Saudi Journal of Biological Sciences 30 (2023) 103707

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Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Enhanced activity of Ellagic acid in lipid nanoparticles (EA-liposomes) against *Acinetobacter baumannii* in immunosuppressed mice

Khaled S. Allemailem

Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraydah 51452, Saudi Arabia

ARTICLE INFO

Article history:
Received 17 April 2023
Revised 3 June 2023
Accepted 9 June 2023
Available online 15 June 2023

Keywords:
Acinetobacter baumannii
Ellagic acid
Liposomes
Immune-suppression

ABSTRACT

Acinetobacter baumannii infections have come to the surface in huge numbers in the recent decades. Furthermore, *A. baumannii* has adopted great ability to nullify the majority of currently available antibiotics. With the purpose of finding a nontoxic and efficient therapeutic agent, we analyzed the activity of Ellagic acid (EA) against the multidrug-resistant *A. baumannii*. EA not only demonstrated its activity against *A. baumannii*, but also inhibited the biofilm formation. Since EA shows poor solubility in an aqueous environment, a lipid nanoparticle-based (liposomal) formulation of EA (EA-liposomes) was prepared and its effectiveness was assessed to treat bacterial infection in the immunocompromised murine model. Therapy with EA-liposomes imparted greater protection to infected mice by increasing the survival and decreasing the bacterial load in the lungs. *A. baumannii* infected mice treated with EA-liposomes (100 mg/kg) showed 60% survival rate as compared to 20% of those treated with free EA at the same dose. The bacterial load was found to be 32778 ± 12232 in the lungs of EA-liposomes (100 mg/kg)-treated mice, which was significantly lower to 165667 ± 53048 in the lung tissues of free EA treated mice. Likewise, EA-liposomes also restored the liver function (AST and ALT) and kidney function parameters (BUN and creatinine). The broncho-alveolar fluid (BALF) from infected mice contained greater quantities of IL-6, IL-1β and TNF-α, which were significantly alleviated in EA-liposomes treated mice. These findings together support the possible implication of EA-liposomes to treat *A. baumannii* infection, especially in immunocompromised mice.

1. Introduction

Acinetobacter baumannii is a menacing bacterial pathogen that is causing grave infections, particularly in immunocompromised individuals. It is a predominant healthcare-associated pathogen causing a global threat due to its robust tendency to acquire multidrug resistance (Harding et al., 2018, WHO, 2017). The propensity of multidrug resistance is much higher in *A. baumannii* as compared to other pathogens (WHO, 2017). It causes a number of infections that include blood stream, soft tissue and catheter- or ventilator-associated infections (Weiner et al., 2016). The patients with diabetes, alcoholism, obstructive pulmonary disorder and cancer are easy targets of *A. baumannii* (Dexter et al., 2015). There are certain bacterial components such as porins, outer membrane proteins and vesicles (OMPs and OMVs), capsular polysaccharides, lipopolysaccharide (LPS), phospholipase C and D (PLC and PLD) that have a critical role in the virulence of the pathogen (McConnell et al., 2013). Besides, it can adopt multiple mechanisms for drug-resistance, including multi-drug efflux pump, enzymatic degradation of antibiotics, alteration of target, permeability defects and the formation of biofilm (Boucher et al., 2009, Kim et al., 2012, Lee et al., 2017).

The use of natural compounds has been suggested to be a safe and effective therapeutic option to fight against drug-resistant *A. baumannii* (Tsai et al., 2018, Hassannejad et. al.,2019). Natural compounds such as thymol and carvacrol showed antibacterial effects against drug-resistant *A. baumannii* (Hassannejad et al., 2019). In another study, Tsai reported the activity of *Scutellaria barbata* against *A. baumannii* (Tsai et al., 2018). Volatile oil from Cinnamon and Eucalyptus camaldulensis essential oil also showed potent antibacterial activity against *A. baumannii* (Intorsoot et al., 2017, Knezevic et al., 2016). Ellagic acid (EA) is mainly found in vegetables and fruits, including pomegranates, strawberries, walnuts and raspberries (Rios et al., 2018). EA has demonstrated many therapeutic benefits such as antioxidant and anti-inflammatory activity, chemo-preventive, anti-apoptotic, neuroprotective and antimicrobial activity (Rios et al., 2018, De et al., 2018, Kha et al., 2021). In spite of loaded with many beneficial effects, the therapeutic use EA is restricted due to its minimal solubility and

E-mail address: k.allemailem@qu.edu.sa

https://doi.org/10.1016/j.sjbs.2023.103707
1319-562X/

decreased bioavailability in an aqueous environment (Alfei et al., 2019). Various approaches, including amorphization, particle size reduction, liposome-based nanoformulations and nano-emulsions have been suggested to enhance the efficacy and bioavailability of the drug (Khan et al., 2021, Nyamba et al., 2021). These formulations successfully increased the *in vivo* efficacy of EA in various disease conditions (Khan et al., 2021, Alhakamy et al., 2020, Hallan et al., 2020, Mady et al., 2017). Liposomes have extensively been studied as the carriers of drugs and vaccines (Khan MA, 2021). Liposome-mediated site specific targeting may reduce the dose of the drug as compared to when free drug is used. Earlier, we suggested various surface modification startegies of liposomes that may increase the therapeutic efficacy of liposomes (Khan et al., 2020). Here, we prepared EA-incorporated liposomes (EA-liposomes) and investigated their activity in the treatment of *A. baumannii* infection in a murine model. EA-liposomes were not only found to be very safe, but were also very effective in the elimination of *A. baumannii* when compared to free EA.

2. Materials and methods

Highly purified lipids such as 1,2-dipalmitoyl-Sn-*glycero*-3-phosphocholine (DPPC), cholesterol were bought from the Avanti Polar Lipids (Alabaster, AL, USA). Cytokines and antioxidants estimation kits were obtained from the Abcam (Cambridge, UK). Whereas Ellagic acid, Cyclophosphamide and RPMI were from MedChem Express (Monmouth Junction, NJ, USA). Nutrient Agar and Tryptic soya broth (TSB) were procured from the HiMedia Company (Mumbai, India).

2.1. Acinetobacter baumannii

A multi-drug resistant strain of *A. baumannii* (ATCC 19606) was used in the current study (Su et al., 2006, Allemailem et al., 2021).

2.2. To determine the antibacterial activity of EA

Antibacterial activity of EA was ascertained in the nutrient agar plates by measuring the inhibition zone. *A. baumannii* inoculum were seeded on nutrient agar (NA) plates and EA (100, 200 and 400 μg) was loaded into each well. The plates were kept at 37 °C for 24 h and EA activity was determined by evaluating the inhibition zone in each plate.

Agar well diffusion is method has limitation to determine the antimicrobial activity of the drug because different drugs diffuse at different rates in agar. Thus the dilution method is employed to estimate the minimum inhibitory concentration (MIC) of EA. Various concentrations of EA (0.5 to 512 μg/ml) in nutrient broth (NB) at 37 °C for 24 h (CLSI, 2016). The amount of EA that inhibited the growth of bacteria was taken as the MIC of the drug.

Microscopic analysis of *A. baumanni* was also performed to determine the antibacterial activity of EA. *A. baumannii* (1×10^6 cells) was taken in NB in 12- well sterile culture plates. After 4 h, A. baumannii was treated with EA at the doses of 16, 32 and 64 μg/ml for 24 h. The well containing the vehicle was considered as a negative control. Each well was washed with PBS and the status of the untreated or treated *A. baumannii* was monitored under the microscope.

The Time-kill study was done with 1×10^6 cells of *A. baumannii* with 32 and 64 μg/ml of EA (Khan et al., 2021). Briefly, *A. baumannii* was grown in TSB and the bacterial suspension (1×10^6 cells) was included in the flask containing TSB (20 ml) and the above EA doses. A 100 μl aliquot was withdrawn at the time intervals of 0, 3, 6, 12, and 24 h. The aliquots were centrifuged, washed and reconstituted in PBS. The bacterial colony forming units (CFUs) were calculated by culturing suspension dilutions onto nutrient agar plates.

2.3. Preparation and characterization of EA- liposomes

Phosphatidylcholine (PC) and, cholesterol in the molar ratio of 7:3 were dissolved in 1:1 (Vol/Vol) mixture of methanol and chloroform (Allemailem et al., 2021). Whereas, EA and the lipids were taken in the molar ratios of 1:20. The solvents were evaporated to form a thin dried lipid film that was dispersed in the sterile PBS. The lipid suspension was sonicated to prepare liposomes with or without EA. The sonicated suspension was centrifuged to separate EA-liposomes from free EA.

The characteristics of liposomes, including the size, polydispersity index (PDI) and zeta potential were determined by the Dynamic Light Scattering (DLS) technique by Malvern Nano Zeta Sizer (Malvern instruments, Southborough, Massachusetts). The quantity of liposomes-entrapped EA was estimated by recording the optical density at 340 nm. EA-liposomes were lysed in DMSO to measure the entrapped EA by the standard curve of EA The entrapped EA was estimated by the following formula:

$$\% \text{ of entrapped EA} = (\text{Liposomal EA} / \text{Total EA}) \times 100$$

2.4. Anti-biofilm activity of free EA or EA-liposomes

In order to determine the anti-biofilm formation efficacy of EA or EA-liposomes, 1×10^5 cells of *A baumannii* were inoculated with 32 and 64 μg/ml of EA or EA-liposomes into 96-well plate at 37 °C (Allemailem et al., 2021c). After 24 h, the wells were washed and the free floating bacterial cells were decanted. After drying the wells, 100 μl of 0.1% crystal violet (CV) was included in each well for 15 min. After washing and drying, 95% ethanol (100 μl) was put to solubilize CV that was recorded at 595 nm.

2.5. Animals

BALB/C mice (female and 12 weeks old) were used in the current study. The experiments in mice were endorsed by the committee of research ethics, Deanship of Scientific Research, Qassim University. The ethical approval number for this study is 5604-cams1-2019–2-2-I.

2.6. The effect of free EA or EA-liposomes against cyclophosphamide-induced immune-suppression

Mice were injected with cyclophosphamide (CYP) at 200 mg/kg dose through intraperitoneal route (Allemailem et al., 2021). After 24 h, free EA or EA-liposomes at the doses of 25 and 50 mg/kg was given for 7 days. On days 5 and 10 after CYP administration, the blood was drained to analyze leukocyte numbers by the automated hematology analyzers.

2.7. A. Baumannii infection in immunosuppressed mice

On day 5 post-CYP administration, 1×10^7 CFUs of A. baumannii via the lateral tail vein as standardized earlier (Allemailem et al., 2021).

2.8. The treatment of infected mice with EA-liposomes

Acinetobacter baumannii infected mice received a dose of free EA or EA-liposomes (50 and 100 mg/kg) for consecutive 7 days. Mice were randomly distributed into six groups: (1) Control, (2) Sham liposomes, (3) EA-50 mg/kg, (4) EA −100 mg/kg, (5) EA-

liposomes 50 mg/kg, (6) EA–liposomes 100 mg/kg. The survival rate was observed everyday for 30 days and the numbers of dead and live mice were noted.

2.9. Determination of the severity of bacterial infection

The effectiveness of the drug formulations was assessed by survival data and bacterial burden (Allemailem et al., 2021). Three mice were taken from each group and sacrificed on day 5 post-treatment. The lungs were detached and were homogenized in PBS by the gentle Mac Dissociator. The dilutions of homogenates were spread on NA plates for 24 h. The CFUs of *A. baumannii* were counted.

2.10. Determination of the safety and toxicity of EAor EA-liposomes

The safety of therapeutic preparations was assessed by analyzing the status of Aspartate transaminase (AST), Alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine in the untreated or treated mice (Allemailem et al., 2021).

2.11. Analysis of the broncho-alveolar fluid (BALF) to measure inflammatory cytokines

The efficacy of EA-liposomes treatment was examined by investigating the status of IL-1β, TNF-α and IL-6 in the BALF from the untreated or treated mice. The BALF was recovered from the lungs and the amounts of the cytokines were analyzed by the ELISA (Allemailem et al, 2021).

2.12. Statistical analyzes

The Kaplan–Meier curve and the Log-rank Chi square test were used to analyze the survival rate and bacterial load. One-way ANOVA and Bonferroni post-test were used to analyze the bacterial burden by GraphPad Prism software. The data are expressed as the mean ± SD of three values.

3. Results

3.1. EA shows activity against A baumannii

EA exerted activity against *A. baumannii* as determined by the dilution method, agar well diffusion method and microscopy. The MIC of EA against *A. baumannii* was determined to be 64 μg/ml.

The antibacterial activity of EA against *A. baumannii* was examined by the microscopic analysis. EA showed dose-dependent activity against *A. baumannii* (Fig. 1A). A sustained decrease in the viability of *A. baumannii* was noticed as the concentration of EA was increased.

In addition to microscopic analysis and the dilution assay, EA also demonstrated its activity against *A. baumannii* as measured by the inhibition zone. The well containing 400 μg of EA showed 28 mm of zone of inhibition, whereas those containing 100 μg and 200 μg exhibited 11 mm and 19 mm of inhibition zones (Fig. 1B).

The results of time-kill assay also substantiated the antibacterial effect of EA against *A. baumannii*. EA at 32 μg/ml of dose inhibited about 99% growth of *A. baumannii* in comparison to the control group (without EA) at 24 h time point. Whereas, EA at 64 μg/ml killed 99.9% of original *A. baumannii* inoculum at 24 h point (Fig. 1C).

3.2. Free EA or EA-liposomes inhibited the biofilm formation

Free EA and EA-liposomes showed distinct efficacies against biofilm formation (Fig. 2). Treatment with Free EA or EA-liposomes at 32 μg/ml decreased the biofilm formation to 72.3% and 60% as compared to the untreated bacteria. Moreover, free EA or EA-liposomes (64 μg/ml) showed higher effectiveness and restricted the biofilm formation to 23% and 13%, respectively (Fig. 2).

3.3. EA-liposomes effectively alleviated CYP-induced immune suppression

The findings demonstrated that the administration of EA-liposomes induced early revival of the leukocytes in CYP-injected mice (Fig. 3). The numbers of white blood cells were significantly reduced to 1851 ± 330 per mm^3 in the blood of mice on day 5 of CYP-injection as compared to 7652 ± 886 in the normal control (Fig. 3). EA-liposomes at a dose of 25 mg/kg induced the recovery of the leukocytes to 2959 ± 238, but the identical formulation at a dose of 50 mg/kg recovered to 3032 ± 503 per mm^3. However, free EA did not induce significant leukocyte recovery (Fig. 3). Interestingly, the revival of leukocytes was found to be more prominent in free EA or EA-liposomes-treated mice on day 8 post post-CYP injection (Fig. 3). On day 10, the leukocyte numbers were found to be 3921 ± 283 per mm^3, this number was elevated to 5247 ± 173 in free EA (25 mg/kg)-treated and 5208 ± 359 in free EA (50 mg/kg)-treated mice (Fig. 3). Importantly, there was higher recovery of leukocytes to 5775 ± 290 in EA-liposomes (25 mg/kg)-treated, and 6040 ± 283 in EA-liposomes (40 mg/kg)-treated mice (Fig. 3). The recovery of the leukocytes was found to be more significant on day 10 in the mice treated with EA-liposomes (50 mg/kg) as compared to those treated with free EA (50 mg/kg) (Fig. 3) ($p < 0.01$).

3.4. EA-liposomes effectively eliminated A. Baumannii infection

Here, free EA (50 mg/kg) did not impart significant protection to *A. baumannii* infected mice as the treated mice in this group died within 30 days. However, it increased the median survival time (MST) from 3.5 days in the untreated mice to 7.5 days in EA (50 mg/kg)-treated mice (Fig. 4A) ($p = 0.0027$). Whereas, free EA at 100 mg/kg imparted 20% survival rate ($p = 0.009$) to the treated mice. On the contrarily, EA-liposomes (50 mg/kg) treated mice had 30% survival rate ($p = 0.0214$), whereas those treated with EA-liposomes (100 mg/kg) had 60% survival rate ($p < 0.0001$). The data showed that EA-liposomes (50 and 100 mg/kg) had remarkably superior therapeutic potential to free EA at the comparable doses ($p = 0.0214$ and $p = 0.0259$, respectively).

The gravity of the infection in mice was established by calculating the CFUs of bacteria in the lungs. The CFUs were found to be 962342 ± 130341 CFUs/gram in the lungs from PBS-treated mice (Fig. 4B), whereas, the treatment with free EA (50 and 100 mg/kg) decreased the bacterial burden to 324239 ± 74548 and 165667 ± 53048 CFUs/gram ($p < 0.001$). Notably, EA-liposomes at 50 and 100 mg/kg of doses lowered the CFUs to 153365 ± 39510 and 32778 ± 12232 ($p < 0.001$). Besides, the therapeutic activity of EA-liposomes was found to be significantly superior to free EA at the equivalent doses ($p < 0.05$).

3.5. Treatment with EA-liposomes alleviated liver and kidney toxicity

The liver inflammatory markers such as AST and ALT were analyzed in *A. baumannii* infected mice in the untreated control or treated with free EA or EA-liposomes (Fig. 5A, 5B). The AST in the normal mice was 17.33 ± 3.2 IU/L that was raised to 162.7 ± 20.4

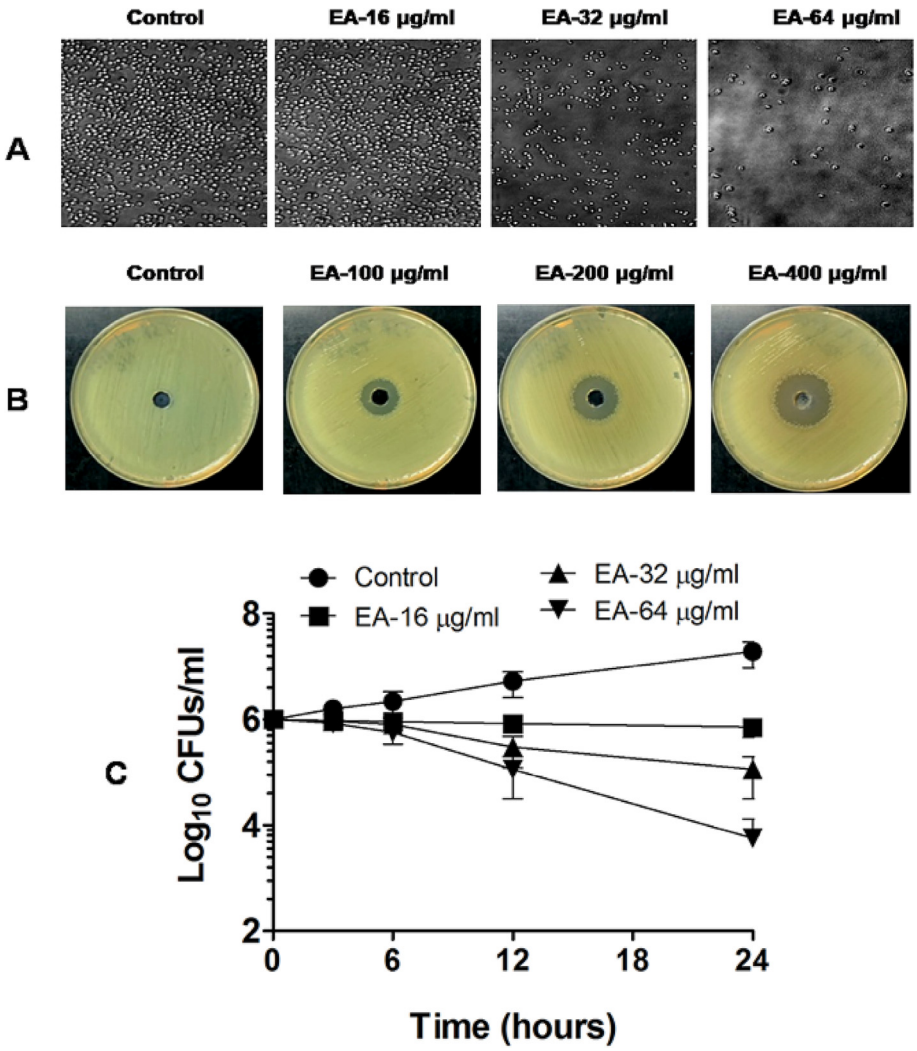

Fig. 1. *In vitro* antibacterial activity of Ellagic acid (EA) against *A. baumannii* by (A) Microscopic analysis (B) Agar well diffusion method, and (C) Time-kill assay.

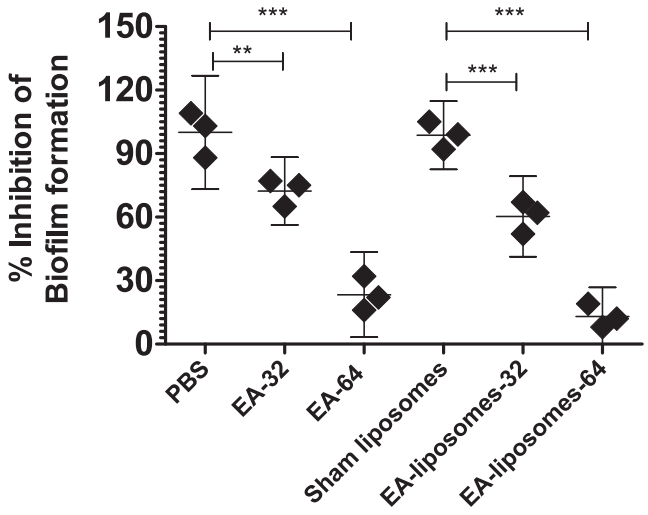

Fig. 2. EA-liposomes significantly inhibited the formation of the biofilm. *** ($p < 0.001$), ** ($p < 0.01$).

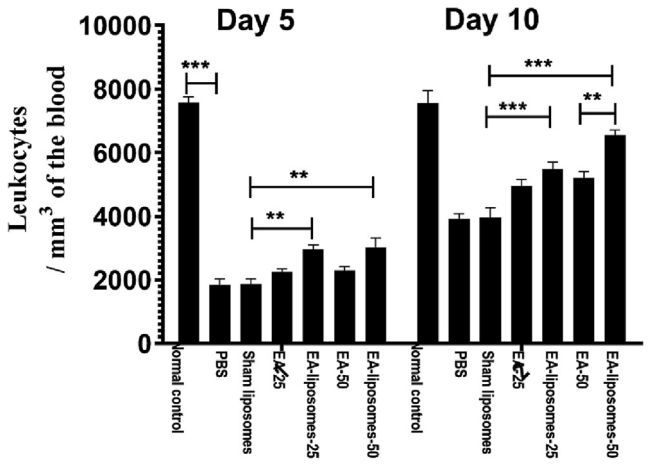

Fig. 3. Treatment with EA-liposomes resulted in greater recovery of leukocytes on days (A) 5 and (B) 10 post-CYP injection. *** ($p < 0.001$), ** ($p < 0.01$).

IU/L in the untreated infected mice. The therapy of EA (50 mg/kg) alleviated the AST to 130 ± 19, whereas EA at 100 mg/kg reduced AST to 96 ± 13 IU/L (Fig. 5A). Whereas, EA-liposomes (50 and 100 mg/kg) significantly lessened the AST levels to 84 ± 15.9 and 48 ± 6 IU/L, respectively (Fig. 5A). In addition, EA-liposomes

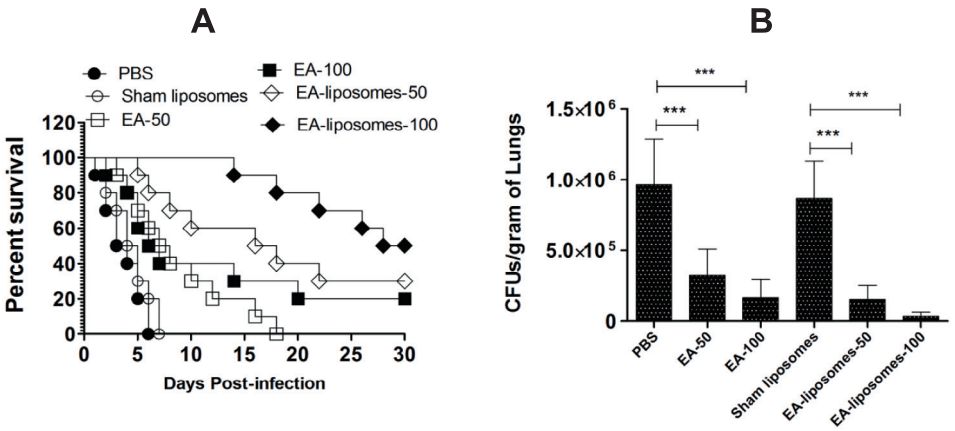

Fig. 4. EA-liposomes showed superior anti-*A.baumannii* activity in immunocompromised mice. (A) Mice were challenged with bacteria and were observed for 30 days. PBS vs EA-50 ($p = 0.009$), PBS vs EA-100 ($p = 0.0027$), PBS vs EA-liposomes-50 ($p = 0.0002$), PBS vs EA-liposomes-100 ($p = 0.0001$), EA-50 vs EA-liposomes-50 ($p = 0.0214$), EA-100 vs EA-liposomes-100 ($p = 0.0259$). (B) The CFUs of *A. baumannii* were assessed as revealed in the methodology part. *** ($p < 0.001$).

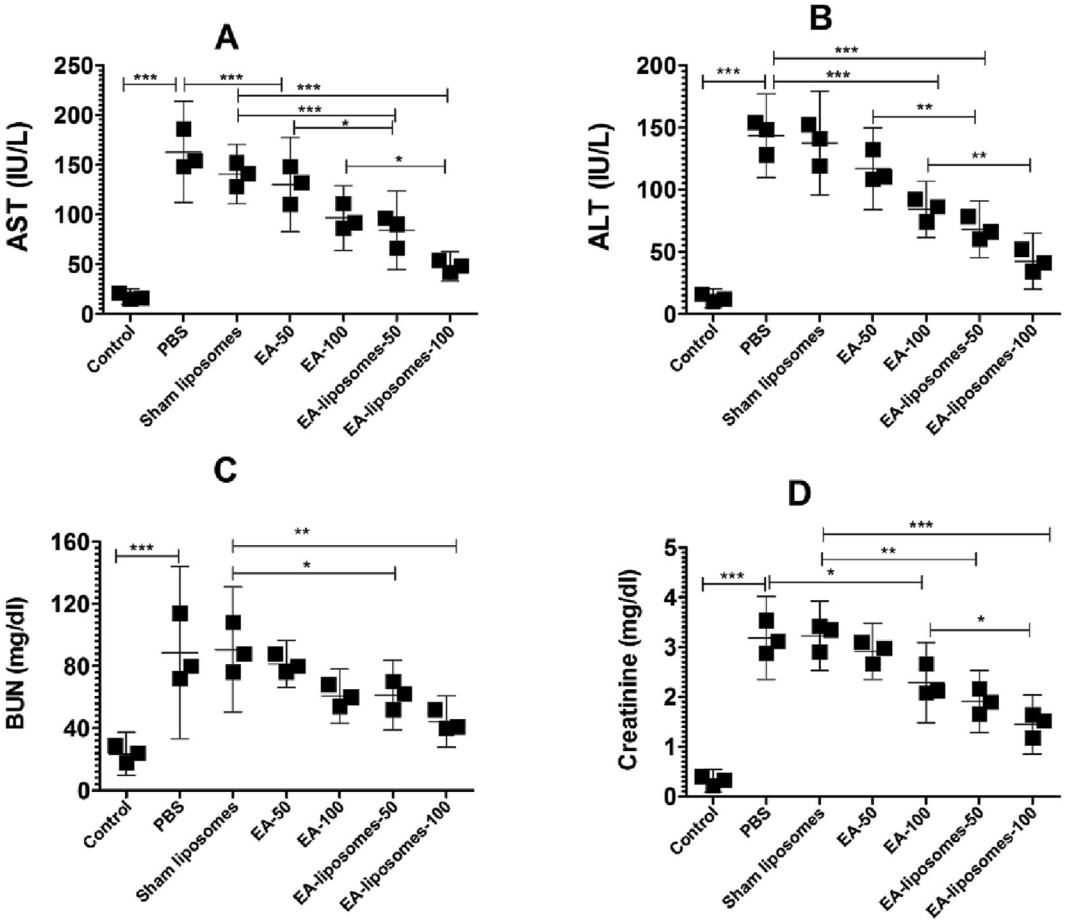

Fig. 5. *A. baumannii* infected mice treated with EA-liposomes showed improved liver and kidney functioning parameters. (A) AST, (B) ALT, (C) BUN and (D) Creatinine. *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$).

demonstrated significantly superior effectiveness in comparison to free EA in improving the AST level ($p < 0.05$).

Besides AST, the ALT level was examined in infected mice followed by the treatment with free EA or EA-liposomes (Fig. 5B). It was detected to be 143 ± 13.6 IU/L in infected mice as compared to 12.67 ± 3 IU/L in the uninfected mice. Free EA (50 and 100 mg/kg) decreased the ALT levels to 116.7 ± 13.3 and 84 ± 9.2 IU/L, whereas the therapy with EA-liposomes alleviated the AST levels to 68 ± 9.2 and 42.3 ± 9 IU/L, respectively (Fig. 5B) ($p < 0.01$).

The kidney toxicity was determined by estimating the BUN and creatinine in the untreated or EA-treated mice (Fig. 5C, 5D). The normal BUN level was 23.67 mg/dl that was elevated to 88.7 mg/dl in untreated infected mice. Therapy with free EA or EA-liposomes decreased the BUN level that was detected to be 44.3 mg/dl in the mice treated with EA-liposomes (100 mg/kg),

which was highly decreased as compared to 90.7 mg/dl in sham liposomes-treated mice (Fig. 5C). In addition to BUN, the creatinine was measured in untreated and treated mice too. The normal level of creatinine was found to be 0.32 mg/dl that was increased to 3.18 mg/dl in the untreated mice (Fig. 5D). Therapy with free EA-100 mg/kg significantly lowered creatinine level to 2.29 m/dl, whereas treatments with EA-liposomes (50 and 100 mg/kg) reduced creatinine levels to 1.91 and 1.45 mg/dl, respectively.

3.6. EA-liposomes reduced the pro-inflammatory markers in BALF

The graveness of infection induced inflammatory responses in mice, which was analyzed by determining IL-6, IL-1β and TNF-α in the BALF. The IL-6 level in PBS-treated mice were detected to be 1334 ± 282 pg/ml that was lowered to 770 ± 107 pg/ml in EA-50, and 591 ± 210 pg/ml in EA-100 treated mice (Fig. 6A). Conversely, EA-liposomes lowered the secretion of the these cytokines in BALF. EA-liposomes therapy at 50 mg/kg decreased IL-6 to 274 ± 68 pg/ml, whereas at 100 mg/kg lowered to 117 ± 28 pg/m l. The amount of IL-1β was also estimated in BALF of *A. baumannii* infected mice. It was elevated to 156 ± 25 pg/ml in infected mice, those were not treated with any formulation. The mice those were treated with free EA-50 and EA-100 had 88 ± 24 and 72 ± 19 pg/ml of IL-1β, respectively (Fig. 6B). Again, EA-liposomes exhibited greater therapeutic value as the IL-1β was 56 ± 9 pg/ml in the BALF of mice treated with EA-liposomes at a dose of 50 mg/kg, whereas 38 ± 6 pg/ml ml in mice treated with 100 mg/kg (Fig. 6B). Like to IL-6 and IL-1β, the level of TNF-α was remarkably increased in infected mice (Fig. 6C). TNF-α was estimated to be 544 ± 126 pg/ml that was reduced to 381 ± 66 in the BALF of EA-50 treated, and 317 ± 30 pg/ml in EA-100 treated mice (Fig. 6C). But, the therapy with EA-liposomes remarkably improved the status TNF-α. The BALF from the mice treated with EA-liposomes (50 mg/kg) contained 234 ± 67 pg/ml of TNF-α, which was further decreased to 114.7 ± 35 pg/ml after treatment with EA-liposomes (100 mg/kg) (Fig. 6C).

4. Discussion

The continued increased frequency of *A. baumannii* infections have worried the clinicians and healthcare workers because of increased mortality of infected persons, particularly those of immunocompromised individuals. It has posed serious challenges to the health care workers to eliminate these infections from the hospital or community-based infections (Lee et al., 2021). A high rate of drug-resistance considerably depleted the available antibacterial drugs. In the contemporary work, we examined the activity of EA against the drug-resistant *A. baumannii*. Since EA is poorly soluble in an aqueous environment that limits its therapeutic implications in the clinical setting. To enhance the therapeutic efficacy of EA, a liposome-based formulation of EA (EA-liposomes) was made and used to treat *A. baumannii* infected mice.

Ellagic acid is a natural phytochemical that possesses many beneficial properties (Sharifi-Rad et al., 2022). The complexes of EA with cyclodextrin or hydroxypropyl- β-cyclodextrin have shown their activity against C. albicans (Sampaio et al., 2021, Gontijo et al., 2019). Earlier, we demonstrated anti-cryptococcal effect of EA, which was increased upon its incorporation into liposomes (Khan et al., 2021). De et al reported that EA possesses therapeutic and preventive effects against *Helicobacter pylori* in mice (De et al., 2018). Moreover, EA was also found to be effective in the treatment of malaria (Oh et al., 2009). EA has been shown to be effective in solving the intracellular leishmaniasis through its immunomodulatory effects (Alves et al., 2020). Furthermore, EA has been shown to enhance amphotericin B activity against cutaneous leishmaniasis (Alves et al, 2020). EA-rich extract of pomegranate also augmented the efficacy of moxifloxacin against methicillin resistance *Staphylococcus aureus* (MRSA) (Dahash et al., 2021). Recently, EA-rich berry extract silver nanoparticles showed their antimicrobial activity against *Enterooccus faecalis* and *C. albicans* (Ekrikaya et al, 2021). Here, we determined the *in vitro* or *in vivo* antibacterial potential of free EA or EA-liposomes against *A. baumannii* that did not respond to common antibiotics. EA inhibited the growth of *A. baumannii* as supported by the findings of antimicrobial studies. The outcomes of the time-kill assay exhibited that EA at the concentration of 32 μg/ml inhibited 99% growth of *A. baumannii*, whereas free EA at 64 μg/ml inhibited 99.9% bacteria.

Acinetobacter baumannii effectively adopts a strategy to form the biofilm in order to thrive under unfavorable conditions. The biofilm helps bacteria to develop the multidrug resistance, because the matrix of biofilm limits the entry of drugs into bacterial cell (Harding et al., 2018, Upmanyu et al., 2022). On the contrary, AdeABC is a drug efflux pump that contributes to the biofilm formation by bacteria (Alav et al., 2018). Earlier we demonstrated that EA can inhibit the biofilm formation by *C. neoformans* (Khan et al., 2021). Here, the findings showed that EA effectively counters the development of the biofilm by *A. baumannii*. Earlier reports demonstrated the inhibitory role of EA against the biofilm formation by *C. albicans*, *C. auris*, *S. aureus* and *E. coli* (Sampaio et al., 2021, Possamai Rosatto et al., 2021, Quave et al., 2012, Hancock et al., 2010).

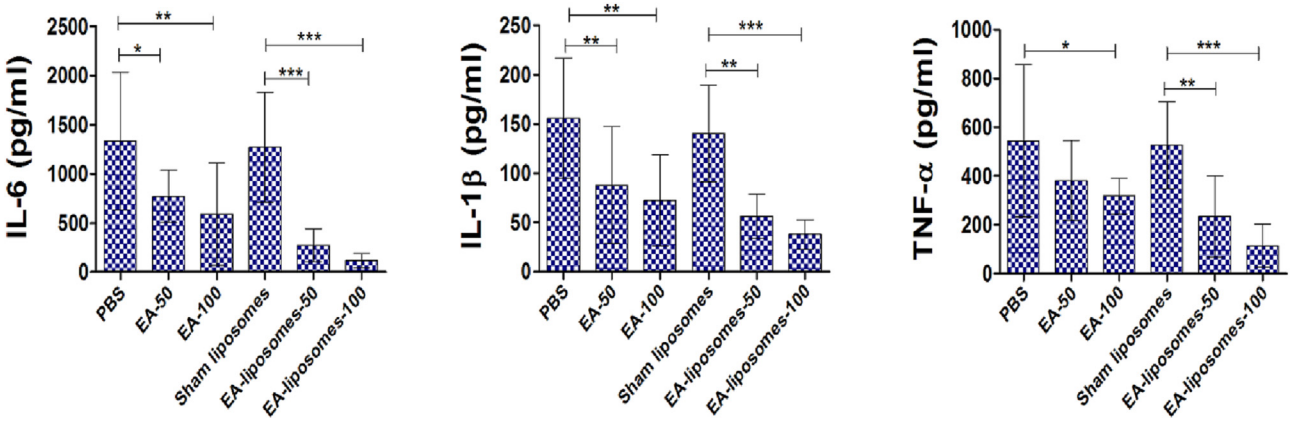

Fig. 6. Therapy with EA-liposomes significantly improved the status of inflammatory cytokines in the BALF of *A. baumannii* infected immunocompromised mice. (A) IL-6, (B) IL-1β and (C) TNF-α. *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$).

The limited bioavailability of EA restrict its implication in a medical setting. Nanoparticle-based formulations of EA have demonstrated greater therapeutic effectiveness in various disease models (Khan et al., 2021, Pirzadeh-Naeeni et al., 2020, Stojiljkovie et al, 2019, Harris et al., 2019). EA-loaded liposomes have effectively alleviated cyclophosphamide-induced toxicity in a murine model (Khan et al., 2021). *A.* baumannii infections have been mainly detected in immunocompromised individuals. A condition of temporarily immune-suppression was developed by injecting cyclophosphamide that significantly reduced the leukocyte numbers in the blood. It is essential to obtain a drug formulation that can be used to treat immunocompromised individuals. Here, we prepared EA-loaded liposomes and used them against the A. baumannii in immunodebilitant mice. EA-liposomes not only rejuvenated the leukocytes, but also effectively eliminated *A. baumannii* from the infected mice. The greater therapeutic efficacy of EA-liposomes is validated by the superior survival rate and the decreased bacterial burden. The reason for the higher effectiveness of the EA-liposomes can be accredited to the higher bioavailability of EA when administered as EA-liposomes. Moreover, liposome-mediated delivery of antibiotics also help in combating the phenomenon of drug-resistance by facilitating the interaction of antibiotics with bacterial membrane (Ferreira et. al., 2021). The incorporation of drugs in the liposomes protects the former against the enzymatic degradation that helps to reduce the chances antibiotic resistance. Interestingly, liposomes can deliver the entrapped drugs into intracellular compartments that targets the shelter sites of the pathogens (Gaspar et al., 2008). Interestingly, the administration of EA-liposomes was found to be safe in immunocompromised mice, because the EA-liposomes treated mice showed improved status of the liver and renal function parameters.

A. baumannii infection induces the production of IL-6, IL-1β and TNF- in the BALF that contribute to severe pathogenesis in the lungs (Harris et al., 2019). Earlier report showed the elevated IL-6 amount in infected mice (Zhang et al., 2021). *A. baumannii* is a lipopolysachhraide (LPS) containing gram-negative bacteria. LPS has the ability to induce the production of IL-6 that is shown to be inhibited by EA (Bensaad et al., 2017). Another pro-inflammatory cytokine IL-1β has been reported to aggravate *A. baumannii* induced pathogenesis as IL-1β deficient mice exhibited lower severity of infection (Kang et al., 2017). Interestingly, EA-liposomes decreased the secretion of IL-1β in BALF. The secretion of TNF-α also contributes bacteria-induced inflammation in the lungs (Qiu et al., 2009). Sun et. al showed that EA inhibited the production of TNF-α through NLRP3 signaling pathway (Sun et al., 2021). Moreover, EA can inhibit allergic airway inflammation by decreasing inflammatory cell numbers and Th2 cytokines in BALF (Zhou et al., 2014). The above findings support a promising therapeutic effect of EA-liposomes in the therapy of infectious or inflammatory diseases.

In conclusion, EA shows an antibacterial activity against *Acinetobacter baumannii*. Moreover, the incorporation of EA in liposomes increased the effectiveness of the drug. Besides, free EA or EA-liposomes also rejuvenates the immune cells in CYP-injected mice. Treatment with EA-liposomes improved the survival and decreased the bacterial burden in the treated mice. EA-liposomes alleviated the liver and kidney toxicity in *A. baumannii* infected immunocompromised mice. The infected mice possessed highly elevated levels of inflammatory cytokines, which were significantly decreased in EA-liposomes treated mice. These results endorse the possible therapeutic role of EA-liposomes against *A. baumannii* infection that is not responding to commonly used antibiotics.

Ethical approval number: 5604-cams1-2019–2-2-I.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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