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# Insights into therapeutic liquid mixtures and formulations towards tuberculosis therapy

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ABSTRACT

Therapeutic liquid mixtures, as deep eutectic systems, are considered a sustainable strategy that can be useful for the modification and enhancement of the pharmacokinetics and pharmacodynamics of different active ingredients. In this study, we assessed the stability and antibacterial activity of therapeutic liquid formulations prepared with anti-tuberculosis drugs. Tuberculosis therapy presents various pitfalls related, for example, to the administration of prolonged regimens of multiple drugs, different severe adverse effects, low compliance of the patient to treatment and the development of drug resistance. During this study, it was possible to assess the physicochemical stability of the formulations for 6 months, by polarized optical microscopy, $^{1}$H NMR and FTIR-ATR. Furthermore, the mixtures present an antibacterial effect against a drug-susceptible *Mycobacterium tuberculosis* strain (H37Rv). This was particularly evident for the mixtures with ethambutol incorporated, making them interesting to pursue with further studies and evaluation of clinical applicability. Upon infection, it was also observed that a single and higher dose appears to be more effective than lower separate doses, which could allow the production of patient-friendly formulations.

## 1. Introduction

Tuberculosis (TB) is an old disease that is thought to have accompanied modern humans up until the present day. It is a bacterial infection caused by the phylogenetically closely related bacteria of the *Mycobacterium tuberculosis* species complex borrowing its name from the most representative agent of human causative agent of *Mycobacterium tuberculosis* (*Mtb*) (Mehta and Dutt, 2016; Smith, 2003; Salvatore and Zhang, 2017). Although it is a preventable and treatable disease it still accounts for one of the most lethal infections worldwide. The incidence of TB has suffered a slow decline over the years, yet the rising proportion of multidrug-resistant and extensively drug-resistant cases of TB has drawn renewed attention to this disease (Geneva: World Health Organization, 2021).

*Mtb* is spread from patient to patient by inhalation of infected aerosols exhaled by infected individuals (Salvatore and Zhang, 2017; Marimani et al., 2018). Briefly, the small droplets containing 1–3 bacilli facilitate the spread from the upper respiratory tract to the deep lung (alveolae), where *Mtb* is phagocyted by alveolar macrophages.

The line of treatment applied for drug-susceptible *Mtb* is the same one established more than 40 years ago and comprises a prolonged treatment with a regimen of four anti-TB drugs (isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB)) for at least 6 months to treat active disease (Jain et al., 2008; Machado et al., 2016; Sarathy et al., 2012; Bahuguna and Rawat, 2020). The treatment for drug-resistant infections is more complex, with an increased time of treatment and administration of more toxic and less effective second-line drugs (Bahuguna and Rawat, 2020; Hussain et al., 2019). The

*Abbreviations:* API, active pharmaceutical ingredient; CA, citric acid; CFU, colony forming units; DES, deep eutectic solvents; DMSO, dimethyl sulfoxide; EMB, ethambutol; FBS, fetal bovine serum; FTIR-ATR, fourier transform infrared spectroscopy with attenuated total reflection; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; INH, isoniazid; $_{L}$-Arg, $_{L}$-arginine; LTTM, low transition temperature mixtures; MBC, minimal bactericidal concentration; MEM, Eagle's minimum essential medium; MIC, minimal inhibitory concentration; MOI, multiplicity of infection; *Mtb*, *mycobacterium tuberculosis*; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; POM, polarized optical microscopy; PZA, pyrazinamide; RIF, rifampicin; RPMI, Roswell Park Memorial Institute 1640 media; TB, tuberculosis; TEER, transepithelial electrical resistance.

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limited ability of the drugs to reach the target, low permeability of the bacteria cell wall (Wallis and Hafner, 2015; Costa et al., 2016), different growth stages and plastic metabolism of mycobacteria contribute to the described longstanding therapy (Jain et al., 2008; Machado et al., 2016; Sarathy et al., 2012), and, consequently, low compliance of the patients to therapy which also favors the development of drug resistance (Marimani et al., 2018; Nathan and Barry, 2015).

The persistence of TB worldwide and the drawbacks previously described foment the need to find new drugs that present improved properties, such as being able to target the infection-site and also different drug regimens that can be patient-friendly. Farah and coworkers enumerate some attractive features, which the design of new anti-TB drugs should consider, such as: (1) be safe and potent with a bactericidal activity in all stages of TB infection; (2) provide shorter treatments with different combinations that can decrease the amount of drugs taken by patients; (3) find drugs with broad mechanisms of action and low toxicity to treat drug-resistant strains; (4) none or reduced drug-drug interactions; (5) find patient-friendly formulations that could be cheaper and available; and also (6) controlled delivery formulations with higher bioavailability and continuous efficacy for extended periods of time, allowing to reduce dose frequency (Farah et al., 2016).

Different strategies have been explored to improve properties of therapeutic compounds, and green and sustainable approaches are recent tools that demonstrate the potential to enhance pharmacokinetic and pharmacodynamic features of active compounds and for the formulation of diverse drug delivery systems. Low transition temperature mixtures (LTTMs) are considered green and sustainable strategies that can be named as alternative solvents, since they describe mixtures that present a large liquid range, great thermal stability, negligible vapor pressure and, generally, environmentally benign and with an acceptable toxicity (Yiin et al., 2016; Perna et al., 2020; Francisco et al., 2013). LTTMs comprise mixtures with low temperature transitions and can include deep eutectic systems (DES) (Perna et al., 2020; Francisco et al., 2013; Durand et al., 2016), that are described as mixtures of two or more compounds that combined in determined molar ratios present a melting point lower than the starting compounds (Francisco et al., 2013; Abbott et al., 2017; Aroso et al., 2016). According to the literature, these mixtures are created mainly by the presence of hydrogen bonds, but also van der Waals and/or electrostatic interactions can be established between the compounds (Francisco et al., 2013; Santana et al., 2019). Thousands of different combinations are possible to formulate (Barros et al., 2017), which turns them very attractive for different applications including pharmaceutical research and drug development. When these eutectic systems include at least one active pharmaceutical ingredient (API) in their composition or are used to dissolve at least one API improving its properties they are called therapeutic deep eutectic systems (Álvarez and Zhang, 2019; Aroso et al., 2015; Roda et al., 2021).

Recently, these types of therapeutic liquid mixtures have been explored in biomedicine, approaching different targets and diseases including TB infection. Zakrewsky and coworkers tested antimicrobial activity against *Mtb* drug-resistant strains of a liquid mixture with choline chloride:geranic acid (1:2) (Zakrewsky et al., 2016; Zainal-Abidin et al., 2019). Also, from previous studies made in the group, it was possible to formulate therapeutic liquid mixtures by combination of EMB and L-arginine, in different molar ratios, with citric acid and water showing a rise on solubility of the active compounds (Santos et al., 2019). In the present study, it was assessed the antimicrobial activity and ability to solubilize different anti-TB drugs in therapeutic liquid mixtures based on EMB, L-arginine, citric acid and water. Additionally, therapeutic liquid formulations with different anti-TB drugs were prepared and their stability, biological and antimicrobial activity against *Mtb* susceptible strain was observed.

**Table 1**
Therapeutic liquid mixtures prepared.

| Therapeutic Liquid Mixtures | Molar Ratio | Abbreviation |
|---|---|---|
| Citric Acid:L-Arginine:$H_2O$ | 1:1:4 (Roda et al., 2020) \| 1:1:5 (Roda et al., 2020) \| 1:1:6 (Santos et al., 2019; Roda et al., 2020) \| 1:1:7 (Santos et al., 2019; Roda et al., 2020) \| 2:1:7 (Santos et al., 2019) \| 2:1:8 (Santos et al., 2019) \| 2:1:9 (Santos et al., 2019) | CA:L-Arg:$H_2O$ |
| Citric Acid: Ethambutol:L-Arginine:$H_2O$ | 2:1:1:7 (Monteiro et al., 2021) | CA:EMB:L-Arg:$H_2O$ |
| Citric Acid: Ethambutol:$H_2O$ | 1:1:5 (Monteiro et al., 2021) \| 2:1:10 (Santos et al., 2019; Monteiro et al., 2021) | CA:EMB:$H_2O$ |

**Table 2**
Different formulations prepared by solubilizing different amounts of anti-TB drugs in the mixture CA:EMB:$H_2O$ (1:1:5).

| | Anti-tuberculosis drugs added | Mass Ratio | Abbreviation |
|---|---|---|---|
| CA:EMB:$H_2O$ (1:1:5) | RIF + PZA + INH | 1:1:1 | CA:EMB:$H_2O$ + RPI (1:1:1) |
| | RIF + PZA + INH | 2:3:1 | CA:EMB:$H_2O$ + RPI (2:3:1) |
| | RIF + PZA + INH | 2:5:1 | CA:EMB:$H_2O$ + RPI (2:5:1) |
| | PZA | | CA:EMB:$H_2O$ + PZA |

## 2. Experimental section

### 2.1. Materials

All reagents were used without further purification. Citric acid monohydrated (CA; CAS: 5949-29-1, ≥99.5% purity), L-Arginine (L-Arg; CAS: 74-79-3, ≥ 98% purity), phorbol-12-myristate-13-acetate (PMA; CAS: 16561-29-8) and IGEPAL CA-630 (CAS: 9002-93-1) were purchased from Sigma Aldrich, MO, USA. Ethambutol (EMB; CAS: 74-55-5, ≥ 98% purity) was obtained from Santa Cruz Biotechnology, Dallas, USA. Isonicotinic acid hydrazide (INH; CAS: 54-85-3, 98% purity), pyrazinamide (PZA, CAS: 98-96-4, 98% purity) and rifampicin (RIF; CAS: 13292-46-1, ≥ 95% purity) obtained from Alfa Aesar, Kandel, Germany.

For cell culture were used Eagle's minimum essential medium (MEM), Roswell Park Memorial Institute (RPMI) 1640 media, fetal bovine serum (FBS), antibiotic-antimycotic solution (penicillin and streptomycin), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and sodium pyruvate from Corning, USA and Gibco, UK. Dimethyl sulfoxide (DMSO; CAS: 67-68-5) was purchased from Fisher BioReagents and Cell Titer96® AQueous One Solution Cell Proliferation Assay from Promega, USA. For *Mtb* culture and assays were used Middlebrook 7H10 agar, Middlebrook 7H9 media and OADC from Becton, Dickinson and Company, USA.

### 2.2. Preparation of therapeutic liquid mixtures and formulations based on the mixture CA:EMB:$H_2O$ (1:1:5)

In previous studies, different therapeutic liquid mixtures were prepared envisaging tuberculosis therapy (Santos et al., 2019; Roda et al., 2020). These mixtures include citric acid, L-Arginine, ethambutol and water combined in several molar ratios (Table 1). The components were mixed in the corresponding molar ratios and kept under stirring at 50–60 °C, until a clear liquid mixture was formed.

To prepare the therapeutic liquid formulations, different amounts of anti-tuberculosis drugs (INH, PZA, RIF) were added to the mixture CA:

EMB:$H_2O$ (1:1:5), according to ratios mentioned in Table 2.

### 2.3. Anti-TB drugs solubilization in PBS using therapeutic liquids mixtures as a carrier

The solubilization of the anti-tuberculosis drugs in the therapeutic liquid mixtures was carried out by dissolving an excess of drug (INH, RIF or PZA) in, approximately, 1 g of different therapeutic liquid mixtures and then 1 mL of PBS solution was added. The mixture was stirred (60 rpm) for 24 h, before analysis. The determination of the solubility of anti-tuberculosis drugs using therapeutic liquid mixtures as carrier was evaluated by UV spectroscopy in a microplate reader (VICTOR Nivo$^{TM}$, PerkinElmer, Waltham, MA, USA). The absorbance of solutions was measured at 263 nm for INH, 270 nm for PZA and 335 nm for RIF. The calibration curve was prepared using the respective drugs as standard in PBS ($R^2$ = 0.9957 (INH), $R^2$ = 0.9919 (PZA), $R^2$ = 0.9977 (RIF). All samples were measured in triplicates.

### 2.4. Polarized optical microscopy (POM)

Optical characterization of the formulations with CA:EMB:$H_2O$ (1:1:5) and anti-tuberculosis drugs were carried out at room temperature using a transmission mode of an BX-53 polarized optical microscope (Olympus, Tokyo, Japan). A droplet of the formulations was placed on a microscope glass slide and then observed, the images were obtained with a digital camera (Olympus SC50) and analyzed with the software Olympus Stream Start 2.4.2 (Olympus, Tokyo, Japan).

### 2.5. Nuclear magnetic resonance (NMR)

NMR spectroscopy was performed to obtain $^1$H NMR and $^1$H–$^1$H-NOESY NMR spectra on a Bruker Avance III 400 spectrometer (Bruker, Billerica, MA, USA) at an operating frequency of 400.13 MHz. The samples were prepared in a 5 mm NMR tube with, approximately, 350 μL of each mixture and 200 μL of DMSO-$_{d6}$ (Euriso-Top, St. Aubin Cedex, France). Chemical shifts were referenced to $Me_4Si$ (δ in ppm) and the data analysis was performed with MestReNova software (11.0.4–18998).

### 2.6. Fourier transform infrared spectroscopy (FTIR-ATR)

Infrared spectra were obtained on a PerkinElmer Spectrum Two (Waltham, MA, USA) with attenuated total reflection (ATR) in the transmittance mode and with a wavenumber between 400 and 4000 $cm^{-1}$. Spectra were acquired from 16 scans with 4 $cm^{-1}$ resolution, at room temperature.

### 2.7. Cell culture

A549 cells are human alveolar epithelial cells from human lung carcinoma (DSMZ, ACC 107) and THP-1 cells are human acute monocytic leukemia cell line (ATCC TIB202). The A549 cells were cultured in MEM media supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. The THP-1 cells were grown in RPMI 1640 media, supplemented with 10% fetal bovine serum, 10 mM HEPES and 1 mM sodium pyruvate. Cells were sub-cultured in humidified atmosphere of 37 °C with 5% $CO_2$ and differentiation of THP-1 monocytes into macrophages was induced using 20 nM phorbol-12-myristate-13-acetate (PMA), overnight, followed by a resting period of 24 h without PMA.

### 2.8. Cell viability assays

Cell viability was evaluated with A549 confluent and non-differentiated cells and confluent macrophages seeded on a 96-well plate (2x$10^4$ cells/well A549; 5x$10^4$ macrophages/well) and after 24 h of seeding the medium was removed and the cells were incubated with different concentrations of pure components (rifampicin diluted in culture media with 5% DMSO), therapeutic liquid mixtures and formulations diluted in culture media, during 20–24 h. After the incubation time, the cells were washed with PBS and cell viability was evaluated using CellTiter96® AQueous One Solution Cell Proliferation Assay based on MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)). The amount of formazan produced was measured at 490 nm in a microplate reader (VICTOR Nivo$^{TM}$, PerkinElmer, USA) and cell viability was expressed in percentage of living cells comparing to the control (untreated cells). The assay was performed with at least three independent experiments, in triplicate, and the data were analyzed by GraphPad Prism 8.0.1.

### 2.9. Permeation studies with transwell permeable supports

The permeability of different combinations of therapeutic liquid mixtures and APIs was evaluated using A549 cell line. The cells were seeded on a 12-well transwell inserts (Falcon cell culture PET inserts, pore size 0.4 μm, area 0.9 $cm^2$) at a cell density of 1.26x$10^4$ cells/well (Vieira et al., 2018). The cells were grown in supplemented and culture media that was added to both apical and basolateral sides. The media was changed every 2 days, for 8 days, until a homogenous monolayer was achieved. The transepithelial electrical resistance (TEER) was measured in all wells and in a well of medium without cells to be considered as blank. The TEER was checked before the assay using a EVOM 3 (epithelial voltohmmeter, World Precision Instruments, USA), equipped with a pair of electrodes placed in apical and basolateral side. The TEER value obtained in this study was 294 ± 14 O/$cm^2$ (mean ± S.D.). The samples were incubated at 37 °C with 5% $CO_2$ for 6 h, and it was collected 0.5 mL of sample from the basolateral side at different time points (0.5, 1, 2, 3, 4, 5 and 6 h), then replaced with same quantity of fresh media. The samples were analyzed and quantified by HPLC (Dionex ICS3000; MERCK Purosphere STAR RP18 250x4mm column; UV detection at 210, 238, 280, 320 and 510 nm; eluents: 20 mM $NAH_2PO_4$ + 0.2% triethylamine at pH 7.0 and acetonitrile). The analyses were performed at 40 °C. The apparent permeability ($P_{app}$) for pure compounds, therapeutic liquid mixtures and formulations was calculated following the equation (Eq. (1)):

$$P_{app(cm/s)} = \frac{\Sigma m_a}{A \times m_d \times t} \quad (1)$$

where $m_a$ is mass of compound permeated each time point (g), $A$ is the effective area of permeation where the monolayer is seeded ($cm^2$), $m_d$ is the initial mass of compound added to the apical side (g) and $t$ is the time of permeation (s). The cellular uptake by A549 cells was estimated by the following equation (Eq. (2)):

$$Cellular\ \ uptake = C_0 - C_{Bf} - C_{Af} \quad (2)$$

where $C_0$ is the initial concentration added on the apical side, $C_{Bf}$ is the final concentration on the basolateral side and $C_{Af}$ is the final concentration on the apical side.

### 2.10. Antimicrobial activity in Mtb (H37Rv)

Microbiologic assays were performed in a Biosafety Level 3 laboratory at the Faculty of Pharmacy of the University of Lisbon, respecting the national and European academic containment level 3, laboratory management and biosecurity standards, based on applicable EU Directives. *Mycobacterium tuberculosis* H37Rv (ATCC 27294), a drug-susceptible strain, was used for determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the therapeutic liquid mixtures, by the broth dilution method with concentrations varying between 0.125 and 16 μg/mL. Briefly, *Mtb* cultures on exponential growth phase were centrifuged and washed with

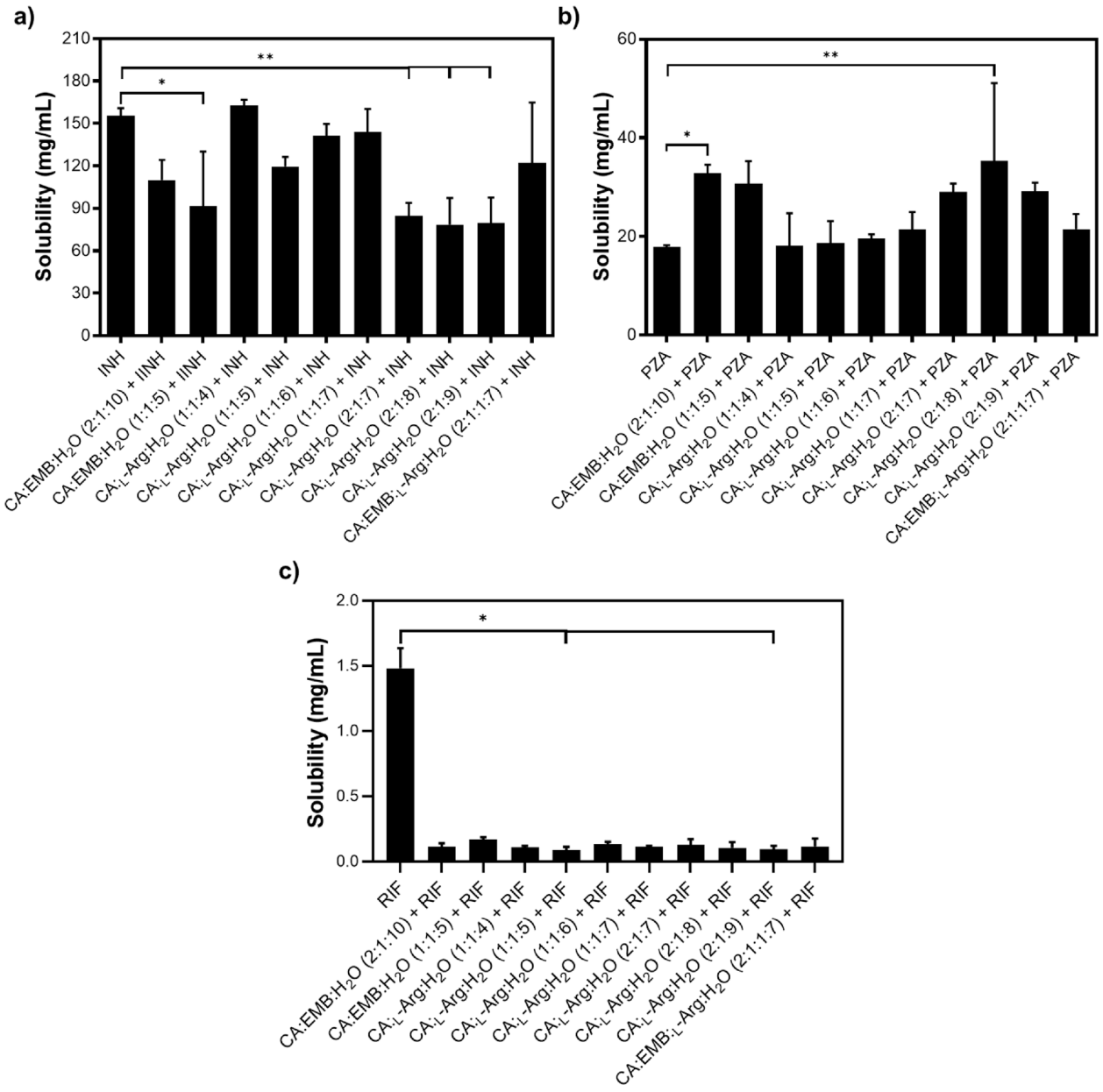


**Fig. 1.** Solubility of different anti-TB drugs (**a)** INH; **b)** PZA and **c)** RIF) in therapeutic liquid mixtures and PBS. Data are presented as mean ± SD (n = 3), *p ≤ 0.05, **p ≤ 0.005.

PBS, then re-suspended in fresh culture medium. Bacterial clusters were dismantled and removed by ultrasonic treatment for 5 min followed by low-speed centrifugation (500 × g) for 2 min. Single cell suspension was verified by fluorescence microscopy. The selected concentrations of therapeutic liquid mixtures were incubated with the bacterial suspensions containing approximately $10^5$ colony-forming units per mL. The MIC was determined at the 10th day of incubation by visual examination of the plates. For MBC determination, the bacterial samples were recovered from MIC test plates, diluted 1:10 in water and platted in 7H10 + 10% OADC solid medium. The MBC corresponded to the concentration that produced no visible colonies on the solid medium.

### 2.11. Macrophages infection with Mtb (H37Rv)

*Mtb* cultures were prepared as described above and resuspended in macrophage culture medium. Macrophages in 96-well plates as described above, were infected with *Mtb* at a MOI of 0.1 for 3 h, at 37 °C with 5% $CO_2$. Following internalization, cells were washed three times with PBS and re-suspended in appropriate culture medium with the respective treatments. At day 3 post infection, half the culture medium was replenished with or without treatment reinforcement.

### 2.12. Intracellular survival of bacteria

When required, infected cells were lysed with a 10 min treatment with 0.05% IGEPAL solution in water, a nonionic, non-denaturing detergent that disrupts eukaryotic cells but does not affect mycobacteria viability, with the goal being to assess the CFU of viable intracellular bacteria. Serial dilutions of the resulting bacterial suspension were plated in Middlebrook 7H10 agar with 10% OADC and incubated for 2–3 weeks at 37 °C before colonies were observable and countable.

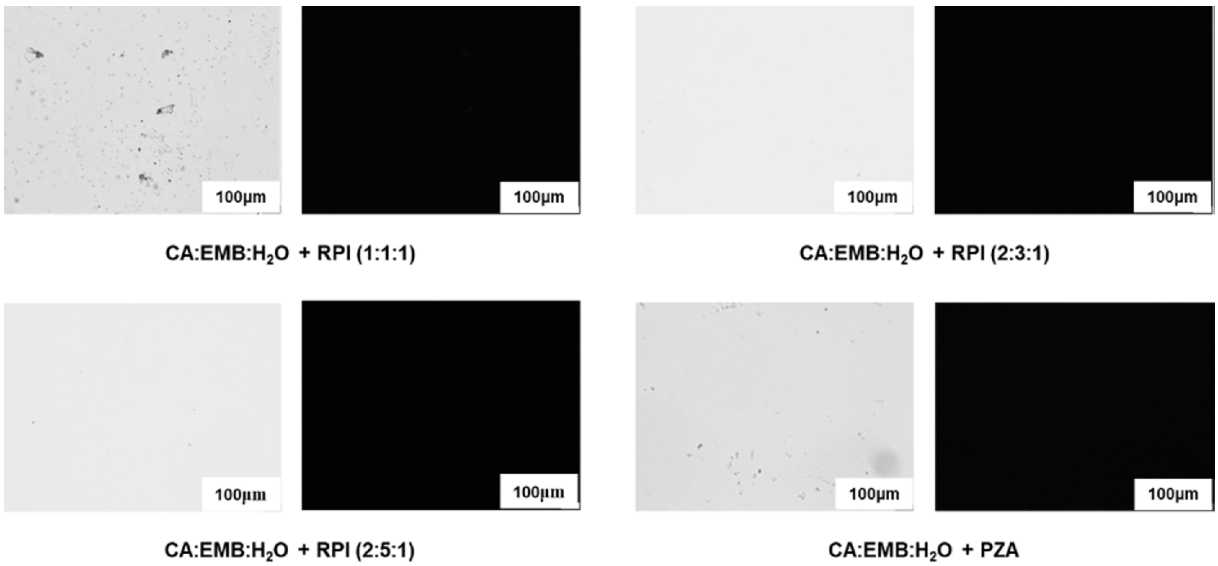


**Fig. 2.** Images of therapeutic liquid formulations based on CA:EMB:$H_2O$ (1:1:5) and anti-TB drugs obtained by POM at room temperature, after 6 months of preparation.

### *2.13. Statistical analysis*

The statistical analysis was performed using GraphPad Prism 8.0.1 software. All measurements were made in triplicate and the data presented as mean ± SD. Normality was evaluated by Shapiro-Wilk test. Data that obey to a normal distribution were tested with one-way and two-way ANOVA analysis with Bonferroni *post-hoc* test for multiple comparisons. Data that do not follow a normal distribution were tested with a non-parametric test Kruskal-Wallis test and Dunn's test was used for multiple comparisons. The differences were considered significant when the $p$ value was $\leq 0.05$.

## 3. Results and discussion

The use of therapeutic liquid mixtures has been explored as a more sustainable approach to modify and improve the characteristics of existing drugs that can, ultimately, enhance their performance and efficacy in the treatment of different diseases. This is achieved, mainly, through the hydrogen bonding interactions that take place between the different counterparts of the mixtures. Some examples of the use of this type of mixtures for therapeutic approaches, have been reported in literature such as the use of mixtures based on choline chloride and ascorbic acid to improve the solubility of dexamethasone; (Silva et al., 2018) use an API to formulate the liquid mixture as ibuprofen (Aroso et al., 2015; Silva et al., 2020; Pereira et al., 2019; Stott et al., 1998; Silva et al., 2021) or acetylsalicylic acid (Aroso et al., 2016) combined with different terpenes which can potentiate the activity and characteristics of the API; combine citric acid and ethambutol or arginine that increase their solubility and permeability; (Santos et al., 2019) or even combine different APIs, like lidocaine and ibuprofen (Mann et al., 2020).

Different ratios in the components of the mixtures can influence their physicochemical characteristics, for example, different water ratios can lead to modification of the viscosity of the media, as well as the polarity and the strength of interactions created between the components of the mixture (Ma et al., 2018; Vilková et al., 2020). Several studies corroborate the hypothesis that the addition of small amounts of water contributes to reinforce the interactions (e.g., hydrogen bonding, van der Walls, electrostatic forces) between the components of the mixtures, maintaining their complex intermolecular structure. However, the addition of high amounts of water (up to 50%) will lead to the dissociation of the molecules of each component, disrupting the network of the mixture and gradually hydrating the molecules and therefore solubilizing the components, forming an aqueous solution (Monteiro et al., 2021; Ma et al., 2018; Vilková et al., 2020; Roda et al., 2021; El Achkar et al., 2019; Dai et al., 2015; Zhekenov et al., 2017; Hammond et al., 2017). Recently, Roda and coworkers focused their attention on understanding the function role of water as a component of liquid mixtures with CA, L-Arg and water, evaluating through different techniques of physicochemical characterization and thermodynamic modelling the role of water in the mixtures. They observed that water will lead to the formation of charged species and, also, act as an hydrogen bond mediator for L-Arg and CA (Roda et al., 2021). In another study, Monteiro and coworkers observed, by molecular dynamics, the existence of interactions between CA and EMB and assumed that water molecules are necessary to form a hydrogen bond network that provides stability to the mixture. They also noticed that by altering the ratios of the components of the liquid mixture, in the case of CA:EMB:$H_2O$ (1:1:5) promotes additional interactions through hydrogen bonds. By molecular docking the authors further explored the internalization of EMB through a mycobacterial ABC transporter (Rv1819c), suggesting a favorable internalization of EMB when in the form of liquid mixture with CA and/or L-Arg (Monteiro et al., 2021).

Considering the studies reported with these mixtures that demonstrated the role of different components of the mixture in the reinforcement of their interactions, particularly for CA:EMB:$H_2O$ (1:1:5). In this work, we formulate two hypothesis, one was the possibility of prepare stable therapeutic liquid mixtures combining anti-TB drugs used as first-line treatment, and the other was to prove the feasibility of these formulations through *in vitro* bioactivity studies, namely if these systems present antibacterial activity against *Mtb*.

### *3.1. Solubility of first-line anti-TB drugs using different therapeutic liquid mixtures as carriers*

Therapeutic liquid mixtures have been described in the literature as mixtures that potentially increase the solubility of APIs, and their overall bioavailability and, consequently, their therapeutic efficacy. Different types of studies can be carried out to evaluate the effect of these mixtures on the drug solubility, depending if the API is incorporated in the liquid mixture (Aroso et al., 2016; Santos et al., 2019; Stott et al., 1998; Duarte et al., 2017) or if it is dissolved in liquid mixture, using that as a carrier (Roda et al., 2021; Silva et al., 2018; Morrison et al., 2009; Tajmir and Roosta, 2020; Li and Lee, 2016). Herein, we used this last approach where we saturate a mixture of therapeutic liquid mixture and PBS

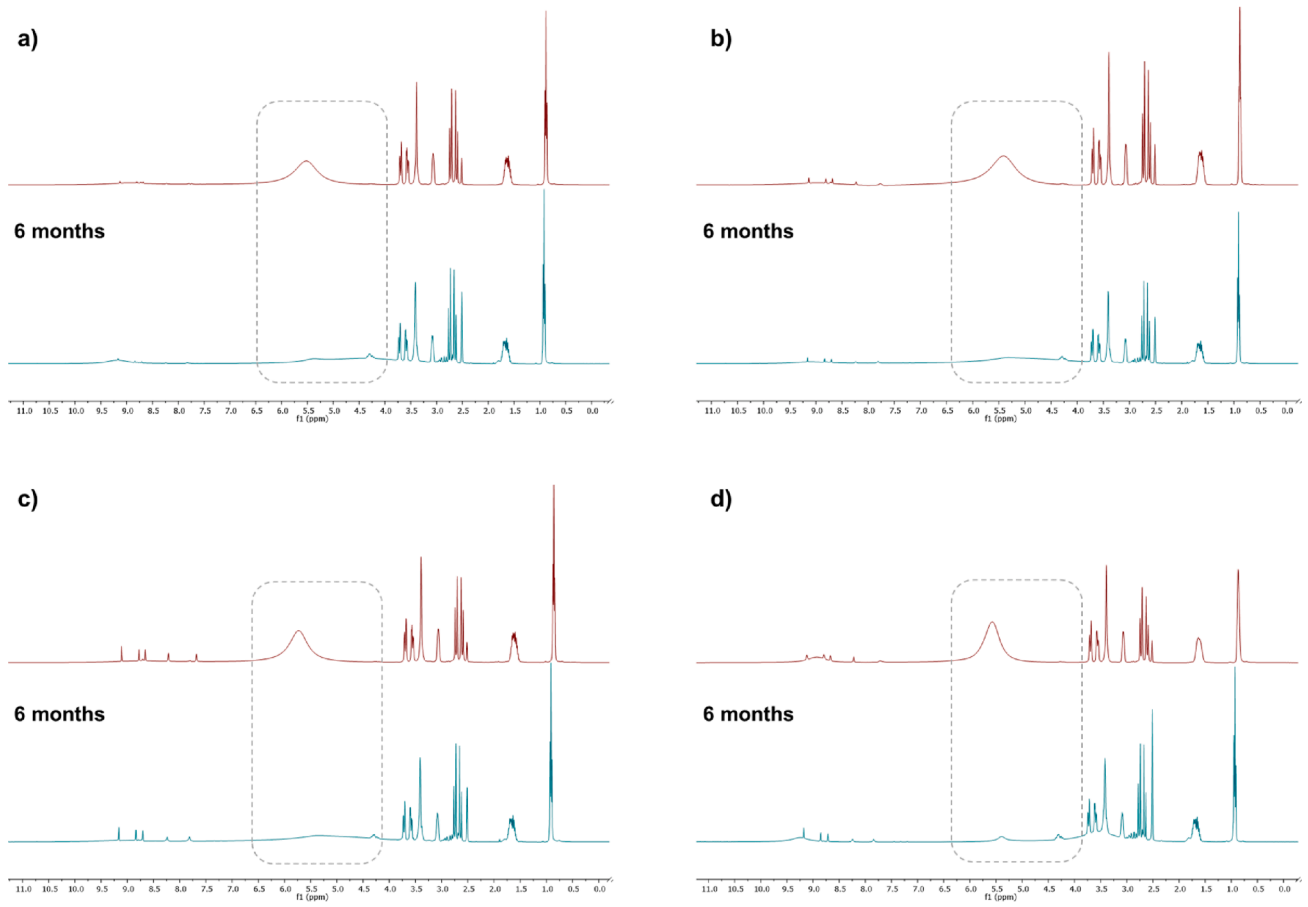


**Fig. 3.** $^{1}$H NMR spectra of the therapeutic liquid formulations with superimpose of spectra from 0 and 6 months of formulation. **a)** CA:EMB:$H_2O$ (1:1:5) + RPI (1:1:1), **b)** CA:EMB:$H_2O$ (1:1:5) + RPI (2:3:1), **c)** CA:EMB:$H_2O$ (1:1:5) + RPI (2:5:1), **d)** CA:EMB:$H_2O$ (1:1:5) + PZA.

(50:50 w/w) with the compounds INH, PZA and RIF (Fig. 1).

Regarding the solubility assays with anti-TB drugs, we can observe that mixtures with higher molar ratios of CA and water present a lower solubilization of INH. Probably the higher decrease in the pH of the systems affects the solubilization of INH. The ones that present better dissolution of INH were CA:$_L$-Arg:$H_2O$ (1:1:4 and 1:1:7), being the formulation 1:1:4 the one that presents a slight increase on solubility comparing to INH itself. Contrarily to INH, PZA presents a better solubilization in mixtures with higher ratios of CA and water, better than PZA itself. Therapeutic liquid mixtures with EMB incorporated are the ones that present increased solubility of PZA, which can be important for a possible readjustment of the dose of PZA used in TB therapy, as this is one of the drugs that requires a higher dose to be effective against *Mtb* (Nahid et al., 2016; Claire du Toit et al., 2006). In the case of RIF, it is not observed an improvement on its solubility, probably due to intrinsic characteristics of RIF as it is a large size molecule with a complex structure and an hydrophobic character (Motiei et al., 2021; Ermondi et al., 2021). However, despite the low solubility of RIF, the systems prepared could solubilize all the first-line anti-TB drugs, which indicate the possibility to formulate therapeutic liquid mixtures with the four anti-TB drugs, as hypothesized in this study. The formulations combining therapeutic liquid mixtures and anti-TB drugs were based on the system CA:EMB:$H_2O$ (1:1:5), owing to previous studies (Santos et al., 2019) and, the molecular dynamics studies that enabled to observe the strengthening of molecular interactions between the components of this system (Monteiro et al., 2021).

### *3.2. Physicochemical stability assays of formulations based on CA:EMB: $H_2O$ (1:1:5) and anti-TB drugs*

To better understand how formulations based on CA:EMB:$H_2O$ (1:1:5) and anti-TB drugs maintain their structure over time, we used different techniques (POM, NMR and FTIR-ATR) to evaluate their physicochemical stability immediately after preparation and 6 months later. The mixtures were maintained at room temperature (20–25 °C), during this period.

Polarized optical microscopy (POM) was used to observe the different mixtures with a cross polarizer to verify the existence of crystals in the mixtures, detecting if the mixtures are homogeneous or present different phases (Aroso et al., 2017). As we can observe in Fig. 2, the images of the liquid formulations under polarized microscopy are uniformly black, which means that they are homogenous liquids and do not present crystals or precipitates in the formulations.

NMR studies were conducted to observe if significant changes in the structure of therapeutic liquid formulations 6 months after preparation occurred. As represented in Fig. 3, the $^{1}$H NMR shows some differences related to the –OH groups, that immediately after preparation present a sharper peak while after 6 months the peak appeared to be broader. In $^{1}$H NMR, the –OH protons, usually, are quite exchangeable and their appearance and intensity in the spectra depend on different factors like temperature of analysis, concentration of the sample, solvent used to prepare the samples, among others (Charisiadis et al., 2014). The broadness of –OH groups can be explained by some exchange of protons of –OH groups with protons from solvent. Also, slight variations in the concentrations of mixtures when the NMR was performed could have contributed to the broadening of peaks from –OH groups that appeared near the baseline in all spectra from 6 months. Despite the differences on

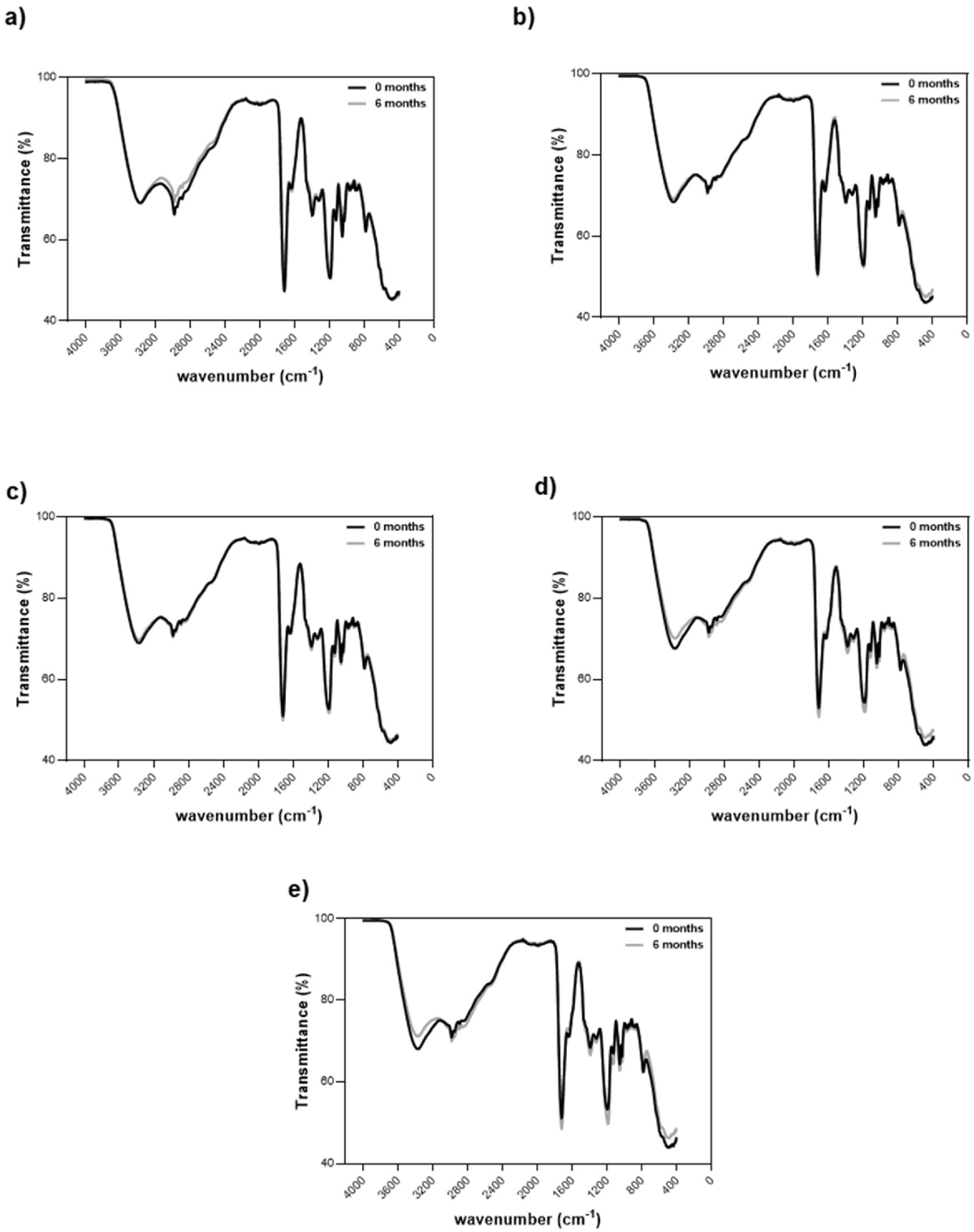


**Fig. 4.** FTIR-ATR spectra of liquid formulations based on CA:EMB:H2O (1:1:5) and anti-TB drugs (**a)** CA:EMB:$H_2O$ (1:1:5), **b)** CA:EMB:$H_2O$ (1:1:5) + RPI (1:1:1), **c)** CA:EMB:$H_2O$ (1:1:5) + RPI (2:3:1), **d)** CA:EMB:$H_2O$ (1:1:5) + RPI (2:5:1), **e)** CA:EMB:$H_2O$ (1:1:5) + PZA.

**Table 3**
$IC_{50}$ values for pure components, therapeutic liquid mixtures and formulations in A549 alveolar epithelial cells. Results are expressed as mean ± SD of at least three independent experiments that were performed in triplicate.

| Compounds | | $IC_{50}$ (mg/mL) in A549 cells |
|---|---|---|
| **Pure Components** | Citric Acid | 1.43 ± 0.13 |
| | $_{L}$-Arginine | 12.30 ± 0.56 |
| | Ethambutol | 21.50 ± 2.94 |
| | Isoniazid | 33.53 ± 1.73 |
| | Pyrazinamide | 31.76 ± 5.59 |
| | Rifampicin | 0.69 ± 0.34 |
| **Therapeutic Liquid Mixtures** | CA:EMB:$H_2O$ (2:1:10) | 2.86 ± 0.60 |
| | CA:EMB:$H_2O$ (1:1:5) | 3.43 ± 0.55 |
| | CA:EMB:$_{L}$-Arg:$H_2O$ (2:1:1:7) | 4.10 ± 0.70 |
| | CA:$_{L}$-Arg:$H_2O$ (1:1:4) | 5.80 ± 1.45 |
| | CA:$_{L}$-Arg:$H_2O$ (1:1:5) | 5.36 ± 1.33 |
| | CA:$_{L}$-Arg:$H_2O$ (1:1:6) | 6.62 ± 1.04 |
| | CA:$_{L}$-Arg:$H_2O$ (1:1:7) | 6.12 ± 0.86 |
| | CA:$_{L}$-Arg:$H_2O$ (2:1:7) | 3.08 ± 0.99 |
| | CA:$_{L}$-Arg:$H_2O$ (2:1:8) | 3.24 ± 0.21 |
| | CA:$_{L}$-Arg:$H_2O$ (2:1:9) | 3.37 ± 0.23 |
| **Therapeutic Liquid Mixtures Formulations** | CA:EMB:$H_2O$ + RPI (1:1:1) | 2.59 ± 1.28 |
| | CA:EMB:$H_2O$ + RPI (2:3:1) | 2.51 ± 1.69 |
| | CA:EMB:$H_2O$ + RPI (2:5:1) | 3.10 ± 1.99 |
| | CA:EMB:$H_2O$ + PZA | 3.45 ± 0.21 |

the broadness of –OH groups, the structure of the liquid formulations appear to be maintained over time.

To further examine the chemical changes that can happen in the structure of liquid formulations and clarify if these formulations are stable over time, infrared spectroscopy was also performed. This method allows to detect the presence of different functional groups and bonds and with attenuated total reflection it is possible to enhance the surface sensitivity and employ liquid samples, like therapeutic liquid formulations. The detection of changes in the frequency of peaks and their intensity can indicate changes in the chemical structure of the mixtures. In Fig. 4, we can observe an overlap of the spectra of the different periods of time (0 and 6 months). The first spectra present the therapeutic liquid mixture without adding any anti-TB drugs (INH, RIF, PZA) and comparing this spectrum with the pure components (Figure S1) it is visible that the therapeutic liquid mixture presents spectra with broad peaks due to presence of water in the mixtures, mainly in the region between 3600 and 2800 $cm^{-1}$. However, it is also possible to identify the –OH stretching of EMB, CA (-C-OH) and water in the same region around 3550–3200 $cm^{-1}$. This confirms the intermolecular interactions between the different components of the liquid mixtures in these groups, and that were previously reported by Santos and coworkers through $^{1}H$–$^{1}H$-NOESY NMR (Santos et al., 2019). The presence of –OH groups of CA (–COOH) was detected, approximately, at 3000 $cm^{-1}$ and -C=O stretch of CA (–COOH) was detected between 1800 and 1700 $cm^{-1}$, the peaks between 1500 and 1400 $cm^{-1}$ appear to correspond to stretching vibrations of heteroatoms groups. Regarding the solubilization of small amounts of anti-TB drugs in the liquid mixture CA:EMB:$H_2O$ (1:1:5) we can observe by FTIR-ATR that the structure of liquid mixture is not changed by the presence of these anti-TB drugs. Concerning the stability of these formulations over time, from our studies no significant differences were detected in the spectra that could affect the chemical structure of the mixtures. Thus, proving that they are suitable to use for at least 6 months without presenting signals of degradation or losing their functionality by the disruption of the intermolecular bonds.

### 3.3. Cell viability studies

The assessment of cell viability is essential to understand the behavior of the mixtures formulated *in vitro*. The half-maximal inhibitory concentration ($IC_{50}$) was assessed in A549 cell line, human alveolar epithelial cells (Table 3). From the results obtained and the calculated $IC_{50}$ we observe that among the pure components used to formulate the mixtures, CA and the anti-TB drug RIF are the ones with lower $IC_{50}$ values. This means less amount of compound is necessary to inhibit by half the cell viability, showing the pure components higher cytotoxicity than when incorporated in the liquid mixtures.

Comparatively, the values obtained for the formulated therapeutic liquid mixtures largely differ from those obtained for the pure components. From the results obtained and despite $_{L}$-Arg presents a lower value of $IC_{50}$ than EMB, when mixed with other components of the mixture (CA and water) the mixtures containing EMB have a slightly lower $IC_{50}$ than mixtures with $_{L}$-Arg. Different molar ratios of the components, also alter substantially the values of $IC_{50}$, being the mixtures with higher ratios of CA the ones that present lower values of $IC_{50}$ and, consequently, higher cytotoxicity, probably due to increase of the acidity of the mixtures. The increase of water ratio in the mixtures, in general, leads to a slight increase on cell viability. For the therapeutic liquid mixtures

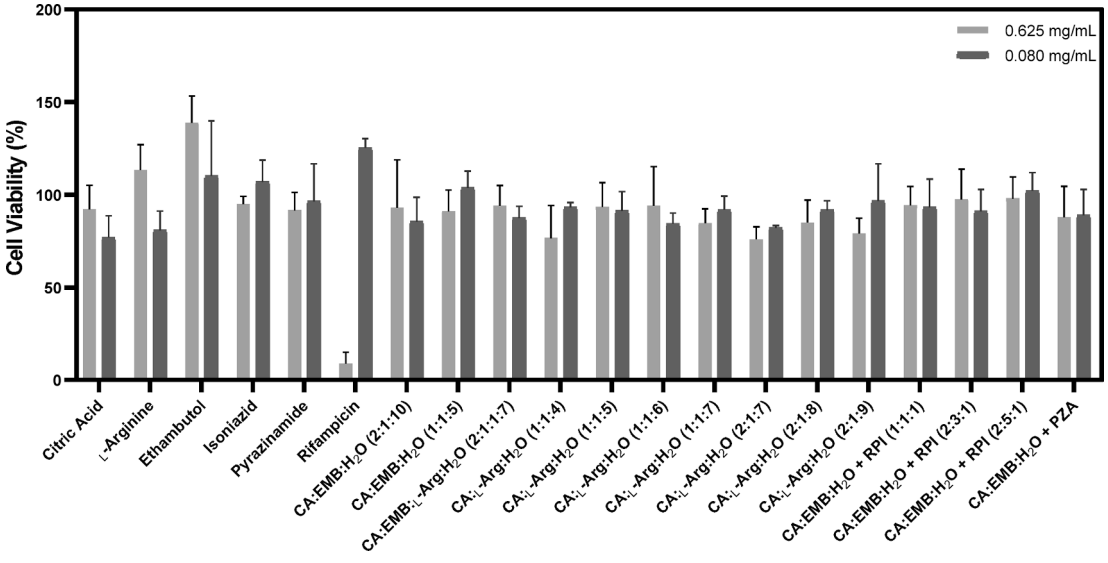


**Fig. 5.** Cell viability evaluated in macrophages at 0.625 mg/mL and 0.080 mg/mL. Data are presented as mean ± SD (n = 3).

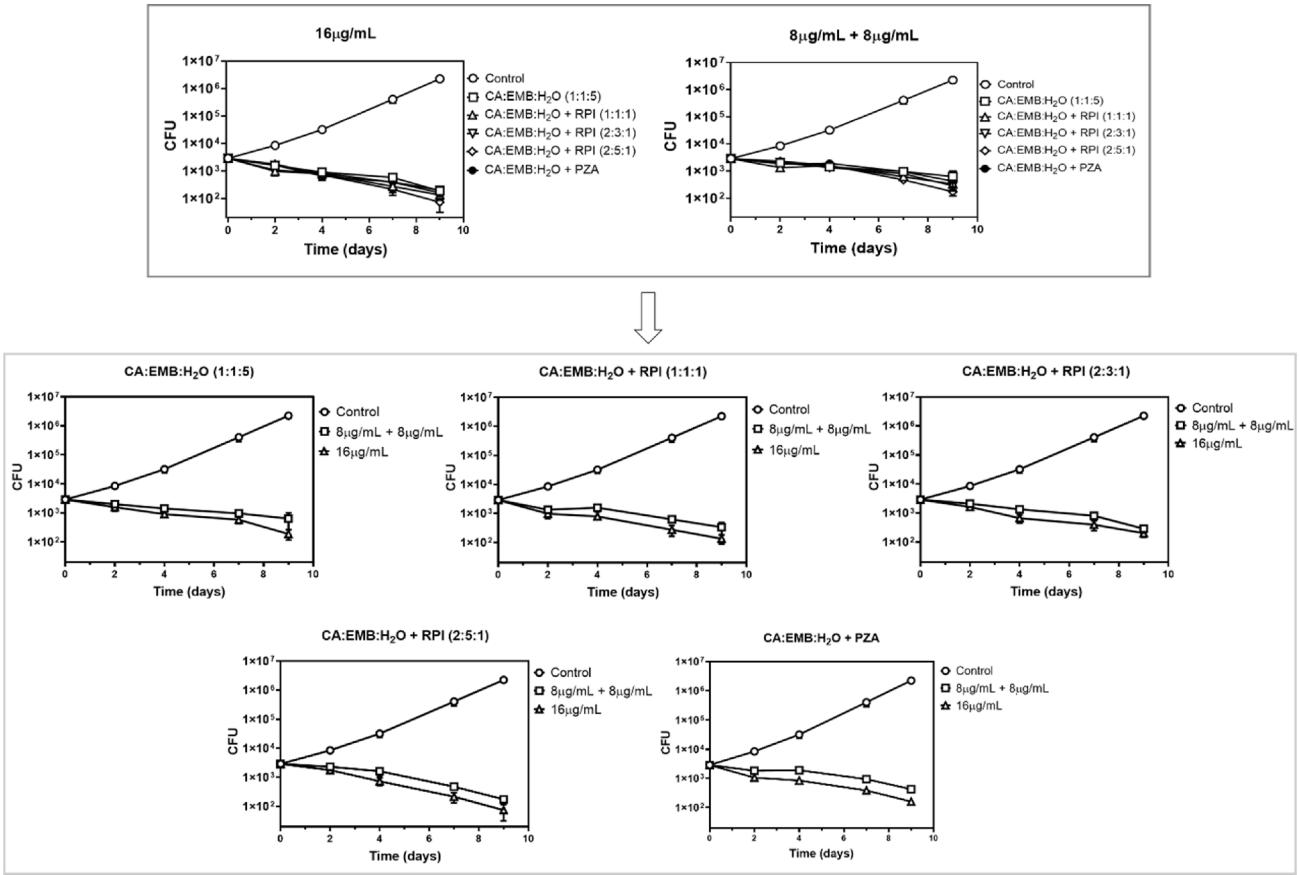


**Fig. 6.** Intracellular activity of therapeutic liquid formulations in macrophages infected with H37Rv. The activity was evaluated at different time points, for 9 days, using different concentrations: 16 μg/mL and 8 μg/mL plus another dose of 8 μg/mL at day 3. Colony-forming units (CFU) were determined by counting the colonies originated from surviving bacteria at each time point. Data are presented as mean ± SD (n = 3), ****p ≤ 0.0001, when compared to the control.

formulations it is observed a decrease on cell viability compared to the initial liquid mixture (CA:EMB:$H_2O$ (1:1:5)), due to the addition of small amounts of different anti-TB drugs (INH, PZA, RIF), however this decrease is not statistically significant.

After evaluating the cell viability in A549 cells, the cell viability in THP-1 macrophages was also evaluated, taking in account the $IC_{50}$ values determined in A549 cells. Since the lower $IC_{50}$ value was found in RIF (0.69 ± 0.34) we tested the cell viability for macrophages with concentrations below $IC_{50}$ determined for RIF to guarantee that we find a limit of non-toxic concentration that can be used in further studies with mycobacteria. Fig. 5 shows the cell viability in macrophages of all the compounds at 0.625 mg/mL and 0.080 mg/mL. From the results obtained we can observe that RIF is cytotoxic at 0.625 mg/mL, yet at 0.080 mg/mL this was not observed. We can, hence, assume that all the liquid mixtures and pure compounds (RIF < 0.080 mg/mL) are non-cytotoxic to macrophages, since they present a cell viability nearly to 100% (see Fig. 6).

### 3.4. Permeation studies upon exposure to therapeutic liquid formulations

The *in vitro* studies concerning the transport of molecules and/or formulations are important to estimate the ability of the molecules to permeate through different models of living barriers, as single monolayers or co-cultures. In order to mimic a pulmonary barrier, we used a transwell system that enables the formation of two different microenvironments (apical and basolateral side). On the apical side were cultured alveolar epithelial cells (A549) until they differentiated and established a cellular monolayer (barrier model), that formed a polarized epithelia and prevented the passage of ion currents along a paracellular pathway. After exposure of therapeutic liquid mixtures to the pulmonary barrier model, for 6 h, we estimated the apparent permeability (Table 4) for each compound and observed that anti-TB drugs (EMB, INH, PZA) tested presented higher permeation than the liquid formulations. Between the therapeutic liquid formulations, small differences were observed. However, there appears to be a tendency for increased permeability with increasing ratios of drug present in the formulation. The formulation with PZA presented the lowest permeability, being almost the only compound retained in the apical side.

Regarding the cellular uptake estimated for each formulation in A549 cellular monolayer, we can infer that a large amount of the compounds studied were internalized by the cells instead of passing through the apical to basolateral side, which justifies the lower permeation of the formulations through the barrier model of A549 cells.

Furthermore, the estimated cellular uptake in A549 cells indicates that, in the case of liquid formulations, a higher concentration of formulation is accumulated inside the cells in a non-toxic concentration instead of permeating from apical to basolateral side, except for formulations with PZA that remain in the apical side. The permeability of the formulations through this pulmonary barrier model is lower compared to the pure drugs, being hypothesized that the formulation stays in the epithelium and is not absorbed to the systemic circulation, remaining at the site of infection.

### 3.5. Antimicrobial activity in H37Rv

The antimicrobial activity was evaluated *in vitro* for drug-susceptible *Mtb* strain H37Rv with concentrations that proved to be non-toxic for cell lines (16 – 0.125 μg/mL), and the values of minimal inhibitory

**Table 4**
Estimate apparent permeability and cellular uptake from transwell assays, using a A549 cellular monolayer as membrane.

| Compound | Papp ($10^{-5}$ cm/s) | Final Concentration on apical side (mg/mL)* | Final Concentration on basolateral side (mg/mL) | Estimated cellular uptake (mg/mL) |
|---|---|---|---|---|
| Ethambutol | 2.484 ± 1.279 | – | – | – |
| Isoniazid | 1.663 ± 0.316 | – | – | – |
| Pyrazinamide | 3.007 ± 0.896 | – | – | – |
| Rifampicin | 0.339 ± 0.068 | – | – | – |
| CA:EMB:$H_2O$ (1:1:5) | 0.714 ± 0.472 | – | – | – |
| CA:EMB:$H_2O$ + RPI (1:1:1) | 0.656 ± 0.318 | 0.016 | 0.009 | 1.237 |
| CA:EMB:$H_2O$ + RPI (2:3:1) | 0.659 ± 0.484 | 0.038 | 0.050 | 1.223 |
| CA:EMB:$H_2O$ + RPI (2:5:1) | 0.715 ± 0.503 | 0.086 | 0.049 | 1.272 |
| CA:EMB:$H_2O$ + PZA | 0.292 ± 0.113 | 1.085 | 0.003 | 0.220 |

*Indicative value, estimated from one replica.

**Table 5**
MICs and MBCs determined *in vitro* in *mycobacterium tuberculosis* (H37Rv), after 7 days of treatment.

| Compound | MIC (μg/mL) | MBC (μg/mL) | Compound | MIC (μg/mL) | MBC (μg/mL) |
|---|---|---|---|---|---|
| Citric Acid | >16 | >16 | CA:$_L$-Arg:$H_2O$ (1:1:5) | >16 | n.d. |
| $_L$-Arginine | >16 | >16 | CA:$_L$-Arg:$H_2O$ (1:1:6) | >16 | n.d. |
| Ethambutol | 1 | 2 | CA:$_L$-Arg:$H_2O$ (1:1:7) | >16 | >16 |
| Isoniazid | <0.125 | <0.125 | CA:$_L$-Arg:$H_2O$ (2:1:7) | >16 | n.d. |
| Pyrazinamide | >16 | >16 | CA:$_L$-Arg:$H_2O$ (2:1:8) | >16 | n.d. |
| Rifampicin | <0.125 | 0.25 | CA:$_L$-Arg:$H_2O$ (2:1:9) | >16 | >16 |
| CA:EMB:$H_2O$ (2:1:10) | 4 | 8 | CA:EMB:$H_2O$ + RPI (1:1:1) | 2 | 4 |
| CA:EMB:$H_2O$ (1:1:5) | 2 | 4 | CA:EMB:$H_2O$ + RPI (2:3:1) | 2 | 4 |
| CA:EMB:$_L$-Arg:$H_2O$ (2:1:1:7) | 4 | 8 | CA:EMB:$H_2O$ + RPI (2:5:1) | 2 | 4 |
| CA:$_L$-Arg:$H_2O$ (1:1:4) | >16 | n.d. | CA:EMB:$H_2O$ + PZA | 2 | 8 |

*n.d. non determined.

concentration (MIC) and minimal bactericidal concentration (MBC) were calculated and are presented in Table 5.

From the determination of MIC and MBC values we observe some variability in the pure components, being as expected anti-TB drugs more effective and with lower MICs and MBCs than CA and $_L$-Arg, with the exception of PZA which is known to only be active at low pH values (Salfinger and Heifets, 1988). Furthermore, the values reported for anti-TB drugs are in accordance with previous studies presented in literature (Alliance, 2008; Alliance, 2008; Alliance, 2008; Alliance, 2008). RIF and INH are the ones that presented a more pronounced effect against *Mtb* (H37Rv). Regarding the therapeutic liquid mixtures the ones that have EMB in their composition are the ones that present better activity against H37Rv, being the one with lower ratio of CA and water (CA:EMB:$H_2O$ (1:1:5)) the one that presents a better performance against H37Rv (2 μg/mL). Regarding the formulations prepared with the therapeutic liquid mixture CA:EMB:$H_2O$ (1:1:5) and different amounts of anti-TB drugs, a similar activity is observed in comparison to the therapeutic liquid alone, with the exception of the formulation in which only PZA was added that requires higher concentrations to inhibit bacteria growth.

### 3.6. Effects on Mtb (H37Rv) during infection in macrophages

The infection of THP-1 macrophages with H37Rv and posterior treatment with the formulations was performed to evaluate the effect of the formulations in intracellular survival of *Mtb*. The selected concentrations tested did not affect the viability of macrophages, as expected from the viability studies shown previously and are in the range of MICs tested. Two approaches were followed. A single dose of 16 μg/mL was given to the macrophages infected as well as two doses of 8 μg/mL, one at day 0 plus a new dose of 8 μg/mL at day 3 post infection. It is observed that compared to the control, the mixture and the formulations presented antibacterial activity against H37Rv decreasing the CFU over the entire time-course of the experiment, inhibiting the growth of the bacteria until at least 9 days of culture. It is also interesting to observe that although the pattern of antibacterial activity presented is similar, the treatment with an initial dose of 8 μg/mL and reinforcement after 3 days with additional 8 μg/mL presented to be less effective than the administration of a single higher dose at the beginning of infection (16 μg/mL). As expected, with the increase of the ratio of anti-TB drugs dissolved in the therapeutic liquid mixture it is observed an increase in the antibacterial activity against H37Rv.

A more pronounced effect against the growth of Mtb was observed with an administration of a single higher dose at the beginning, when compared to the control, which could be an advantage to adjust the therapeutic regimen and, for instance, to enhance the compliance of the patient to treatment.

## 4. Conclusion

Therapeutic liquid mixtures as deep eutectic systems have been studied as a more sustainable approach to address tuberculosis therapy, improving properties of the existing anti-tuberculosis drugs. As it was proven by this study, therapeutic liquid mixtures and their formulations presented a physicochemical stability up to 6 months, therapeutic activity against *Mtb* (H37Rv) and it was also improved the solubility of pyrazinamide using therapeutic liquid mixtures. Pursuing to answer the drawbacks of tuberculosis therapy, these mixtures present themselves as more sustainable approaches that can potentially contribute to the treatment of TB.

## CRediT authorship contribution statement

**Filipa Santos:** Conceptualization, Investigaition, Methodology, Data curation, Witting – original draft. **David Pires:** Conceptualization, Investigaition, Methodology, Data curation, Witting – original draft. **Elsa Anes:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Validation, Writing – review & editing. **Ana Rita C. Duarte:** Conceptualization, Formal analysis, Funding acquisition, Supervision, Validation, Writing – review & editing.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.122862.

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