymeric characteristics, aphrodisiac properties, and valuable components in food as well as pharmaceutical products [20].

This study aims to develop and characterize a novel anti-biofilm electrospun nanofibrous pad by integrating nanoethosome-encapsulated AJ_{EO} uses a modified thin-film hydration technique into a SA_{MU} –PVA matrix. The innovation lies in the double encapsulation approach—where lipid-based nanoethosomes are embedded within polymeric nanofibers via electrospinning—to enhance the stability, controlled release, and bioactivity of AJ_{EO} . This multifunctional system uniquely integrates: (i) a natural and underutilized bioactive AJ_{EO} , (ii) a nanostructured lipid-based delivery system, and (iii) a natural polysaccharide-based nanofiber matrix. We hypothesize that this innovative system will show significant anti-biofilm efficacy against both reference and clinical strains of *Acinetobacter baumannii* as a ubiquitous pathogen capable of causing both community and HAIs.

2 Experimental

2.1 Chemicals and Reagents

Aerial parts (Leaves) of *Allium jesdianum* Boiss. and the palmate tuber Salep (Fig. 1A) were acquired, respectively,

from Lorestan and Kurdistan Provinces, Iran. The reagents utilized in this study included crystal violet (with a dye content of at least 90%), sterile phosphate-buffered saline (PBS), L- α -soybean lecithin (comprising > 94% phosphatidylcholine and < 2% triglycerides), and 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT), all sourced from Sigma-Aldrich, USA. All chemicals, organic solvents, and reagents, including dichloromethane, methanol, and ethanol, were analytical grade and obtained from Merck, Germany. Quelab, Canada supplied Tryptic Soy Broth (TSB), Mueller–Hinton Broth (MHB) and Mueller–Hinton Agar (MHA). The A375 and HSF cell lines were acquired from the National Cell Bank of the Pasteur Institute in Iran. Additionally, Roswell Park Memorial Institute (RPMI 1640) culture media, enriched with 10% fetal bovine serum (FBS), was obtained from Gibco, UK. The Merck, Germany also provided the commercial PVA (medium molecular weight = 72,000 Da) and a dialysis bag with a molecular Weight cut-off of 12 kDa. All chemicals were utilized in their primary form without undergoing any further purification processes.

2.2 Extraction of AJ_{EO}

The fresh leaves were carefully cleaned and dried in the shade and then hydrodistilled in a glass Clevenger-type apparatus for 3 h to obtain its essential oil (AJ_{EO}) as described by Ekrami et al. [16]. After drying AJ_{EO} with anhydrous sodium sulfate, it was preserved in a sealed chamber at 4 °C.

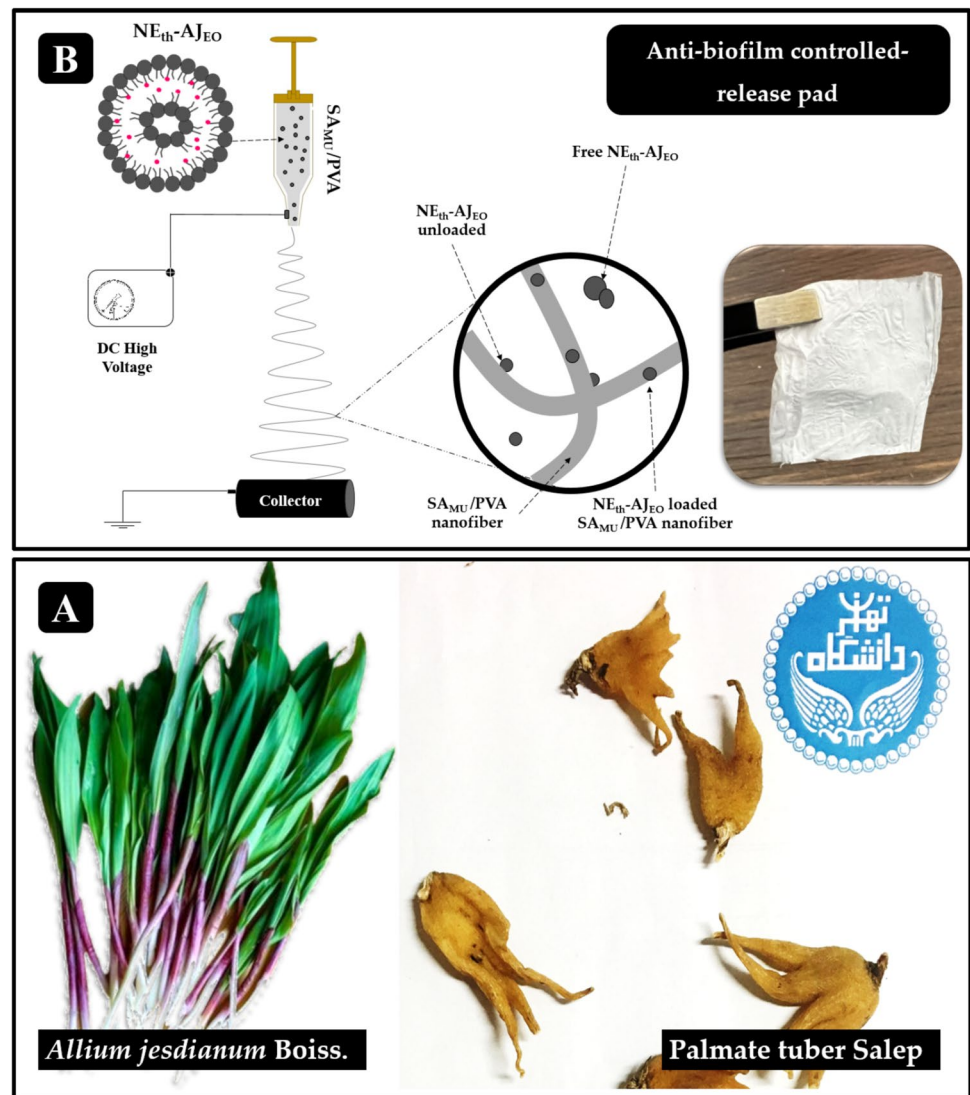
2.2.1 Identification of AJ_{EO}

GC–MS (Hewlett-Packard, USA) was set up for the identification of AJ_{EO} components according to Ekrami et al. [21]. The classification of compounds was achieved by comparing the mass spectra of the recorded chromatographic peaks against the Wiley 275 MS database.

2.2.2 Cell Cytotoxicity

The cytotoxicity of AJ_{EO} on A375 and HSF cell lines was evaluated through a modified MTT assay, as outlined by Nazari et al. [22]. The cell lines were maintained at 37 °C at specific atmosphere containing 5% CO_2 , with RPMI 1640 (serving as a culture medium). AJ_{EO} was introduced into a 96-well plate, which was subsequently sterilized using ethylene-oxide gas. After three passages, the cell lines were enumerated and seeded at a density of 15×10^3 cells/well. AJ_{EO} was tested at concentrations of 0–2000 $\mu\text{g/mL}$. Control groups included untreated cells (negative control) and cells treated with the solvent alone (DMSO at concentrations $\leq 0.1\%$, as vehicle control). Following a 24 h incubation period for each group, the culture medium was substituted

Fig. 1 **A** Photographic images of aerial parts (Leaves) of *Allium jesdianum* Boiss. and the palmate tuber Salep. **B** Schematic representation of electro-spinning technique utilized for obtaining SA_{MU}-PVA nanofiber containing NE_{th}-AJ_{EO} as anti-biofilm controlled-release pad



with 50 μL of MTT solution, and the cells were further incubated at 37 $^{\circ}\text{C}$ for an additional 4 h. Furthermore, the MTT assay is a standard and reliable method for assessing cell viability by Measuring the metabolic activity of living cells to evaluate cytotoxicity. After this incubation, 200 μL of fresh medium supplemented with dimetilsulfoxido (DMSO) was added to each well to replace the previous medium, and the mixture was gently agitated for 5 min. Then the absorbance of the resulting formazan product was quantified at 570 nm using an enzyme-linked immunosorbent assays (ELISA) reader (TECAN, Australia), and cell viability was assessed according to Eq. (1):

$$\text{Cell viability (\%)} = \left(\frac{\text{ABS}_{\text{Sample}}}{\text{ABS}_{\text{Control}}} \right) \times 100 \quad (1)$$

where $\text{ABS}_{\text{Sample}}$ and $\text{ABS}_{\text{Control}}$ are the absorbances of the sample and control, respectively.

2.3 Preparation of NE_{th}-AJEO Suspension

The NE_{th}-AJ_{EO} suspension was prepared using the cold method as described by Kumar et al. (2024) with certain adjustments [23]. Initially, lecithin (5% w/v) was mixed with ethanol (25–45%v/v) and heated in a closed water-bath vessel up to 30 ± 1 $^{\circ}\text{C}$. Subsequently, the extracted AJ_{EO} (25–2500 $\mu\text{g}/\text{mL}$) was slowly added to the ethanolic lipid solution under continuous stirring at 1000 rpm with a magnetic stirrer (IKA, Germany). The mixing process continued for an additional 5 min and the resulting vesicular dispersions were allowed to cool down to room temperature (25 ± 1 $^{\circ}\text{C}$). Finally, the mixture was homogenized for 5 min by using a high-speed (15,000 RPM) homogenizer (IKA, Germany) for further particle size reduction. Then, a probe-type sonicator (Hielscher, Germany) operating at 400 W-24 kHz with 10-cycle propulsion, a duration of 30 s/cycle, and a cycle interval of 15 s was utilized.

2.3.1 Size and Zeta Potential Determination

The NE_{th}-AJ_{EO} suspension was analyzed for mean particle size (z-average in intensity), polydispersity index (PDI) as size distribution, and zeta-potential using a Zetasizer (Malvern Nano Zetasizer, UK) under the voltage of 3.9 V; the electric charge on the suspension droplets was Measured at pH 7.4 and 25 °C. Double-distilled water was used to dilute the samples 50 times, and measurements were taken in triplicate.

2.3.2 Encapsulation Properties

The encapsulation efficiency (EE) and loading capacity (LC) of NE_{th}-AJ_{EO} suspension were assessed following the method of García-Moreno et al., with some modifications [24]. A total of 5 mg nanocarriers (nanoethosome or nanofiber mat) were added to a 10 mL mixed solvent (methanol-hexane 1:1) for 15 min at 100 rpm under shaking. The solution was filtered, liquid absorbance was measured at $\lambda=275$ nm using a UV/Vis Spectrophotometer (Cecil Instruments Ltd., UK), and AJ_{EO} amounts were estimated using a calibration curve. Equations (2) and (3) were applied to calculate EE and LC values:

$$EE(\%) = \frac{EO_i - EO_f}{EO_i} \times 100 \quad (2)$$

$$LC(\mu g/g) = \frac{EO_i - EO_f}{NC_w} \quad (3)$$

where EO_i and EO_f are respectively the total theoretical and free amounts of AJ_{EO} in the collection solution. NC_w is the weight of the nanocarriers. Measurements were carried out in triplicate.

2.3.3 Field Emission Scanning Electron Microscopy (FESEM)

The dimensions of NE_{th}-AJ_{EO} particles were assessed through FESEM (TESCAN, Czech Republic) equipment, where suspended DMSO-particles were analyzed after they were gold-sputtered and dried.

2.3.4 Thermodynamic Stability

The thermodynamic stability of NE_{th}-AJ_{EO} was assessed with some thermal shocks by using 4 steps of phase separation method as outlined by Sharma et al. [25]:

1st: NE_{th}-AJ_{EO} dispersion was stored at 25 °C for 30 days; 2nd: Centrifugation at 1500 × g for 30 min; 3rd: 6-cycle heating up to 45 °C followed by cooling down

to 4 °C; 4th: 3-cycles of freezing at −21 °C by subsequent thawing at 25 °C. Samples that exhibited instability were discarded, categorizing them as thermodynamically unstable.

2.4 Fabrication of Anti-Biofilm Pad

The Salep mucilage solution for the fabrication of the anti-biofilm pad was prepared using our previous method [26]. The two aqueous solutions of SA_{MU} (2% w/v) and PVA (10% w/v) were prepared by 1 h magnetic stirring respectively at 35 °C and 80 °C. Then both solutions were homogenized using a ultra-turrax digital high-speed homogenizer (IKA, Germany) at 12,000 rpm for 3 min followed by hydration overnight at 4 °C [27]. Later, NE_{th}-AJ_{EO} was mixed with SA_{MU}:PVA (v/v) solutions using tween 80 (5% v/w) to facilitate its emulsification and the resulting mixture subsequently was sonicated utilizing a probe-type ultrasonic (Hielscher, Germany) at 24 kHz, amplitude 50%, duty cycle 0.5 for 5 min. The neat SA_{MU}:PVA nanofiber was prepared as a control without including tween 80 and NE_{th}-AJ_{EO}.

The electrospinning apparatus used at 25 ± 1 °C and relative humidity of 30–35% (as illustrated in Fig. 1B). This setup incorporated a dosing pump (Terumo, Tokyo, Japan) with a feeding rate from 0.01 to 10 mL/h, utilizing a 1-mL syringe fitted with a 21G needle. Additionally, the system was powered by a high-voltage supply capable of converting 220 V AC to a maximum output of 30 kV DC. In this configuration, the positive electrodes were affixed to the tip of the needle, while an aluminum foil, serving as the negative electrode, was attached to the cylindrical rotating collector.

The optimum conditions obtained (after running different tests) for fabricating an anti-biofilm pad containing NE_{th}-AJ_{EO} with a minimum diameter (288.1 nm) were 60:40%v/v SA_{MU}:PVA, 0.5 mL/h pump rate, 17.5 cm distance (between tip and collector), 20 kV applied voltage, and 10% (w/w) NE_{th}-AJ_{EO} concentration. SA_{MU}:PVA nanofibers loaded with NE_{th}-AJ_{EO} was identified as anti-biofilm controlled-release pad.

2.4.1 The Morphological Characterization

The morphology of neat SA_{MU}:PVA nanofibers and the anti-biofilm pad was determined using scanning electron microscopy (SEM) (TESCAN, Czech Republic) and atomic force microscopy (AFM) in tapping mode (Nanoscope, CA, USA). ImageJ (version 1.5) and DME-SPM (version 2.1.1.2) software were used to calculate nanofiber diameter (based on 100 random measurements per SEM image) distribution and 2D/3D surface images.

2.4.2 Mechanical Properties

The mechanical characteristics of neat SA_{MU}-PVA nanofibers and anti-biofilm pad were assessed using a universal testing machine (Testometric, England) with a load capacity of 0.5 N and a tensile speed of 5 mm/min. The 3D of randomly selected areas of nanofiber mats (sectionized approximately into 10 × 1 × 0.1 cm) were measured by a digital caliper with at least five replicates to obtain their accurate dimensions and provide a comprehensive evaluation.

2.4.3 FTIR Analysis

The functional and chemical groups of AJ_{EO}, NE_{th}-AJ_{EO}, and anti-biofilm pad were examined using ATR-FTIR (Bruker, Germany). Data acquisition was performed via attenuated total reflection employing a zinc selenide crystal plate, achieving a nominal resolution of 2 cm⁻¹ for enhanced analytical precision.

2.4.4 Temperature-Induced Release

The evaluation of the release profile of AJ_{EO} from NE_{th}-AJ_{EO} and anti-biofilm electrospun pad was conducted through a cumulative release methodology. Initially, a sample of 1 mg was suspended in 3 mL of pH 7.4 buffer (PBS) as release medium at 4, 25, 37, and 45 °C and subsequently placed in a dialysis bag, where it was agitated at 100 rpm. Then, 3 mL of the release medium was extracted, and AJ_{EO} concentration was quantified using a UV/Vis Spectrophotometer at a wavelength of 275 nm. The cumulative released-percentage of AJ_{EO} was calculated using Eq. (4):

$$\text{Cumulative release (\%)} = (M_t / M_0) \times 100 \quad (4)$$

where M_t is the cumulative amount of released-AJ_{EO} at each point in the sampling time, and M_0 is the initial weight of AJ_{EO} loaded in the sample. All data were obtained in triplicate.

2.5 Anti-biofilm Properties

2.5.1 Bacterial Strains and Culture Conditions

The bacterial strains utilized in this investigation include the reference strain *Acinetobacter baumannii* ATCC-19606 and a multidrug-resistant (MDR) clinical isolate of *Acinetobacter baumannii*. To facilitate biofilm development, these strains were cultivated in TSB culture enriched with 1% sucrose and 0.5% yeast extract, followed by incubation at 37 °C for 24 h in an orbital shaker (IKA, Germany) operating at 150 rpm. The selection of the MDR isolate was

conducted in accordance with the Clinical and Laboratory Standards Institute, employing the disk diffusion method on MHA. The cultures were maintained at 37 °C for a duration of 24 h, with MDR characterized as the acquired non-susceptibility to at least one antimicrobial agent across three or more distinct categories.

2.5.2 Minimum Inhibitory Concentration (MIC)

The MIC of AJ_{EO} against various strains of *Acinetobacter baumannii* was determined using the broth microdilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) standard M7-A10 [28]. Briefly, bacterial strains were cultured overnight in MHB. The bacterial suspension was adjusted to a turbidity equivalent to 0.5 McFarland standard (OD₆₂₅ ≈ 0.09), corresponding to approximately 1.5 × 10⁸ CFU/mL. Serial two-fold dilutions of AJ_{EO} (final concentration range: 25–2500 µg/mL) were prepared in 96-well sterile polystyrene microtiter plates with a final volume of 100 µL per well. Then, 100 µL of the diluted bacterial inoculum was added to each well, resulting in a final volume of 200 µL per well. The plates were incubated at 37 °C for 24 h. After incubation, bacterial growth was assessed by measuring optical density using a multi-mode microplate reader (Biotek, USA). The MIC was defined as the lowest concentration of AJ_{EO} that inhibited visible bacterial growth.

2.5.3 Minimum Biofilm Inhibitory Concentration (MBIC)

The MBIC of AJ_{EO} against various strains of *Acinetobacter baumannii* was assessed using a modified broth microdilution method in accordance with CLSI guidelines and previously published protocols [29]. The sterile 96-well polystyrene plates were filled with 200 µL of supplemented TSB with AJ_{EO} (25–2500 µg/mL). The stock solutions of the AJ_{EO} was prepared in DMSO. Then, 2 µL of the bacterial strains inocula of density corresponding to 0.5 McFarland standard, were added to each well. After the 24 h incubation at 37 °C, the formed biofilm was rinsed twice with sterile phosphate buffer saline (PBS) to remove the non-adherent cells. The attached bacteria were stained with safranin (for 10 min). Then the biofilm was rinsed with PBS solution to remove the dye. Sodium chloride (100 µL) was added to each well and the plate was sonicated to resuspend the bacterial cells. Absorbance measured at =490 nm using a multi-mode microplate reader, was expressed as optical density (OD₄₉₀). The minimal biofilm inhibitory concentration (MBIC) was calculated by comparing the obtained values of OD₄₉₀ with those for the negative control. The experiments were carried out in triplicates. Data are presented as the mean ± SD from at least three independent experiments.

2.5.4 Air–Liquid Interface Biofilm Formation Assay

The impact of the anti-biofilm pad on the biofilm development of *Acinetobacter baumannii* strains at the air–liquid interface was evaluated using Selvaraj et al. (2020) method with some modifications [30]. Specifically, an overnight culture was introduced into 2 mL of supplemented TSB medium, both with and without the anti-biofilm pad. The microtubes and polystyrene Petri dishes were then incubated for 48 h at 37 °C. Following the incubation period, the test of microtubes and Petri dishes were subjected to three sequential PBS washes and subsequently stained using a 0.1% crystal violet solution.

2.5.5 Microscopic Analyses

A biofilm assay was conducted using a 24-well plate that contained glass slides, with experiments carried out both with and without the application of an anti-biofilm pad over a 24 h period at 37 °C. Following this incubation, the glass slides were rinsed with sterile PBS and allowed to dry with ambient air. For subsequent light microscopic examination, the slides were stained with a 0.1% crystal violet solution and analyzed at a magnification of $\times 400$ (Nikon, Japan). In addition, for confocal laser scanning microscopy (CLSM) analysis, the glass slides were stained with a 0.1% acridine orange solution and observed at a magnification of $\times 200$ (Carl Zeiss, Germany). The biofilm characteristics, including thickness, volumetric biomass, and the surface-to-volume ratio of *Acinetobacter baumannii* strains, were evaluated using Comstat software to facilitate a comprehensive analysis of the biofilm in both experimental conditions.

2.5.6 The Extracellular Polysaccharides (EPS) Quantification Assay

To quantify EPS [30], cultures of *Acinetobacter baumannii* were incubated for 24 h at 37 °C with and without the presence of an anti-biofilm pad. Following incubation, the cultures underwent centrifugation at $10,000\times g$ for 15 min to separate the cell pellets from the cell-free culture supernatant (CFCS), allowing for the extraction of both cell-bound and secreted EPS. The cell pellets were resuspended in an isotonic buffer solution (Tris/HCl (pH 8.0), EDTA, and NaCl). These samples were incubated at 4 °C for 16 h before being centrifuged at $8000\times g$ for 10 min, resulting in the collection of cell-bound EPS in the supernatant, which was subsequently combined with the previously obtained CFCS. Following this, one volume of the CFCS containing both cell-bound and secreted EPS was mixed with three volumes of ice-cold ethanol and stored at -20 °C overnight. After this incubation period, the mixture was centrifuged at $12,000\times g$ at 4 °C for 15 min to precipitate its carbohydrates,

which were then collected as a pellet. This pellet was resuspended in 70% ethanol and dried under vacuum, yielding the final dried EPS, which was subsequently weighed. The percentage of EPS inhibition was calculated by the following Eq. (5):

$$\text{EPS inhibition (\%)} = \left(\frac{\text{Control weight} - \text{Treated weight}}{\text{Control weight}} \right) \times 100 \quad (5)$$

This assay was performed in triplicate, and the results are presented as mean $\pm$ standard deviation.

2.5.7 Microbial Adhesion to Hydrocarbons (MATH) Assay

The impact of the anti-biofilm pad on the cell surface hydrophobicity (CSH) of *Acinetobacter baumannii* strains was evaluated using the MATH assay [30]. In this procedure, the strains were cultivated both in the presence and absence of the anti-biofilm pad. Following cultivation, the cells were harvested, washed with PBS, and then resuspended in the same buffer. The cell suspensions were adjusted to an OD of 0.9 at 600 nm, after which 1 mL of toluene was introduced. The mixture was vortexed for 2 min and subsequently stored at 4 °C for 24 h to facilitate the separation of the two phases. Upon completion of the separation, the OD of the aqueous phase was measured at 600 nm, and the CSH was determined using the following Eq. (6):

$$\text{CSH(\%)} = \left(1 - \frac{\text{OD}_{600\text{nmAftervortex}}}{\text{OD}_{600\text{nmBeforevortex}}} \right) \times 100 \quad (6)$$

3 Results and Discussion

3.1 Evaluation of AJEO

3.1.1 Identification of Components

After using Clevenger-hydrodistillation extraction for *Allium jesdianum* Boiss. close to 0.2% light brown essential oil (AJ_{EO}) was obtained AJ. Figure 2A shows the main components of AJ_{EO} recognized by GC–MS chromatogram. Different sulfide compounds including dimethyl trisulfide, dimethyl tetrasulfide, and dipropyl trisulfide were identified in AJ_{EO}. In addition, hexadecanoic acid acts as the main saturated fatty acid, and Neral or Z-Citral acts as oxygenated monoterpenes in the AJ_{EO}. The main components documented in this study were in good agreement with previous researches. The main components of AJ_{EO} were trisulfide, Hexadecanoic acid, and Neral compounds [31, 32]. However, the main chemical composition of AJ_{EO} can vary significantly due to different factors such as drought

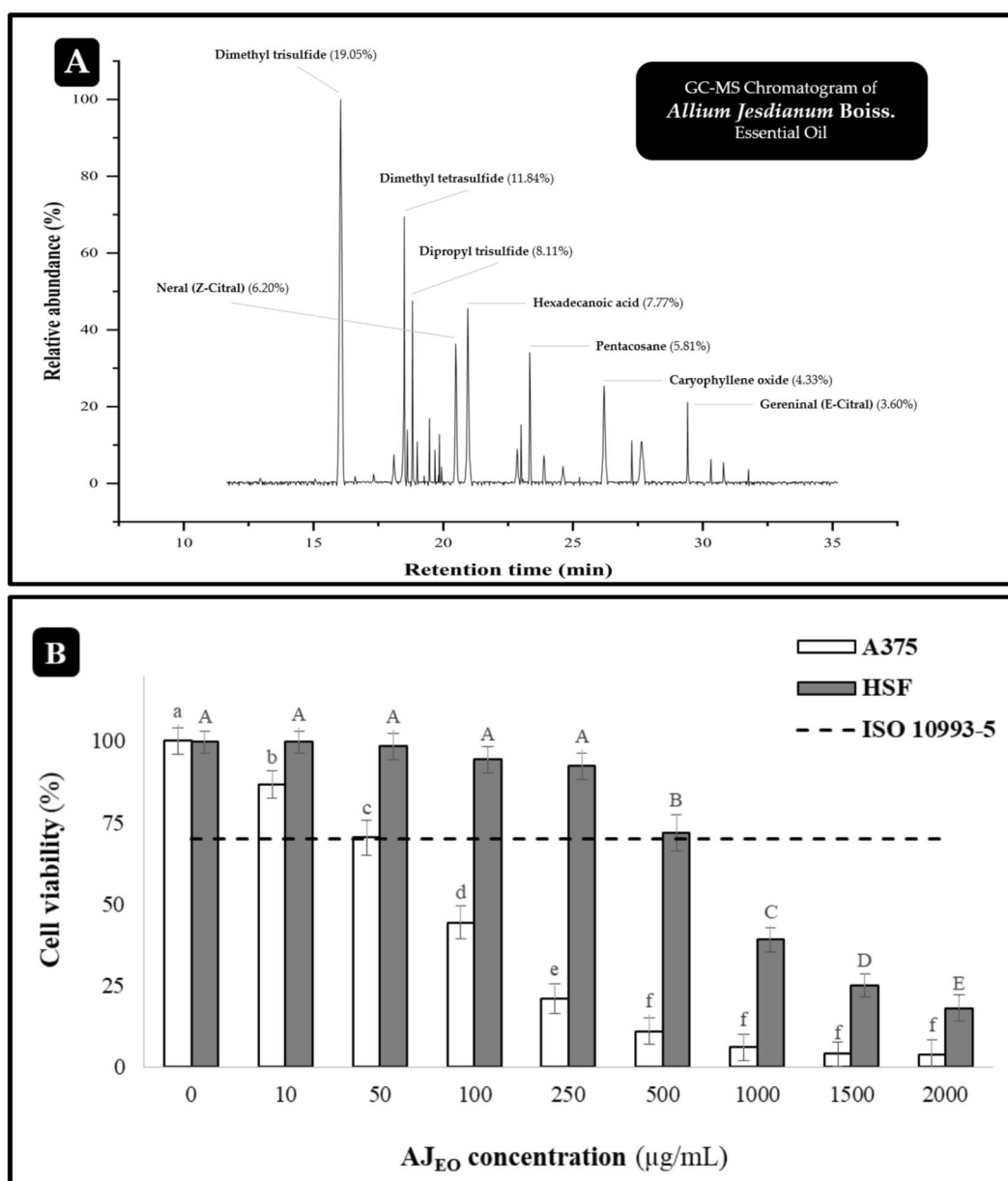


Fig. 2 **A** GC–MS chromatogram and **B** cytotoxicity activity of essential oil extracted from *Allium jesdianum* Boiss

stress, climate, soil composition, salinity stress, plant organs, extraction conditions, and other variables [33].

3.1.2 AJ_{EO} Cell Cytotoxicity

The increasing popularity of plant-derived antimicrobials has raised significant safety concerns, particularly regarding their cytotoxic effects. Limited research has been conducted on the cytotoxicity of plants from the genus *Allium*. Notably, pure AJ_{EO} demonstrated a pronounced cell-dependent cytotoxic impact on the A375 and HSF cell lines with statistical significance ($p < 0.05$) observed as AJ_{EO} concentration

increased (Fig. 2B). Considering the applications of multifunctional pads in medicine, the food industry, and public health, the sensitivity of normal (HSF) and cancerous (A375) skin cell lines is important to ensure their safety and effectiveness.

AJ_{EO} was identified as the most cytotoxic agent, with a concentration of 50 $\mu\text{g/mL}$ reducing A375 cell viability to 70%, the threshold ISO 10993–5 defined for a substance to be classified as non-cytotoxic. In contrast, HSF cells maintained their viability at concentrations of up to 500 $\mu\text{g/mL}$ under the same experimental conditions. This indicates that AJ_{EO} 's cytotoxicity depends on several factors, including

treatment dosage, incubation duration, and the specific cell type involved. In a related investigation, Naeini et al. demonstrated that the *Allium jesdianum* aqueous extract can be used to increase nitric oxide production as macrophage functions up to 1000 µg/mL [34]. Alidadi et al. (2021) reported cytotoxicity of *Allium jesdianum* hydro-alcoholic extract on the growth of HT-29 human colorectal cancer cell line at 25–100 µg/mL [35]. These findings suggest that AJ_{EO} may possess selective cytotoxic properties against cancerous A375 cells while exerting minimal effects on normal HSF cells at comparable concentrations. Although this selective cytotoxicity is promising and points toward a potential anti-cancer application, further studies are necessary to elucidate the underlying mechanisms, assess apoptosis induction, and validate its efficacy through in vivo and clinical investigations.

3.2 Characterization of NE_{th}-AJEO

3.2.1 Optimization of Preparation Factors

The present investigation involved the preparation of NE_{th}-AJ_{EO} through the addition of varying amounts of AJ_{EO} (25–2500 µg/mL) and ethanol (35–45% v/v) using a cold method combined with ultrasonic-ultra-turrax treatment.

According to the findings illustrated in Fig. 3A, the size of NE_{th}-AJ_{EO} notably rose from 105 to 351.9 nm as the concentration of AJ_{EO} increased. Nevertheless, once the AJ_{EO} concentrations surpassed 500 µg/mL, a precipitate emerged in the nanoethosome suspension, indicating the inadequate stability of nanoethosomal nanoparticles when AJ_{EO} is in excess. In a study by Wei et al. (2023), it was noted that the size of ethosomal nanoparticles containing cinnamaldehyde increased from 57.7 to 265.9 nm with escalating cinnamaldehyde concentration [36]. Also, the size of NE_{th}-AJ_{EO} decreased with an increase in the proportion of ethanol in combination. Rakesh and Anoop (2012) confirmed the decrease in ethosomes of cromolyn sodium with increasing ethanol concentration [37]. Meanwhile, the polydispersity index (PDI) also changed from 0.13 to 0.67 as a result of increasing AJ_{EO} concentration (Fig. 3B).

The particle sizes and PDI distributions of lipid-based nanocarriers are critical physical attributes that must be taken into account during the formulation of food-grade or pharmaceutical-grade products [38]. The findings shown in Fig. 3B show that up to 500 µg/mL AJ_{EO}, the PDI value corresponds to the ideal range for lipid nanostructures because a PDI value of around 0.3 indicates a narrow size distribution, and the sample can be classified as monodisperse. Additionally, changes in ethanol concentration have no effect on the PDI value [39].

The zeta potential plays a crucial role in determining the electrostatic stability of particles and connecting of

NE_{th}-AJ_{EO} to the target cell membrane. Figure 3C shows the zeta potential in NE_{th}-AJ_{EO} ranging from −11.4 to −36 mV. In solutions with low ionic strength, the phosphatidyl group (negative charge) is oriented towards the outside, while the choline group (positive charge) is leaning towards the inside of the bilayers [40]. The zeta potential of NE_{th}-AJ_{EO} was expected to increase with AJ_{EO} concentration, because this parameter has a negative nature in essential oil of AJ plant [41].

The encapsulation efficiency (EE) and loading capacity (LC) of NE_{th}-AJ_{EO} (as shown in Fig. 3D and E) were found to be above 70% and 7500 µg/g, respectively, when 500 µg/mL of AJ_{EO} and 35% ethanol:water (v/v) were used. However, there was a noticeable decline in both EE and LC as the concentration of AJ_{EO} increased [40]. Lipid structures possess a notable capacity to encapsulate bioactive compounds; however, an increase in the concentration or quantity of the core composition may lead to a reduction in the efficiency and capacity of the nanolipid carriers [42]. Additionally, the FESEM analysis revealed the micromorphology of NE_{th}-AJ_{EO} (500 µg/mL AJ_{EO} and 35% v/v ethanol) showed the resulting nano-particles had an oblate spherical shape with an average size of ~120 nm and a well-dispersed population (as depicted in Fig. 3F). The variation in size is attributed to the coexistence of larger AJ_{EO}-loaded nanoethosomes and smaller unloaded vesicles. The size distribution histogram shows that approximately 50% of the particles range from 100 to 125 nm, 75% fall between 75 and 150 nm, and nearly 90% lie within 50–175 nm, indicating a desirable and well-controlled size range for nanoethosomal delivery systems. The particle sizes are crucial characteristic of nano-delivery systems, as they directly impacts the physicochemical properties and bioactivity of nanomedicines [43]. Wang et al. (2018) documented that the biological activity of avermectin and its release rate from the nano-delivery system exhibited a gradual increase as the particle size decreased. This phenomenon was attributed to the larger surface area of the particles, which facilitated enhanced adhesion and penetration of each drug [44].

3.2.2 Thermodynamic Stability

Thermodynamic stability shows valuable perceptions for the long-term physical and thermal integrity of lipid-based systems. The characteristics of stable NE_{th}-AJ_{EO} composition, as detailed in Table 1, showed no visible signs of macroscopic instability, such as creaming or phase separation, following centrifugation. The presence of lecithin as an emulsifier likely contributed to steric hindrance, which facilitated droplet repulsion, while the effects of homogenization and sonication effectively minimized droplet size and regulated size distribution. The combined impact of ultrasonication and the emulsifier appears to have played a crucial role in

Fig. 3 Counter plots for the concentration effects of AJ_{EO} and ethanol **A** on the mean size of particles in nm, **B** polydispersity index, **C** zeta potential in mV, **D** encapsulation efficiency, and **E** loading capacity of NE_{th} - AJ_{EO} in $\mu\text{g/g}$. **F** The field emission scanning electron microscopy (FESEM) image of NE_{th} - AJ_{EO} at specific concentrations of 500 $\mu\text{g/mL}$ of AJ_{EO} + 35% v/v ethanol along with the size distribution of the resulting particles

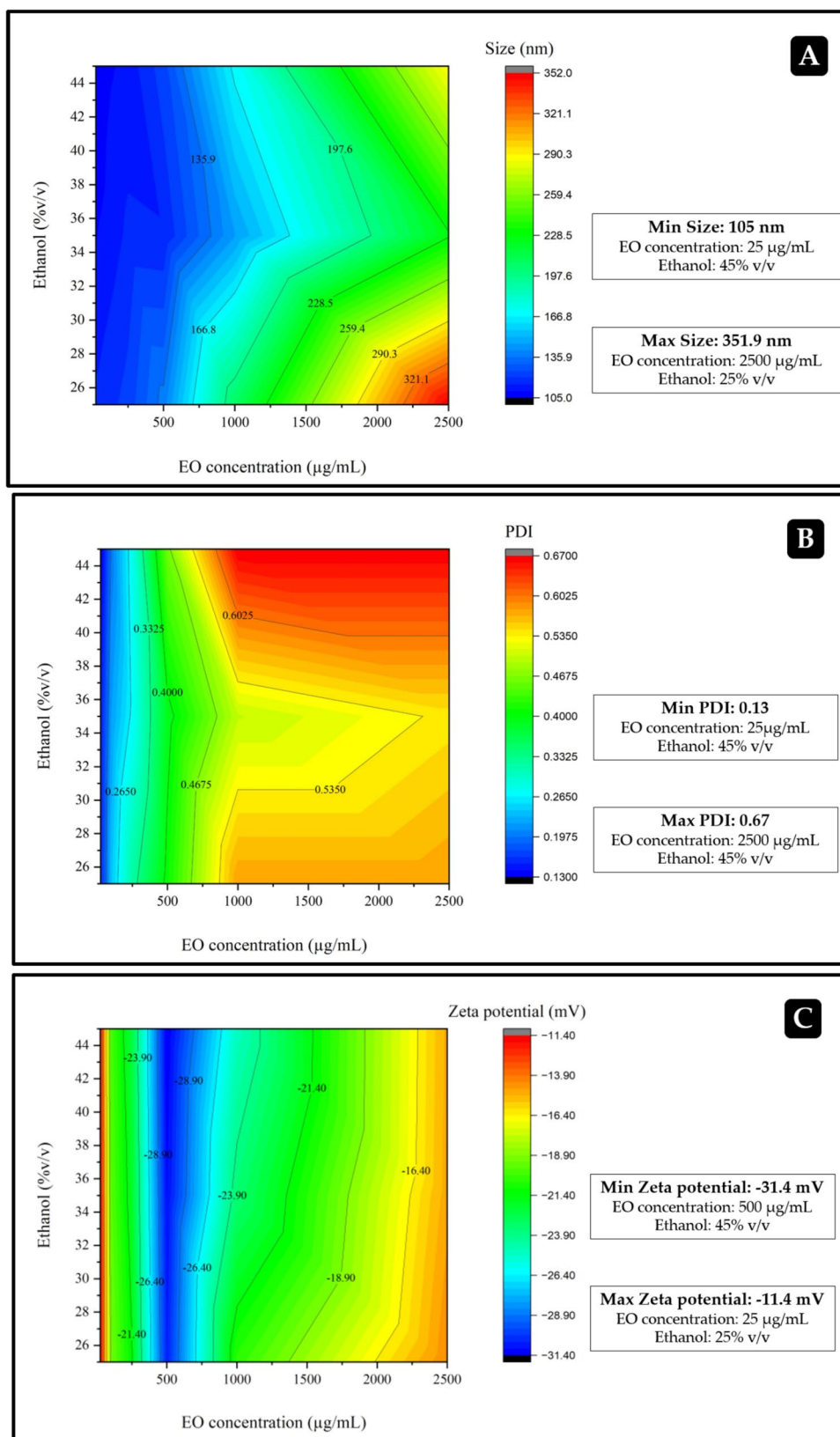


Fig. 3 (continued)

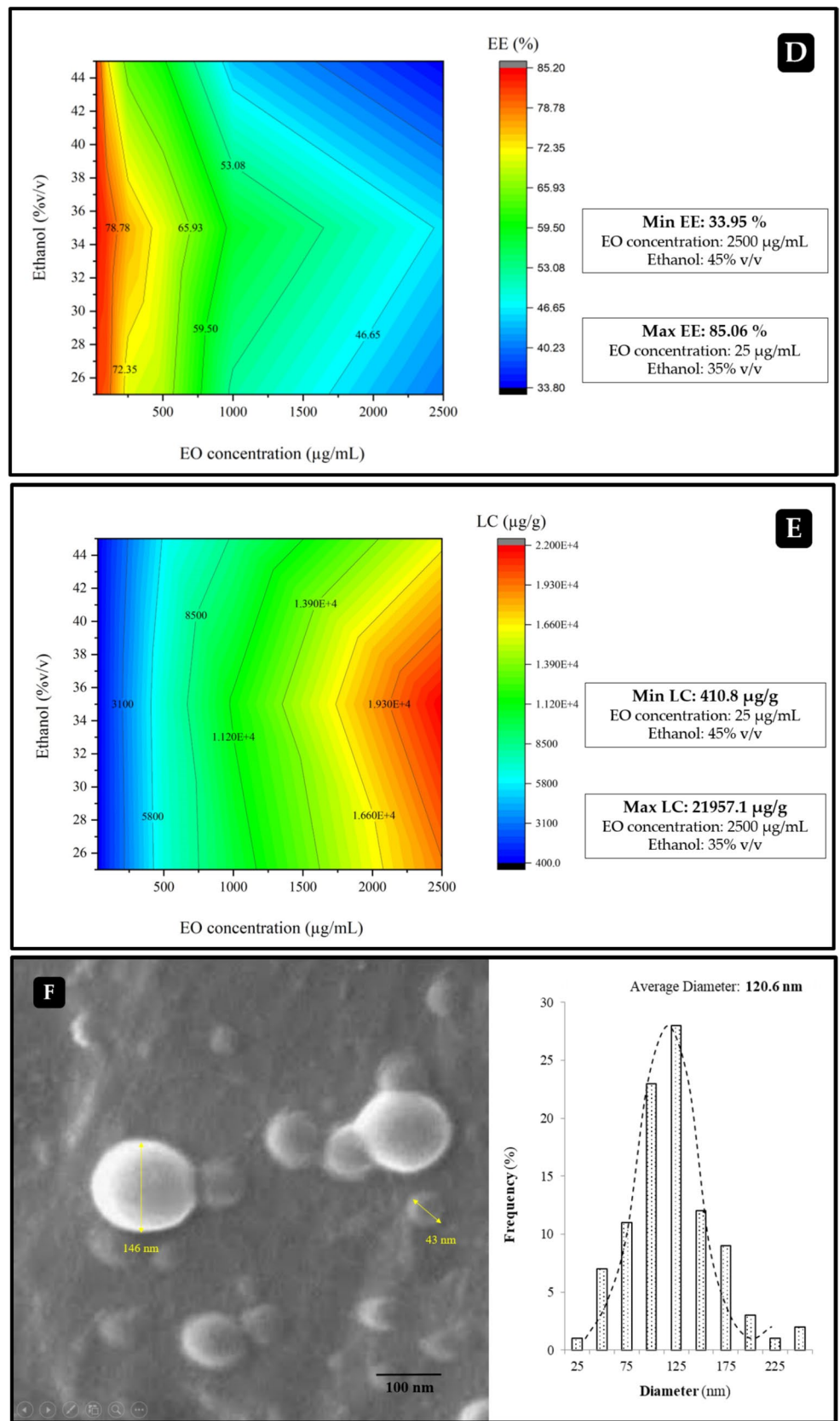


Table 1 Physico-chemical characteristics and thermodynamic stability of optimized NE_{th}-AJ_{EO}

Properties	Stability				
	Initial conditions	25 °C at 30 days	Centrifugation	Heating–cooling	Freezing–thawing
Size (d.nm)	115.7 ± 5.2 ^b	115.9 ± 4.4 ^b	117.3 ± 8.8 ^{ab}	120.8 ± 6.3 ^a	121.2 ± 7.5 ^a
Zeta potential (mV)	− 31 ± 2.4 ^a	− 30.8 ± 6.3 ^a	− 30.1 ± 5.5 ^a	− 28.4 ± 6 ^a	− 28.2 ± 5.7 ^a
Polydispersity index (PDI)	0.27 ± 0.02 ^a	0.27 ± 0.05 ^a	0.28 ± 0.06 ^a	0.30 ± 0.04 ^a	0.31 ± 0.07 ^a
pH	6.71 ± 0.28 ^a	6.71 ± 0.36 ^a	6.69 ± 0.30 ^a	6.61 ± 0.43 ^a	6.6 ± 0.25 ^a
Encapsulation efficiency (%)	70.88 ± 4.05 ^a	70.80 ± 7.21 ^a	69.05 ± 6.33 ^a	66.40 ± 5.97 ^{ab}	63.38 ± 5.23 ^b
Loading capacity (µg/g)	7017.8 ± 167.3 ^a	7009.5 ± 146.3 ^a	6945 ± 128.4 ^a	6824.2 ± 197.2 ^{ab}	6701.4 ± 113 ^b

Values are mean ± SD. Different letters indicate significant differences ($p < 0.05$)

maintaining the stability of the dispersion under the shear forces experienced during centrifugation [45].

Quantitative analysis revealed non-significant changes in particle size, PDI, and zeta potential following centrifugation, indicating good physical stability under mechanical stress. Similarly, EE and LC exhibited non-significant reductions, confirming structural integrity of the system post-treatment.

Furthermore, during repeated heating–cooling and freezing–thawing cycles, the NE_{th}-AJ_{EO} formulation demonstrated no visible phase separation, suggesting that the formulation maintained its colloidal balance under thermal stress. In both conditions, a significant ($p < 0.05$) increase in particle size was observed, accompanied by a slight but non-significant increase in PDI. The zeta potential showed a non-significant decrease, remaining within the range considered acceptable for stable nanoemulsions. However, EE and LC underwent significant ($p < 0.05$) reductions, likely due to structural stress and partial release of active compounds. Sharma et al. (2021) indicated that phase separation in lipid-based systems could arise from various physicochemical changes induced by freezing, including lipid crystallization, ice formation, surface phase transitions, and alterations in surface layer structure and chemical interactions [25]. The freezing process compels EO droplets to come into closer proximity, and if the repulsive forces at the droplet surfaces are insufficient, coalescence may occur [46].

The NE_{th}-AJ_{EO} formulations that exhibited no phase separation during experimentation were subsequently selected for further investigation into their morphological properties and in vitro release profiles.

3.3 Evaluation of Anti-biofilm Pad

3.3.1 Encapsulation Capacity

The encapsulation efficiency (EE) and loading capacity (LC) of the anti-biofilm pad were found to be above 93% and 700 µg/g of AJ_{EO}, respectively (when 10% wt. of NE_{th}-AJ_{EO} was infused). However, considering the selective

cytotoxicity profile of AJ_{EO}—where 50 µg/mL reduces the viability of cancerous A375 cells to 70% while normal HSF cells remain viable up to 500 µg/mL—it is crucial to carefully control the dosage and release rate of AJ_{EO} in the formulation. These safety considerations emphasize the importance of maintaining encapsulation and controlled release to maximize antimicrobial efficacy while minimizing potential cytotoxic effects on normal cells. Compared to other studies employing electrospinning for double bioactive encapsulation, our result clearly suggests that higher encapsulation efficiencies of the electrospinning – nanoethosome structure can be accomplished with this strategy. This can be attributed to (1) the uniqueness of the fabrication method and (2) SA_{MU}:PVA nanofibers containing NE_{th}-AJ_{EO} could control the incorporation efficiency and release behavior of AJ_{EO} due to the electrostatic interaction.

3.3.2 Surface Morphology

Figure 4, section A, presents the SEM images and fiber size distribution of (1) SA_{MU}:PVA nanofibers and (2) the anti-biofilm pad containing 10 wt% NE_{th}-AJ_{EO}, while section B displays their corresponding 2D and 3D AFM topographic images. The scanning electron microscopy (SEM) images reveal that the diameter of the electrospun nanofibers experienced a modest increase from 235.5 to 288.7 nm following the incorporation of NE_{th}-AJ_{EO}. This alteration is linked to variations in the viscosity and conductivity of the electrospinning solution. Typically, an increase in the viscosity of the spinning solution correlates with a rise in the diameter of nanocarriers; however, a significant reduction in conductivity can lead to the formation of larger beads, resulting in chain-like structures. The observed increase in nanofiber diameter can be attributed to the diminished electrical conductivity caused by the additives. The electrospinning process necessitates a careful balance between solution viscosity and conductivity, particularly at a defined surface tension. This behavior can be elucidated by the molecular interweaving that facilitates the formation of denser structures [47]. This finding is consistent with the observations

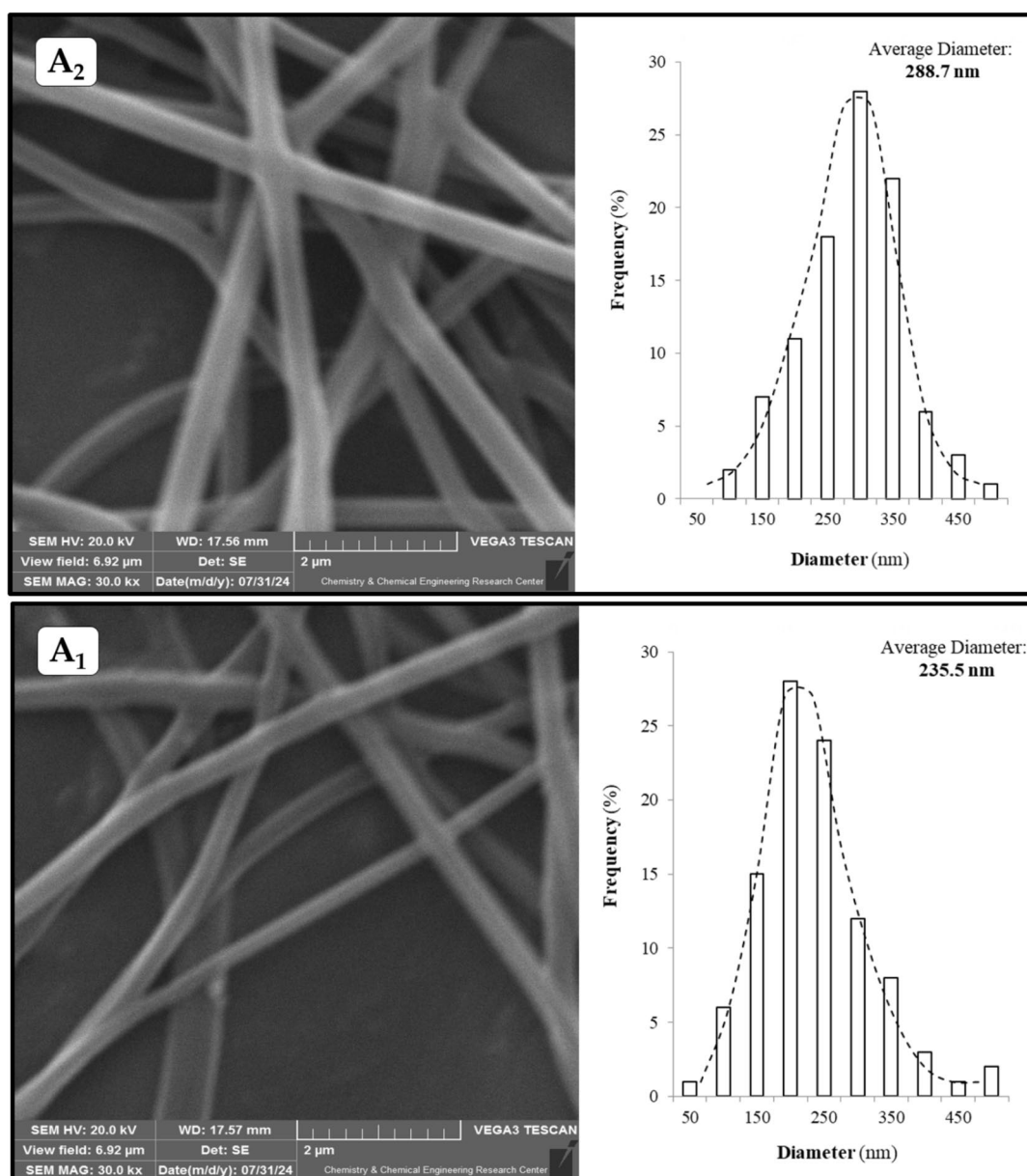


Fig. 4 Representative **A** Two images of SEM along their size distributions, **B** Two 2D/3D images of SA:PVA nanofibers (1) SA_{MU} :PVA nanofibers and (2) anti-biofilm pad, and **C** stress–strain curves of neat SA_{MU} :PVA nanofibers and anti-biofilm pad (containing 10%wt. NE_{th} - AJ_{EO})

by Mansouri Boroujeni et al. (2022) in their study on cefazolin-loaded niosomes onto electrospun chitosan nanofibrous membrane [48].

The analysis conducted using AFM confirmed the observations made through SEM regarding the morphology, diameter, uniformity and distribution of nanofibers of the nanofibers [49]. Variations in the types of bioactive compounds and the methods employed for encapsulation significantly influence the morphological attributes of nanocarriers, including their shape and surface texture. The AFM data indicate that the incorporation of NE_{th} - AJ_{EO} into SA_{MU} :PVA nanofibers alters the properties of the carriers, potentially affecting their

uniformity. Furthermore, during the nanoethosomal-electrospinning process, the encapsulation and subsequent release of active ingredients are hindered by the formation of large, heterogeneous beads due to the accumulation of NE_{th} - AJ_{EO} , which in turn diminishes the specific surface area of the functional SA_{MU} :PVA nanofibers.

3.3.3 Mechanical Properties

The mechanical properties of neat SA_{MU} :PVA nanofibers and those loaded with NE_{th} - AJ_{EO} (utilized as an anti-biofilm pad) are illustrated in Fig. 4C. The incorporation of

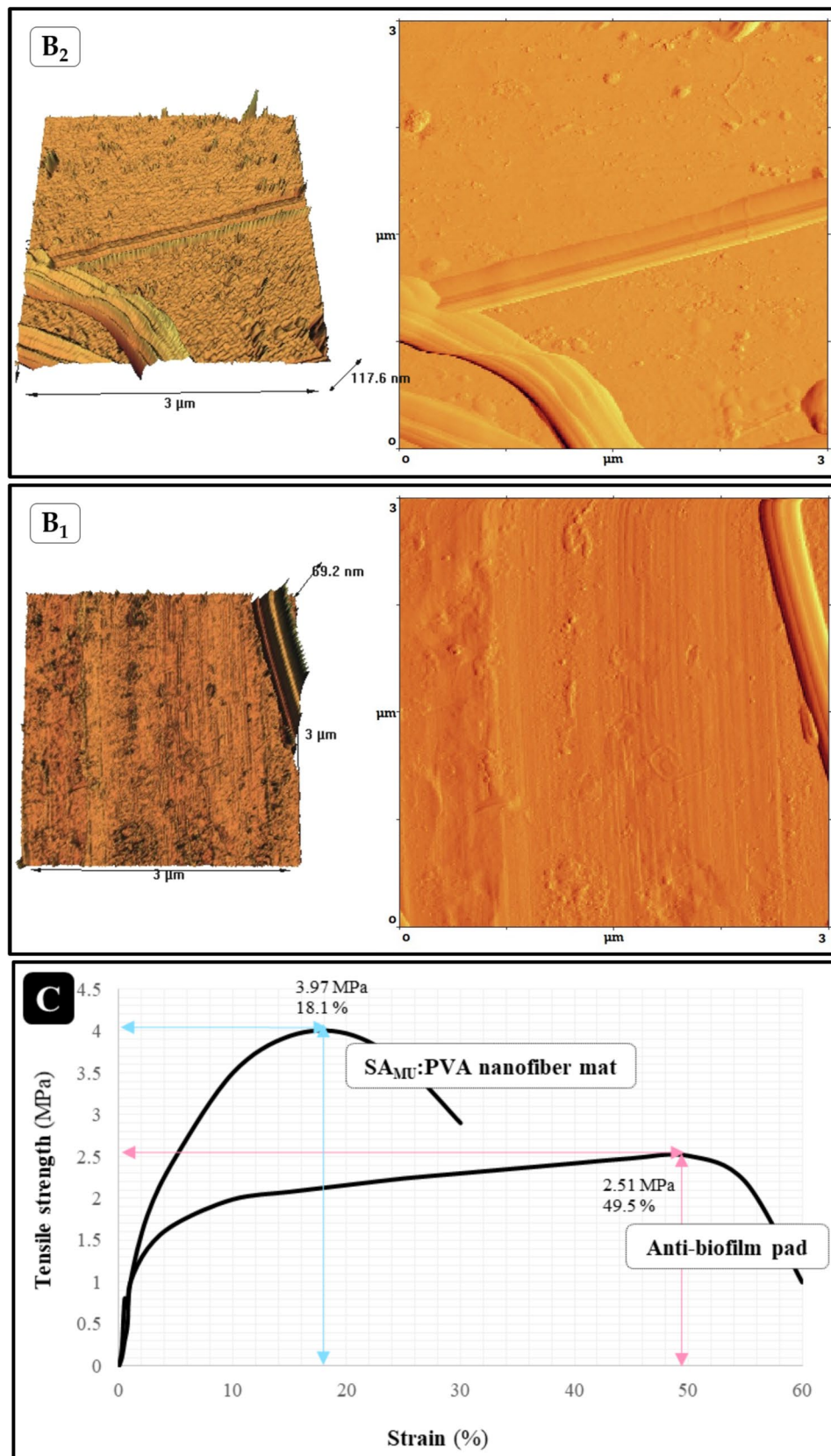


Fig. 4 (continued)

NE_{th}-AJ_{EO} significantly influenced the mechanical attributes of the resultant hybrid nanofiber pad, as evidenced by the data presented in the graph. Specifically, the addition of NE_{th}-AJ_{EO} resulted in a reduction of tensile strength while simultaneously enhancing the elongation at break when compared to the neat SA_{MU}:PVA nanofiber mat. Furthermore, the introduction of NE_{th}-AJ_{EO} led to an increase in the average fiber diameter. The structural characteristics and morphology of the nanofiber mat in different forms (woven, non-woven, or directional) plays a crucial role in determining the mechanical properties of the final pad [50]. The mechanical properties of nanofibers are influenced by several key factors including the polymer chemistry, fibers' diameter, the processing conditions, and the surrounding environmental factors. A comprehensive understanding of how these elements interact is essential for effective tailoring the properties of electrospun fibers. This can be achieved by judiciously selecting materials, adjusting the composition of the solution, modifying processing techniques and parameters, or implementing treatments after the spinning process [51]. Nanofibers characterized by a smaller diameter exhibit a less woven architecture, which markedly enhances the mechanical characteristics of the resultant pad. The incorporation of NE_{th}-AJ_{EO} as a lipophilic bioactive component leads to a reduction in porosity, prompting the nanofibers to realign and the structure to evolve into a more layered configuration. This transformation increases elasticity; however, the reduction in interweaving concurrently leads to a decline in mechanical strength. The incorporation of NE_{th}-AJ_{EO} as a lipophilic bioactive component leads to a reduction in porosity and increasing elasticity. However, the reduction in interweaving concurrently leads to a decline in mechanical strength.

3.3.4 FTIR Analysis

The FTIR spectra of AJ_{EO}, NE_{th}-AJ_{EO}, SA_{MU}:PVA nanofibers, and anti-biofilm pad (containing 10%wt. NE_{th}-AJ_{EO}) are shown in Fig. 5.

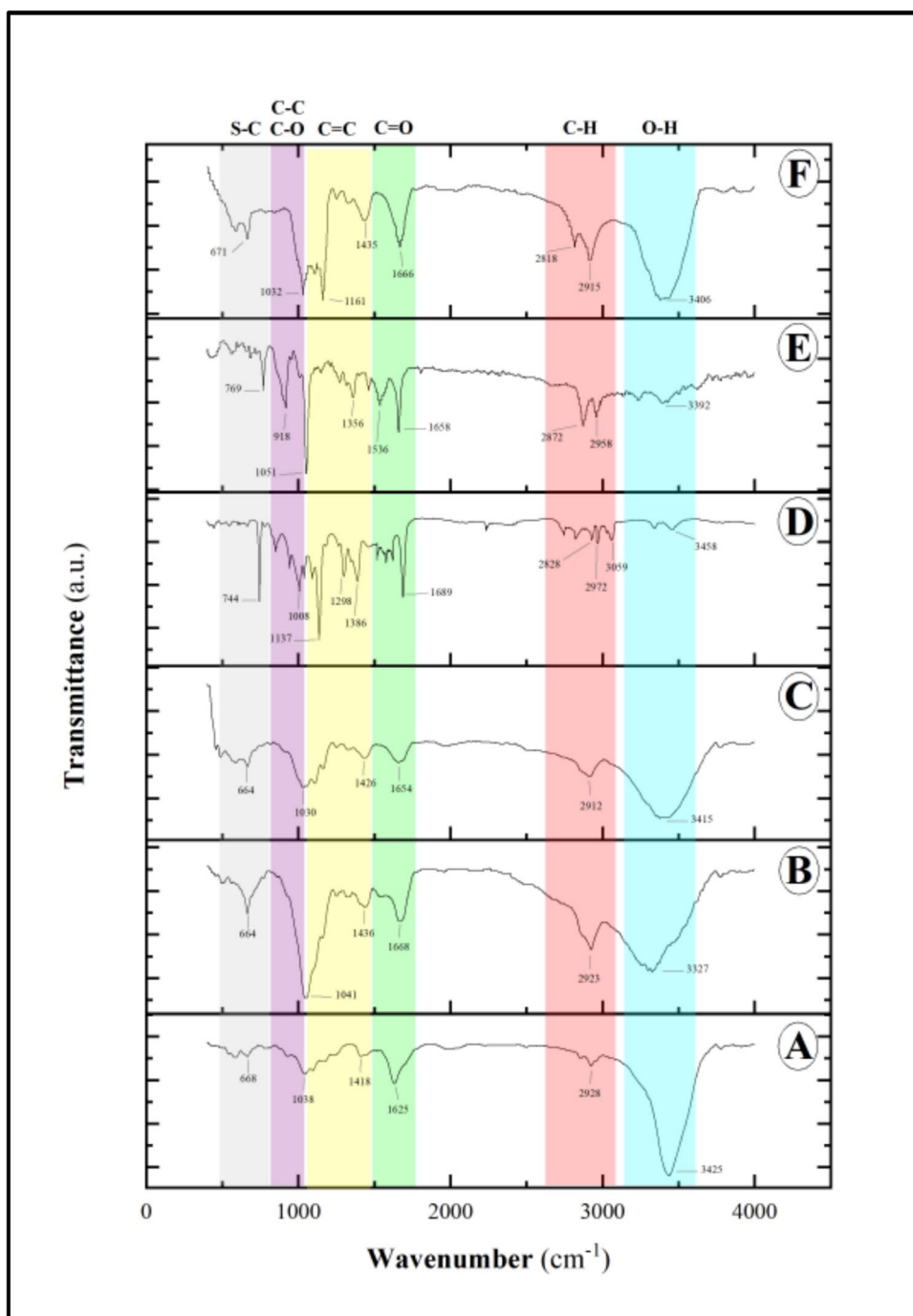
The comparative FTIR analysis of PVA, SA_{MU}, and their blended nanofiber matrix is presented in Fig. 5A and B. In all samples, a broad absorption band related to O–H stretching was observed within the range of 3000–3500 cm⁻¹. In pure PVA, this band appeared at 3425 cm⁻¹, while in SA_{MU} it was centered at 3327 cm⁻¹ [52]. In the SA_{MU}:PVA nanofibers (Fig. 5C), this band shifted to 3415 cm⁻¹, indicating hydrogen bonding interactions between the hydroxyl groups of both components [53]. The C–H stretching bands were also present across the spectra. In PVA and SA_{MU}, they appeared at 2928 cm⁻¹ and 2923 cm⁻¹, respectively, whereas in the nanofiber matrix, the band shifted slightly to 2912 cm⁻¹, suggesting minor structural rearrangements during blending [54]. C=O stretching vibrations, observed at

1625 cm⁻¹ in PVA and 1668 cm⁻¹ in SA_{MU}, were present at 1654 cm⁻¹ in the SA_{MU}:PVA nanofibers, further supporting molecular-level interactions without the formation of new chemical bonds [55]. The C–O–C and C–O stretching vibrations, characteristic of both polymers, were also evident. For instance, peaks at 1041 cm⁻¹ (SA_{MU}) and 1038 cm⁻¹ (PVA) merged in the composite spectrum, showing a broad band around 1030 cm⁻¹, indicating physical compatibility and intermolecular interactions [54]. The comparison of spectra confirms that while no new covalent bonds were formed in the SA_{MU}:PVA nanofibers, notable shifts and broadening of key absorption bands—especially in the O–H and C=O regions—support the existence of strong hydrogen bonding and favorable interactions between the polymer chains. These results align with Ebadi Ghareh Koureh et al. (2023), who reported similar FTIR shifts and band broadening in salep-PVA nanofibers, indicating strong hydrogen bonding and good molecular compatibility without new covalent bonds [27].

The FTIR spectra of AJ_{EO} (Fig. 5D), NE_{th}-AJ_{EO} (Fig. 5E), and the anti-biofilm pad containing 10% NE_{th}-AJ_{EO} (Fig. 5F) reveal significant spectral modifications that provide insight into the molecular interactions occurring during coating and integration into the nanofibrous matrix. In the pure AJ_{EO} spectrum (Fig. 5D), characteristic peaks appear at 3059 cm⁻¹ (aromatic C–H), 2972 and 2828 cm⁻¹ (asymmetric and symmetric C–H stretching of methyl/methylene and aldehyde groups), 1689 cm⁻¹ (C=O stretching of aldehyde groups), and bands at 1386 and 1298 cm⁻¹ (C–O and CH₂ bending) [56, 57]. Additionally, intense peaks at 1137 and 744 cm⁻¹ correspond to S–S and S–C stretching, indicating the presence of organosulfur compounds typical of Allium-derived EOs. These peaks confirm the complex phenolic and sulfur-rich composition of the AJ_{EO} [58, 59]. Upon nanoencapsulation (Fig. 5E), distinct changes are evident. The C=O stretching band at 1689 cm⁻¹ shifts to 1658 cm⁻¹, indicating hydrogen bonding between the oil components and phospholipids in the ethosomal vesicles. The broad O–H stretching band centered at 3392 cm⁻¹ is more intense than in the pure oil, further supporting hydrogen bond formation [60]. New bands appear at 1356 cm⁻¹ (P=O stretching) and 918 cm⁻¹ (P–O–C stretching of phosphate esters), which are attributed to lecithin components in the ethosomal structure. Meanwhile, the C–H stretching bands shift slightly and appear at 2958 and 2872 cm⁻¹, indicating structural reorganization of hydrocarbon chains within the vesicles. The reduced intensity of certain AJ_{EO}-specific peaks (e.g., 744 and 1137 cm⁻¹) suggests partial encapsulation and shielding of sulfur-containing groups [61].

In the final anti-biofilm pad (Fig. 5F), the FTIR spectrum shows additional spectral transformations. The O–H stretching band is further broadened and slightly shifted to 3406 cm⁻¹, indicative of enhanced hydrogen

Fig. 5 FTIR spectra of **A** PVA, **B** SA_{MU}, **C** SA_{MU}:PVA nanofibers, **D** AJ_{EO}, **E** NE_{th}-AJ_{EO}, and **F** anti-biofilm pad (containing 10%wt. NE_{th}-AJ_{EO})



bonding between the polymeric matrix (SA_{MU}:PVA) and NE_{th}-AJ_{EO}. The C–H stretching vibrations appear at 2915 and 2818 cm^{−1}, with clearly reduced intensity compared to AJ_{EO} and NE_{th}-AJ_{EO}, confirming successful incorporation and restricted mobility of the oil within the fiber matrix. The C=O band is observed at 1666 cm^{−1}, closely aligned with the NE_{th}-AJ_{EO} signal but with decreased sharpness, suggesting physical entrapment rather than covalent modification [17]. Importantly, multiple AJ_{EO}-specific peaks disappear entirely, including those at 3059, 2972, 2828, 1386, 1298,

1137, and 744 cm^{−1}, which strongly suggests that the essential oil components are no longer present in free form but are instead fully encapsulated or bound within the matrix structure. This aligns with Sreedharan et al. (2022), who confirmed curcumin's successful incorporation and compatibility in nanoethosome formulations through FTIR analysis [62]. These spectral features—including peak shifts, intensity reduction, loss of characteristic bands, and appearance of new peaks—collectively support the occurrence of intermolecular interactions such as hydrogen bonding and

electrostatic attraction, as well as successful encapsulation of NE_{th}-AJ_{EO} in the polymeric nanofiber network. This confirms the structural integration of the essential oil and validates the effectiveness of the electrospinning process for producing bioactive pads with sustained release potential.

3.3.5 Temperature-Induced Release

Successful encapsulation fundamentally relies on the effective delivery of bioactive compounds to their designated target sites [63]. The In-vitro release kinetics of AJ_{EO} from the NE_{th}-AJ_{EO} and anti-biofilm pad (containing 10%wt. NE_{th}-AJ_{EO}) is shown in Fig. 6.

AJ_{EO} was released rapidly from NE_{th}-AJ_{EO}, and the cumulative release reached > 99% at 37 and 45 °C. On the contrary, the AJ_{EO} released a uniform and time-dependent anti-biofilm pad compared to the NE_{th}-AJ_{EO}. In addition, the results indicated that in NE_{th}-AJ_{EO}, the diffusion rates increased with temperature due to partial softening or increased molecular mobility of the lipophilic wall materials within the range of 37–45 °C, enhancing permeability. This led to an initial increase in the release of AJ_{EO} molecules. However, as the temperature rose further, swelling of the nanofiber matrix occurred, which acted as a barrier and reduced the overall release from the anti-biofilm pad. Therefore, although the nanoethosomal structure showed increased permeability, the swelling effect of the nanofiber wall counteracted this, resulting in a net decrease in release rate at higher temperatures. The persistent release caused by the hybrid encapsulation was protected in the nanofibers for longer time. A key feature of delivery systems is their ability to dissolve and break down time-dependently in the target environment, thereby targeting the release of their entrapped active components. This capability is a significant promise for various medical, pharmaceutical, and food industries [16]. The low release at all temperatures is greatly desired since it shows that the anti-biofilm pad is effectively shielded throughout storing conditions.

3.4 Anti-biofilm Evaluation

3.4.1 The Minimum Inhibitory Concentration (MIC) and Minimum Biofilm Inhibitory Concentration (MBIC) of AJEO

A microtiter plate method coupled with a crystal violet quantification assay was employed to assess the MIC and MBIC of AJ_{EO} against both reference and MDR clinical isolate strains of *Acinetobacter baumannii*. The findings indicated that the MIC values for AJ_{EO} were 250 µg/mL for the reference strain and 500 µg/mL for the MDR clinical isolate strain, as illustrated in Fig. 7A. The MBIC is defined as the lowest concentration of a substance that achieves over

50% inhibition of biofilm formation without significantly impacting bacterial growth. At a concentration of 50 µg/mL, AJ_{EO} demonstrated substantial anti-biofilm activity against both reference and MDR clinical isolate strains, as shown in Fig. 7B, while maintaining < 50% effect on growth. Previous research has indicated that organosulfides possess notable anti-biofilm properties [64, 65]. Our results support that organic trisulfide contributes to the antibacterial efficacy of AJ_{EO} and exhibits a pronounced ability to disrupt biofilm formation. Furthermore, the inhibitory effect of AJ_{EO} on *Acinetobacter baumannii* strains and their biofilms was found to be highly dependent on the dosage applied.

3.4.2 Air–Liquid Interface Biofilm Formation

Acinetobacter baumannii demonstrates a notable capacity for biofilm formation at air–liquid interfaces, primarily by adhering to the upper regions of liquid media. Consequently, the impact of an anti-biofilm pad on the adherence of this bacterium to polystyrene surfaces and the development of ring biofilms was evaluated. To further investigate the influence of the anti-biofilm pad on the three-dimensional architecture of *Acinetobacter baumannii* biofilms, CLSM analysis was conducted, with results summarized in Table 2. Control samples exhibited multi-layered biofilms with substantial biomass, while the biofilm produced by the MDR strain was significantly greater than that of the reference strain. The persistence of *Acinetobacter baumannii* in various environments can be partially attributed to its ability to form biofilms on both abiotic and biotic surfaces, which also plays a critical role in the pathogenesis of device-related infections [66]. Notably, the presence of an MDR phenotype correlates with an increase in biofilm formation [67]. A significant decrease in biofilm biomass, surface area coverage, and thickness was noted in *Acinetobacter baumannii* strains treated with an anti-biofilm pad containing 10% wt. NE_{th}-AJ_{EO}. The findings indicated that the anti-biofilm pad had a pronounced impact on key parameters, including biomass, maximum thickness, and the surface-to-volume ratio [68].

In contrast, control samples exhibited a highly intricate ring structure with multilayered cell arrangements, demonstrating strong adherence to the polystyrene surface, as detailed in Fig. 8A. Conversely, the microtubes treated with the anti-biofilm pad displayed dispersed and disintegrating cell clumps along with collapsed micro-colonies. Additionally, the study by Bajpai et al. corroborated a significant reduction in both the biomass and the average and maximum thickness of biofilms in samples treated with nitrogen-phosphorus-doped carbon nanodots, highlighting their effective anti-biofilm properties against *Listeria monocytogenes* [69]. The inclusion of AJ_{EO} in the pad significantly diminished biofilm formation at the air–liquid

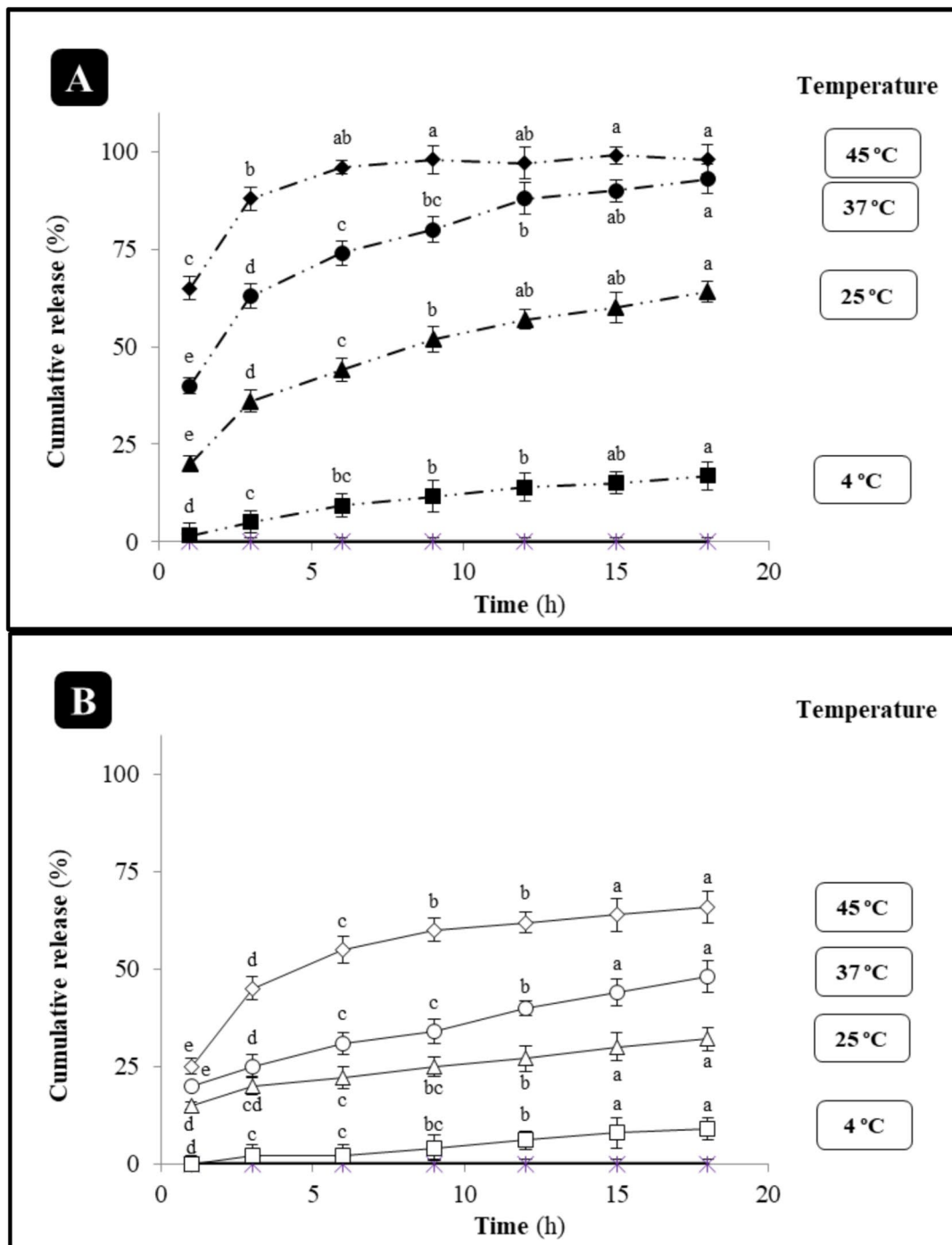


Fig. 6 Effects of different temperatures on the cumulative release profile of AJ_{EO} from **A** NE_{th}-AJ_{EO} and **B** anti-biofilm pad (containing 10%wt. NE_{th}-AJ_{EO}). Values are mean $\pm$ SD. Different letters indicate significant differences ($p < 0.05$)

interface across all tested strains of *Acinetobacter baumannii*, as illustrated in Fig. 8B. Furthermore, the anti-biofilm pad effectively decreased the attachment of these strains to the surface of polystyrene Petri dishes, a finding documented by crystal violet staining. Light microscopy

revealed that control samples exhibited highly aggregated and well-structured biofilms. In contrast, samples treated with the anti-biofilm pad displayed a marked reduction in biofilm density, characterized by dispersed cellular arrangements across all strains tested, as shown in Fig. 8C.

Fig. 7 Effect of AJ_{EO} at various concentrations (1–2500 $\mu\text{g/mL}$) on the **A** growth and **B** biofilm inhibition of *Acinetobacter baumannii* reference and MDR clinical isolate strains. Values are mean $\pm$ SD. Different letters indicate significant differences ($p < 0.05$)

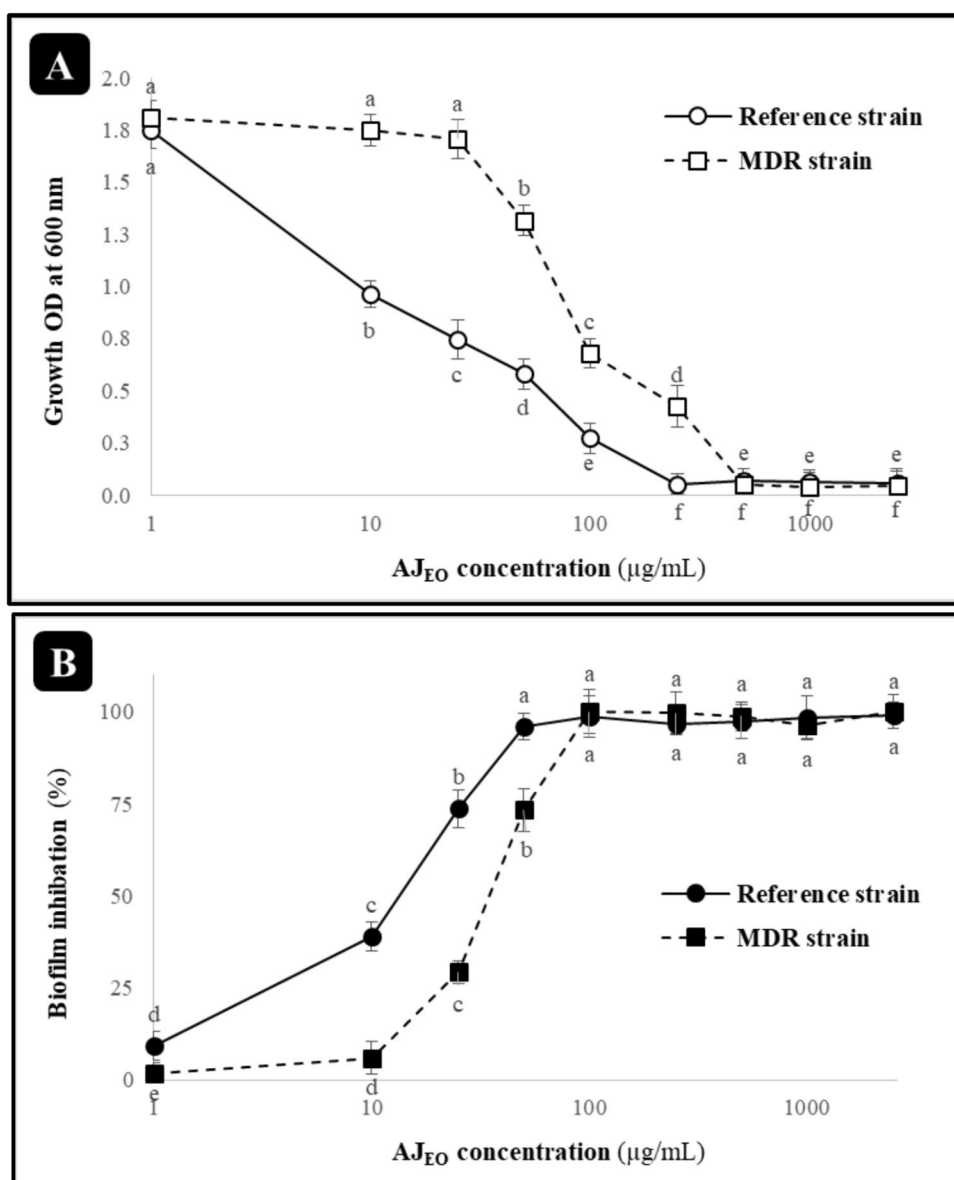


Table 2 Analysis of biofilms formed by *Acinetobacter baumannii* strains in the absence and presence of anti-biofilm pad (containing 10%wt. NE_{th} - AJ_{EO})

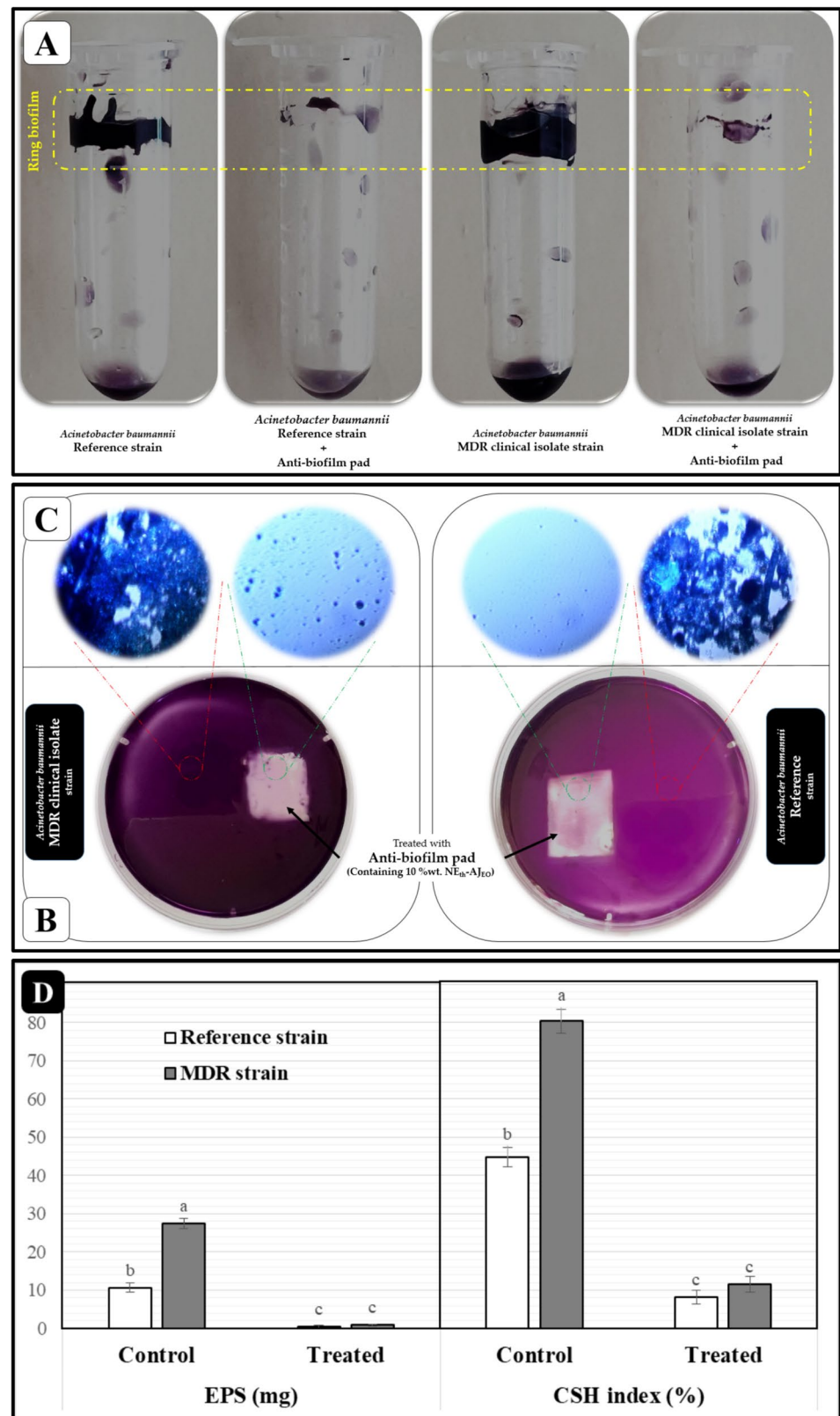
<i>Acinetobacter baumannii</i> strains	Sample	Maximum thickness (μm)	Surface to volume ratio ($\mu\text{m}^2/\mu\text{m}^3$)	Biofilm (μm^3)
Reference	Control	20.33 ± 0.98	1.14 ± 0.27	18.68 ± 1.14
	Treated	0.94 ± 0.12	8.25 ± 0.67	0.85 ± 0.16
MDR clinical isolate	Control	52.84 ± 2.45	0.94 ± 0.20	26.02 ± 1.60
	Treated	1.16 ± 0.09	7.03 ± 0.99	0.91 ± 0.18

3.4.3 The Extracellular Polysaccharides (EPS) and Cell Surface Hydrophobicity (CSH) Inhibition

Biofilms consist of different microorganisms communities that attach to both biotic and abiotic surfaces, encapsulated within a matrix of EPS. This matrix serves to shield the microbial cells from adverse environmental

conditions, thereby enhancing their survival and resilience. The production of EPS in *Acinetobacter baumannii* is closely linked to the formation and structural integrity of biofilms [70]. Quantitative mass analysis of EPS in samples treated with an anti-biofilm pad revealed a notable decrease in EPS levels (Fig. 8D) specifically, at 10%wt. NE_{th} - AJ_{EO} for the anti-biofilm pad, a reduction

Fig. 8 **A** Ring biofilm formation, **B** adherence on polystyrene surface, **C** light microscopy image of anti-biofilm pad against *Acinetobacter baumannii* reference and MDR clinical isolate strains and **D** Inhibitory effect of anti-biofilm pad (containing 10%wt. NE_{th}-AJ_{EO}) against EPS production and CSH of *Acinetobacter baumannii* reference and MDR clinical isolate strains. Values are mean $\pm$ SD. Different letters indicate significant differences ($p < 0.05$)



exceeding 90% in EPS was recorded across all tested strains of *Acinetobacter baumannii* when compared to the control samples.

In *Acinetobacter baumannii*, hydrophobicity has previously been correlated with increased adherence to abiotic surfaces. Additionally, the CSH of *Acinetobacter baumannii*

plays a crucial role in its ability to adhere to both living and non-living surfaces [71]. The investigation for the impact of the anti-biofilm pad on the CSH of *Acinetobacter baumannii* demonstrated a consistent decline in CSH among the treated strains relative to the control samples.

3.5 Conclusion

A multifunctional pad for the controlled release of essential oil derived from *Allium jesdianum* Boiss. AJ_{EO} was designed in this research. This pad was successfully developed through the integration of its nanoethosome encapsulation with Salep mucilage and electrospinning techniques. The hybrid encapsulation of the nanoethosome/nanofiber structure not only enhanced the stability of the AJ_{EO} but also improved the persistent release of hydrophobic components within the anti-biofilm pad. Notably, the incorporation of an appropriate ratio of NE_{th}-AJ_{EO} into the nanofiber matrix was found to promote drug release in response to bacterial phospholipase activity, particularly in the presence of *Acinetobacter baumannii*. Such stimulus-responsive systems are increasingly favored in research due to their ability to facilitate controlled drug release under specific conditions. In addition to inhibiting the biofilm formation of *Acinetobacter baumannii*, the anti-biofilm pad containing 10% wt. NE_{th}-AJ_{EO} also influenced biofilm-related adherence factors of *Acinetobacter baumannii* strains including its extracellular polysaccharides and cell surface hydrophobicity. Furthermore, the materials utilized in this formulation align with contemporary trends in anti-biofilm strategies. The findings of this study suggest a promising approach for the management of HAIs.

Author Contribution Mohammad Ekrami: Methodology, data analysis, and writing—original draft preparation, Ali Ekrami: Data collection (Anti-biofilm pad preparation and characterization section), Negar Roshani-Dehlaghi: Data collection (Nanoethosome preparation and characterization section), Nima Mobahi: Data collection (Essential oil preparation and characterization Sect. 0, Zahra Emam-Djomeh: Supervision, project administration and funding acquisition, Mohammadreza Salehi: Validation data and visualization, Reza Ovissipour: Conceptualization and visualization, Ahmad Kalbasi-Ashtari: Writing—review and editing.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval Not applicable.

Competing interests The authors declare no competing interests.

Research Involving Humans and Animals Statement None.

Informed Consent None.

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