The honey: an alternative for antibiotic-resistant infections?

An experimental study

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I per a que així consti a tots els efectes oportuns, signo el present document en Girona a 27 de gener de 2020.

Signat: Dr. Enrique Verdú Navarro

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ABBREVIATIONS

AMPs: antimicrobial peptides

AZDAST: Ameri-Ziaei double antibiotic synergism test

BSL: biosafety level

CFU: colony-forming unit

DMSO: dimethyl-sulfoxide

GC-MS: gas chromatography-mass spectrometry

HPLC: high performance liquid chromatography

i.p.: intraperitoneally

MDR: multidrug-resistant

MGO: methylglyoxal

MIC: minimum inhibitory concentration

MRSA: methicillin-resistant *Staphylococcus aureus*

PBP 2a: penicillin binding protein 2a

PBS: phosphate-buffered saline

RR: resistant to rifampicin

SMIF: synergistic multiple ingredients factor

TB: tuberculosis

UAM: Autonomous University of Madrid

WHO: World Health Organization

ABSTRACT

Background: Tuberculosis (TB) is the major cause of death per infection cause, affecting around of 10 million people per year with a more than a million death per year. This problem is aggravated by the resistance spread around the world of *M. tuberculosis* against the current treatment used nowadays, making more difficult to treat the patients who suffered and died from TB. This data is translated as a social problem because TB affects mainly the African and the Asian continent, the poor people and a huge proportion of young adults, with a negative economic impact in the countries and in the own population. Being studied possible alternatives for this problem and existing such evidence of honey and its antibacterial activity, it has been considered as a possible candidate to treat resistant TB cases.

Objective: the aim of this study is to evaluate if there is a synergistic effect of the honey and honey compounds with current drugs used to treat *M. tuberculosis* resistant strains in petri dishes (*in vitro*) and in animal models (*in vivo*).

Design: blinded and randomised experimental study will be made in Severo Ochoa Molecular Biology Center of the Autonomous University of Madrid (UAM) since January 2021 to December 2023.

Methods: the *in vitro* study will be made in dishes plates by the Ameri-Ziaei double antibiotic synergism test (AZDAST) method. The *in vivo* study will be made with 310 Balb/c-OlaHsm (ENVIGO) mice strains divided in different experimental groups, will be infected by *M. tuberculosis* resistant strains and will receive different treatments (antituberculosis drugs, honey and/or honey compounds). At different time of the follow-up, mice will be sacrificed, and the lung infection will be studied.

Keywords: animal models, antibiotic resistance, honey, lungs, methylglyoxal, phenolic compounds, synergism, tuberculosis

INTRODUCTION

1. Epidemiology of infectious diseases

Infectious diseases are the second cause of death in the world (Mathers et al 2009), being one of the 15 leading causes of death worldwide, highlighting among them infections of the low respiratory tract, AIDS, malaria and tuberculosis (TB) (Mathers and Loncar 2006). Actually, it is estimated that communicable diseases still cause more than 13 million deaths per year (Khyatt et al 2014).

1.1. TB: an overview around the world

Being the first infectious disease to be declared as a global emergency from the World Health Organization (WHO) in a press release (WHO 1994), TB is one of the top 10 causes of death worldwide and the main death cause from a single infectious agent. Indeed, it is estimated that in 2017 TB caused 1.6 million deaths. At the same time, millions of people continue to fall sick with TB each year, estimating 10 million people develop TB disease every year (WHO 2019).

Geographically, around 65% of the new TB cases have produced in eight countries, situate in Africa and Asia (*figure 1*). In the other hand, only 6% of global cases were in Europe and America (WHO 2019).

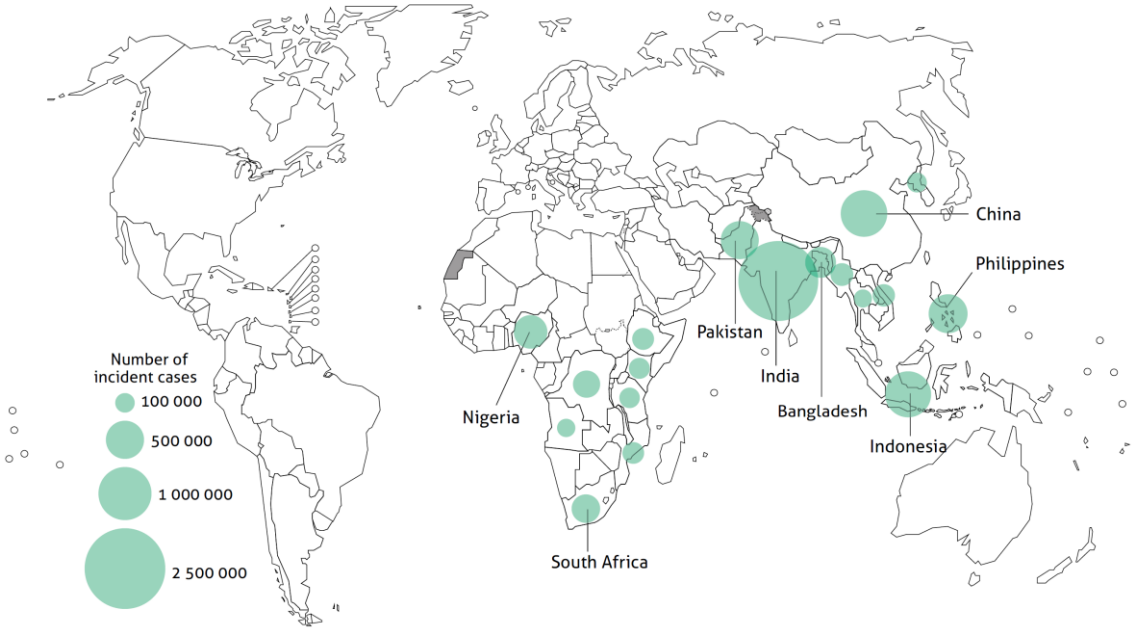


Figure 1. *Estimated TB incidence in 2018, for countries with at least 100,000 incident cases;* ***from*** *WHO 2019.*

2. Current treatment of infections

Antibiotics have a long history, beginning in the 1928 with the discovery of penicillin by Alexander Fleming (Fleming 1929), and since 1940 until 1960s more than 20 new classes of antibiotics were created (Coates et al 2011). After these discoveries, antibiotics have saved millions of lives and eased the suffering of patients of all ages (IDSA 2004).

However, the antibiotic use had not been the adequate: in the past 60 years, millions of tons of antibiotics have been produced and distributed in various products for many purposes (Levy and Bonnie 2004), exposing the population to antibiotics even through their own water (Venkatesan and Halden 2014). At the same time, 50% of prescribed antibiotics had been unnecessary (CDC 2013). This antibiotic overuse has led to an exacerbation of the spread and appearance of the antibiotic resistance (Barbosa et al 2000).

As a result, nowadays we are experiencing an important crisis of antibiotic resistance worldwide: bacterial pathogens have developed resistance to antibiotics introduced into clinical practice, including the major last resort drugs (Levy and Bonnie 2004), and this resistance is increasing around the world in an alarming rate, making infections more difficult to treat, or even untreatable in some cases (Akova 2016).

This situation has a significant impact on morbidity and mortality in the population (Spellberg et al 2008). For example, about 50% of *E. Coli*, *K. pneumoniae* and *S. Aureus* infections have shown resistance to commonly used antibiotics (WHO 2014), hospital infections caused by *P. aeruginosa* and *A. baumannii* are sometimes resistant to all, or all but one, antibiotics (Levy and Bonnie 2004), most bacteria that cause infections in hospitals are resistant to at least one antibiotic (Blair et al 2009), being this pathogens the major cause of death in hospitals (Hammond et al 2016). In fact, antibiotic-resistant infections double the duration of hospital stay and the mortality compared with drug-susceptible infections (Levy and Bonnie 2004). In USA, over 2 million people have an infection associated with antibiotic-resistant organism, and more than a 23,000 of them die annually (IDSA 2004). Finally, it is estimated that at least 700,000 people die annually from

drug-resistance infections around the world, and this number could rise to 10 million at 2050 (O'Neill 2014).

2.1. TB and drug resistance

The era of modern effective TB chemotherapy began in 1952. Since then, strains of *M. tuberculosis* have acquired resistance to various drugs (Wood and Iseman 1993). For example, TB resistance to streptomycin emerged in the community soon after the discovery of this antibiotic (Crofton and Mitchison 1948). Between 1882 and 1985, existed a declining incidence of TB in some places, but since 1985, this situation changed, trending to an increasing incidence (Bloom and Murray 1992).

In 2016, there were 600,000 new cases of resistant to rifampicin (RR), resistant to the most effective first-line drug, requiring treatment with second-line drugs. From these 600,000 cases, 490,000 of them were multidrug-resistant (MDR), in other words, resistant to rifampicin and isoniazid. Later, in 2017, had been detected more than 558,000 cases of RR and MDR, remaining treatment success low, at 55% globally (WHO 2018).

3. TB in Morocco

3.1. Epidemiology

Morocco, a country with a population of 36 million inhabitants, has 36,000 TB cases estimated with an incidence of 99 cases per 100,000 (*figure 2*), 3,000 deaths and a mortality rate of 8.2 per 100,000 population per year (WHO 2019).

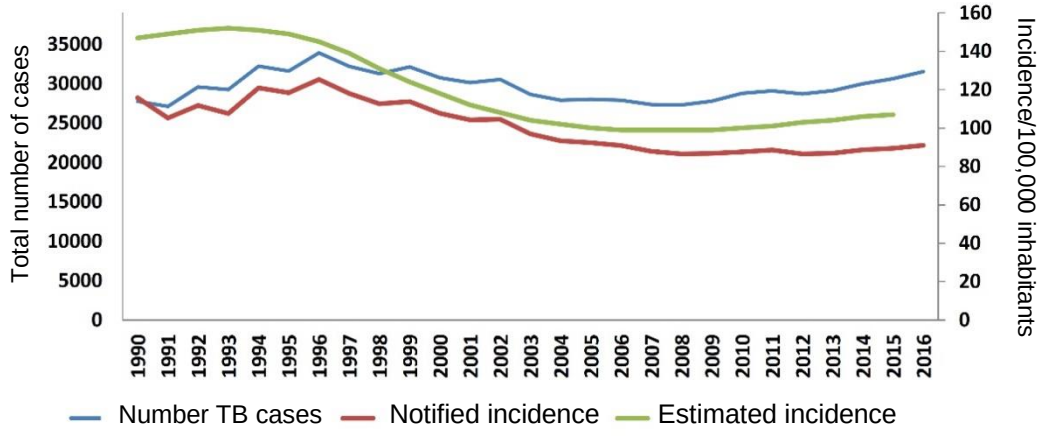


Figure 2. *Total TB cases and TB incidences across the time in Morocco since 1990 to 2016;* ***adapted*** *from Maaroufi 2018.*

Even with a 1-2% decreasing incidence per year and reduction of death cases during the last years, the TB infection and death rates remain high, being an important public health problem in Morocco (Maaroufi 2018, *Ministrére de la santé* 2015, *Ministrére de la santé* 2018, WHO 2019). In fact, according to the Moroccan Health Ministry, “at this decrease pace, the decline in TB burden is likely to remain significant for many of the coming years” (*Ministrére de la santé* 2018). At the same time, the MDR/RR-TB cases remains substantial and in raise during the last years, with 530 new cases per year and an incidence of 1.5 per 100,000, being the proportion of TB cases with MDR or RR 1% in TB new cases and 10% in previously treated TB cases (WHO 2019).

The incidence varies between the different Moroccan administrative regions, being over 70% of the cases concentrated in 4 regions (Casablanca-Settat, Rabat-Salé-Kénitra, Tanger-Tétouan-Al Hoceima and Fès-Meknès) (*Ministrére de la santé* 2018).

In general, is more common in the male gender, and 70% of cases reported are between 15 and 44 years old, affecting the economically productive young adults (*Ministrére de la santé* 2018) (*figure 3*).

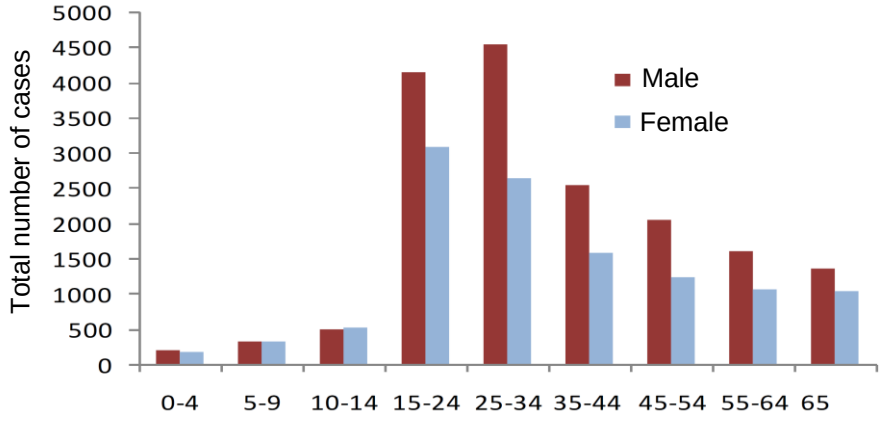


Figure 3. *Distribution of TB cases by sex and age in Morocco;* ***Adapted*** *from Ministrére de la santé 2015.*

3.2. Healthcare system and TB

The implication of the Moroccan government against TB has been considerable. For example, the state funding to fight against TB has increased the recent years, with more than a 10 million US$ since 2011 and more than 20 million US$ in 2019 (WHO 2019).

This implication has been traduced with treatment improvement of the patients, with more than 85% of therapeutic success. To achieve this aim, the Moroccan Health Ministry guaranties a detection coverage with 60 Tuberculosis and Lung Disease Diagnostic Centres around the country, with an easy access to microscopy, radiology, culture and molecular tests in every centre, and also offers for free the antituberculosis drugs to all patients (*Ministrére de la santé* 2018).

The current treatment used against TB in Morocco consist in 2 months of isoniazid, rifampin, pyrazinamide, and ethambutol followed by 4 months of rifampin and isoniazid treatment (Tachfouti et al 2013).

However, the rate of therapeutic success decreases considerably in the MDR/RR, remaining the success percentage, similar to the worldwide average percentage, at 56% (WHO 2019), and as has been seen before, TB infection remains a health problem, being de MDR and RR TB infections a serious problem (El Hamdouni et al 2019).

In front of this situation, alternative therapeutic interventions are urgently needed.

4. Alternative treatments for infectious diseases

Alternative antimicrobial strategies to antibiotic treatment have led to re-evaluation of the therapeutic use of ancient remedies, including honey.

The use of traditional medicine to treat infection has been practiced since the origin of mankind, and honey produced by *Apis mellifera* is one of the oldest traditional medicines considered to be important in the treatment of several human illnesses (Mandal and Mandal 2011). Honey has been used as a treatment and prevention since the earliest times in many cultures for its medicinal properties (Alvarez-Suarez et al 2014). With the advent of antibiotics, the clinical application of honey was abandoned in modern Western medicine (Kwakman and Zaat 2012). However, honey is well known of its antibacterial activity, and the activity of honey against antibiotic-resistant bacteria have resulted renewed interest for its application (McLoone et al 2016), because diluted and undiluted natural honey has an important antibacterial activity, with

bacteriostatic and bactericidal effects (Atwa et al 2014, Lin et al 2010, Bouchikh 2014).

In vitro studies have found that honey can reverse antimicrobial resistance and reduce microbial pathogenicity, suggesting that honey used in combination with antibiotics may have an additional therapeutic effect. For example, sublethal concentrations of honey (5% [w/v] manuka honey) have a marked effect in enhancing the susceptibility of methicillin-resistant *Staphylococcus aureus* (MRSA) to oxacillin. This result is justified for the downregulation of sensor/transducer MecR1 (*figure 4*): MecR1 and MecI regulate MecA gene, which codes for the low-affinity penicillin binding protein 2a (PBP 2a), an enzyme working to allow cell-wall synthesis despite the presence of β-lactam antibiotics. So, blocking the MecR1 pathway it restores antibiotic susceptibility in MRSA (Hou et al 2011, Jenkins and Cooper 2012).

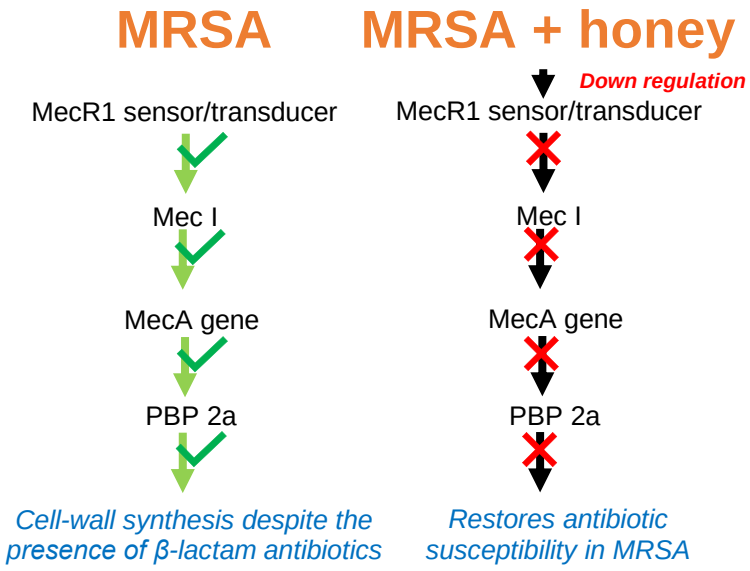


Figure 4. *Comparison of MecR1 pathway in methicillin-resistant Staphylococcus aureus (MRSA; left) and the MecR1 pathway under honey effect (right); ✔: stimulation, X: inhibition;* ***adapted*** *from Archer 2001.*

Interestingly, no honey-resistant microbial strains have emerged to date (McLoone et al 2016). An *in vitro* study, with short-term resistance training with reference cultures and resistance training of clinical isolates by long-term exposure to honey, have proved that *S. aureus* and *P. aeruginosa* exposed continuously to low concentrations of honey has not change their susceptibility to the honey, indicating an absence of honey resistance (Cooper et al 2010).

4.1. Chemical composition of honey

Honey is a sweet and flavourful food produced by bees (*A. mellifera*) from nectar of flowers (Osés et al 2016). It contains more than 200 constituents, mainly composed of sugars (the principal component), water (<18%), proteins, organic acids, vitamins, minerals and aromatic substances (*Annex 1*) (Ciulu et al 2016; Da Silva et al 2016; Kwakman and Zaat 2012). The composition varies depending on geographical regions, botanical origin and climate (*table 1*).

Table 1: honey composition according different parameters

Parameter	Composition variation
Geographical area	Mineral concentration: related with the soil type, because de geochemical and geological characteristics of soil affect the mineral composition of flower nectars, and therefore, of honey (Berriel et al 2019, Bogdanov et al 2007). Floral source: honeybee preference for specific flora is based on its availability in a specific region (Warui et al 2019). Indeed, geographical origin of honey can be established through the pollen content, which reflects the floristic composition of the vegetation zone where is produced (Aina et al 2015).
Botanical origin	Floral source provides nectar and pollen needed for honey (Warui et al 2019), causing the following alterations: Sugars composition: fructose, glucose and reducing sugars content varies among the different botanical sources (Rodríguez-Flores et al 2019). Free acidity values: his variation is attributed to differences in sugar concentration of the nectar (Warui et al 2019). Phenolic compounds: qualitative and quantitative differences in phenolic profile are a direct consequence of these compounds in the different floral sources which they originate (Ciulu et al 2016). Color: varies according the floral source. For example, chestnut honeys tend to be higher red and yellow (Rodríguez-Flores et al 2019).
Climatic conditions	Enzyme content: warm weather can cause overheating of honey, with low enzyme content as a consequence (Warui et al 2019).

At the same time, during storage, through processes such as the specific enzymes present in honey, high temperature and long storage time, honey may change and degrade into new products such as furans, alcohols and phenolic compounds. These can also be affected by the production of secondary reactions, such as the Maillard reaction, when the compounds of furan derivatives are heated or stored for a long time (*figure 5*) (Da Silva et al 2016).

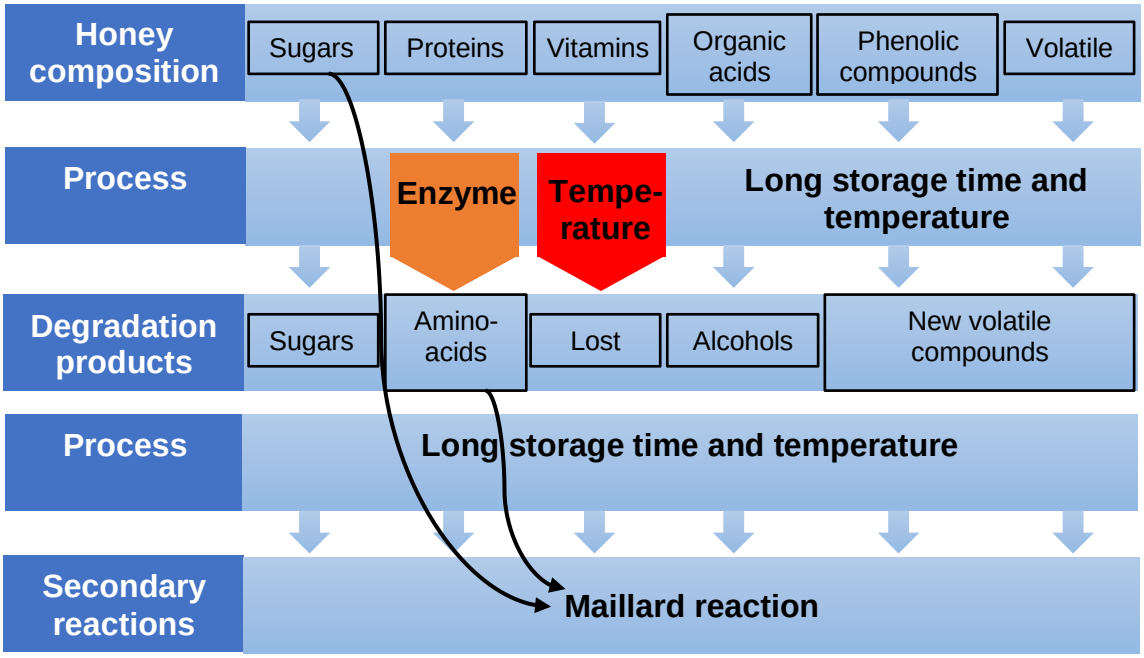


Figure 5. *Compounds present in honey, the processes that influence their composition, degradation products and secondary reactions that may occur.* ***Adapted*** *from Da Silva et al 2016.*

The authenticity of honey is defined internationally by the Codex Alimentarius, which establish the essential quality requirements of honey intended for direct human consumption (*table 2*) (Codex standard 2001).

Table 2: minimum quality requirements

Parameter	Values	Parameter meaning
Sugars	Minimum amount of fructose and glucose: 60g/100g. Maximum value of sucrose content: 5g/100g.	-
Moisture	Maximum moisture content: 20g/100g.	Influences physical properties of honey such as viscosity, crystallization, colour, flavour, taste, solubility and conservation (Escuredo et al 2013).
Free acidity	Maximum value: 50.00meq/kg.	Higher values may indicate fermentation of sugars into organic acids (Da Silva et al 2016).
Electrical conductivity	Maximum value: 800.00mS/cm.	Related with mineral content, acidity and colour. Reveals the presence of ions, organic acids and proteins (Karabagias et al 2014).
Colour	Nearly colourless to dark brown.	An important parameter in the quality, acceptance and preference of consumers (Da Silva et al 2016).

Table 2: minimum quality requirements		
Parameter	**Values**	**Parameter meaning**
5-hydroxy-methylfurfural (5-HMF)	Maximum value for the mixture or processed honey: 10.00mg/kg. Maximum value of honeys from tropical climate: 80.00mg/kg.	Formed by the decomposition of monosacharides or the *Maillard* reaction, its concentration increases across the time and under heat. Is an indicative of honey deterioration and it's falsification by adding invert syrup (Capuano and Fogliano 2011, Tornuk et al 2013).
Diastase activity	Minimum value: 8 Schade Units. Minimum value in honeys with low enzyme content: 3 Schade Units.	Sensitive to heat (thermolabile) indicates overheating of the honey and inadequate storage conditions (Ahmed et al 2013).

The identity and quality parameters of honey are analysed by different honeys samples from different continents, and all of them have values below those allowed by de Codex Alimentarius Committee (Da Silva et al 2016).

4.2. Antibiotic properties of honey

Honey has not a specific compound with antibiotic effect. Indeed, honey has a numerous bioactive substance that interact and work in synergy and is what is designed as a *Synergistic multiple ingredients factor* (SMIF). This hypothesis proposed by Ajibola (Ajibola 2013) is supported by other researchers, who state that most of honey compounds work together to provide a synergistic effect (Eteraf-Oskouei and Najafi 2013). This may account for the inability of bacteria to develop resistance to honey in contrast to the rapid induction of resistance observed with antibiotics (Ajibola 2015), because bacteria are unlikely to acquire resistance if they are treated with compounds with different therapeutic target (Silver and Bostian 1993).

The main components that justify the antibiotic effect of honey are:

Sugars: honey is a super-saturated solution of sugars (Molan 1992) and this high concentration of sugars causes osmotic stress against the microbial cells (Csonka 1989, Kwakman and Zaat 2012). The strong interaction of theses sugar

molecules with water molecules leaves very few of the water molecules available for microorganism (Molan 1992). At the same time, bacteria are bounded by semipermeable cytoplasmic membranes, often including aquaporins, that allow the water flux among the intracellular compartment (cytoplasm) and the extracellular space (environment). The integrity and hydration of the cell and its compartments are determinate by their solute contents and the osmotic pressures of their environment. So, an increase in external osmotic pressure, like exposing bacteria to honey, causes water efflux and dehydration (***figure 6***) (Wood 2015).

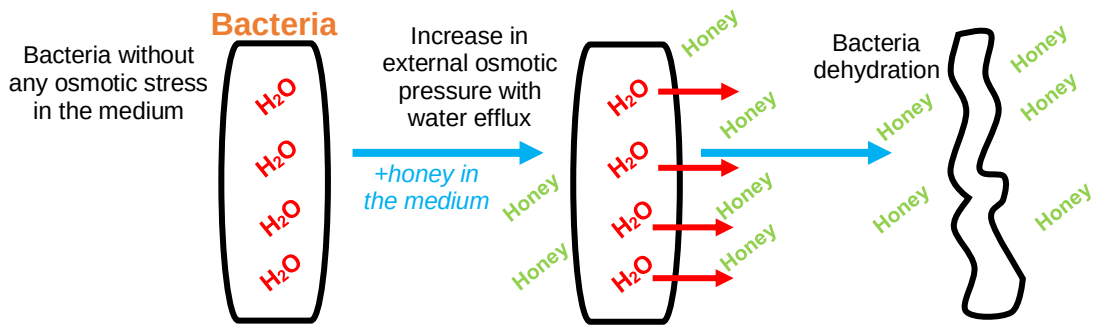


Figure 6. *Graphical representation of the honey osmotic effect against bacteria.* ***Own elaboration.***

Simultaneously, water fluxes also perturb many cellular properties: cell volume (or the relative volumes of the cytoplasm), turgor pressure, cell wall strain, cytoplasmic membrane tension, as well as individual uncharged solute and salt ion concentrations (Wood 2015).

However, the high sugar content is not the only responsible for antibacterial properties of honey (Simon et al 2009), because even diluting the honey in a non-osmotic concentration, it continues with antibacterial activity (Viuda-Martos et al 2008). Naturally, this activity is attributed to other compounds, as it is exposed below.

Hydrogen peroxide (H_2O_2): converted from glucose to H_2O_2 and gluconic acid by the enzyme glucose oxidase, added by honey bees during production of honey, H_2O_2 levels in honey depends by the difference between the rate of its production and its destruction by catalases, an enzyme of flower pollen origin that hydrolyses hydrogen peroxide to oxygen and water (Weston 2000).

The main factors that determine the levels of these enzymes are:

- Glucose oxidase: age and health status of the bees and the richness and diversity of floral resources (Alaux et al 2010, Pernal and Currie 2000).

- Catalase: the amount of pollen collected by the bees, the floral source of the pollen and the catalase activity of that pollen (Weston 2000).

Hydrogen peroxide was identified as a major antibacterial compound in honey at 1960 and enhances the antibacterial capability of honey (Kwakman and Zaat 2012). Really, H_2O_2 is a toxic to many microbes (McLoone et al 2016) with a bacteriostatic effect (Brudzynski et al 2011).

As a low-molecular-weight oxidative biocide, can cross the cell membranes and cause intracellular damage. Once inside the cell, H_2O_2 can react with internal cellular components, leading to apoptotic and necrotic cell death (Finnegan et al 2010). The multiple targets within a cell (*figure 7*) include: DNA degradation (Brudzynski et al 2011), peroxidation and disruption of membrane layers, oxidation of oxygen scavenger's ant thiol groups in enzymes, proteins and bacterial cell membrane, enzyme inhibition, oxidation of nucleosides, impaired energy production, disruption of protein synthesis and, finally, cell death (Finnegan et al 2010).

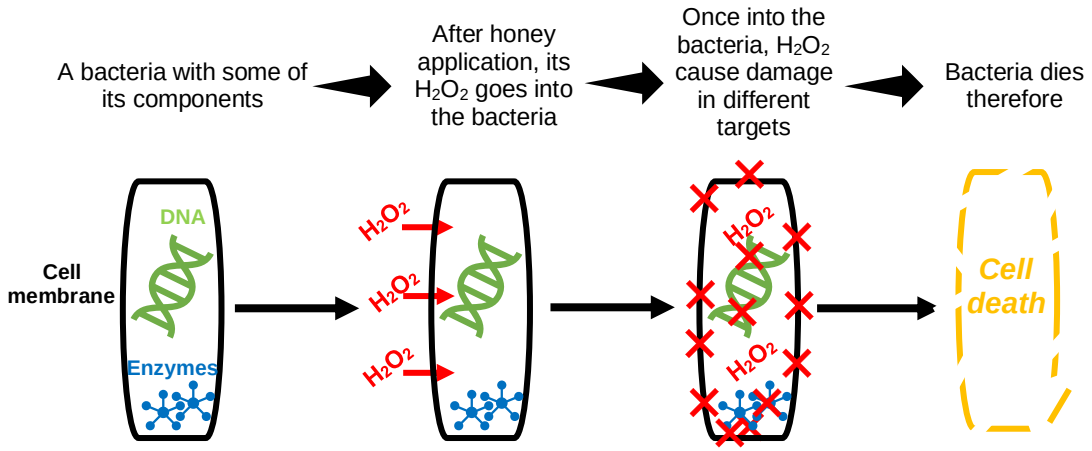


Figure 7. *Schematic representation of the H_2O_2 effect and the different targets in the bacteria; X: damage location.* ***Own elaboration.***

Low pH (3.2-4.5): the acidity is due primarily to the content of gluconolactone and gluconic acid presents as the result of enzymatic action in the ripening nectar (Molan 1992), representing the organic acids 0.57% of honey content (Olaitan et al 2007).

This acidity is unfavourable for the growth of many bacteria (Molan 1992). Bacteria are critically dependent upon pH homeostasis because most proteins have distinct ranges of pH for function and the proton concentration is involved in cellular bioenergetics (Krulwich et al 2012).

Methylglyoxal (MGO): as a cytotoxic substance (Ferguson et al 1998), has been demonstrated a positive linear relationship between his concentration and antibacterial activity of honey (Cokcetin et al 2016). The exact mechanism by which MGO inhibits bacterial growth and causes cell death is unclear, but is believed to be due to its ability to interact with the nucleophilic centres of macromolecules such as DNA, RNA and proteins. In fact, it is possible that there is no single target that is responsible that justifies his toxicity, because *in vitro* studies have shown to react with amino acids and nitrogenous bases (Ferguson et al 1998).

Antimicrobial peptides (AMPs): peptides with antibacterial activity, have been shown to kill Gram-negative and Gram-positive bacteria, including resistant strains, and mycobacteria, including *M. tuberculosis* (Dai et al 2010):

- Abaecin: with a delayed action, is active against most Gram-negative and Gram-positive bacteria (Casteels et al 1990).

- Apidaecin: with an immediate effect, is selectively bactericidal for Gram-negative bacteria (Casteels et al 1990).

- Bee defensin-1: produced from the bees (Majtan et al 2012), is a cationic antibacterial peptide with a potent activity only against Gram-positive bacteria (Kwakman and Zaat 2012). It kills bacterial cells by permeabilization of their cytoplasmic membrane (Klaudiny et al 2012).

- Cecropin: exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria (Guo et al 2012).

Phenolic compounds: defined as substances that possess an aromatic ring bound with one or more hydrogenated substituents (Viuda-Martos et al 2008), is a plant derived secondary metabolite (Ciulu et al 2016). The antibacterial activity is attributed to main following honey phenolic compounds:

- Pinocembrin and caffeic acid: they inhibit the bacterial RNA polymerase (Takaisi-Kikun and Schilcher 1994), presenting the first one antituberculosis effect (Songsiang et al 2009) and defined as one of the most effective phenolic compounds against *M. tuberculosis* (Chou et al 2011).

- Galangin (3,5,7-trihydroxyflavone): degrades the cytoplasm membrane of the bacteria (directly or weakening the cell wall and, thereby, causing osmotic lysis), which leads to a loss of potassium ions, and the damage caused provokes cell apoptosis (*figure 8*) (Cushnie and Lamb 2005).

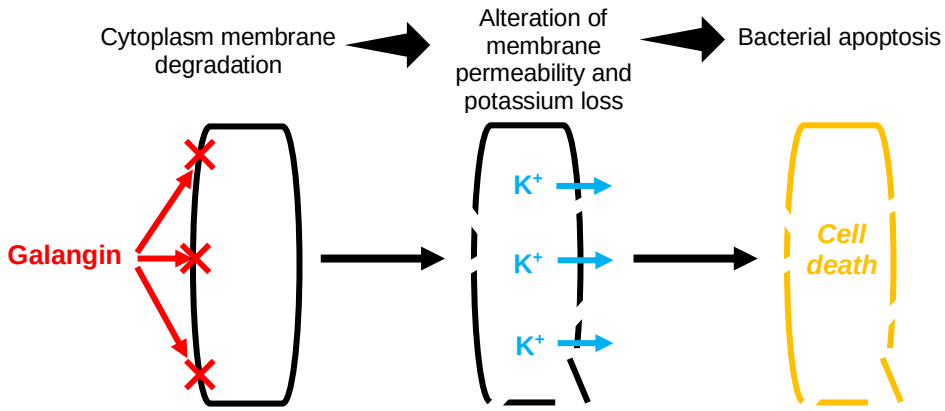


Figure 8. *Schematic representation of galangin action mechanism; X: damage location.* ***Own elaboration.***

- Quercetin: has an important role inhibiting bacterial pathogenesis and the development of infection, causing inhibition of bacterial motility, and has an antimicrobial effect increasing membrane permeability, that dissipates its potential, leading the bacteria to lose their capacity to synthesis ATP, their membrane transport and causing bacterial death (Mirzoeva et al 1997).

4.3. Honey and *M. tuberculosis*: actual evidence

In vitro studies have shown that honey inhibits the growth of *M. tuberculosis* by adding 10% honey to a Standard Lowenstein-Jensen media (Asadi-Pooya et al 2003). Even if they are resistant, honey also inhibits MDR-TB strains at concentrations 1% to 5%, being the accumulative susceptibility at 2% of 86% and being totally effective, with a 100% inhibition, at concentrations equal o superior at 3% (Hannan et al 2014).

In the best of our knowledge, there is no study in animal models that evaluates the honey activity against *M. tuberculosis*.

4.4. Honey against infections: scientific evidences

There is so much evidence that honey is used for its antibacterial properties in *in* vitro studies, in animal models and in human beings, including clinical assays and even in the clinical practice to treat some infections, as will be developed next.

In wounds infection, honey has shown antibacterial activity *in vitro* against pathogens obtained from infected wounds (Cooper et al 1999), even being resistant bacteria (Jenkins and Cooper 2012). In animal models, honey has cured infected wounds by MRSA (Medeiros et al 2016). In a clinical practice, infected wounds that had failed to cure with conventional treatment had shown a clinical improvement after honey dressing treatment and have been successfully healed (Costeloe et al 2018, Sylvester-Hvid et al 2019, Vardi et al 1998, Cooper et al 2001). In fact, honey dressings have been useful to treat diabetic wounds in foot patients, decreasing the amputation rate (Makhdoom et al 2009). There is also a random controlled clinical trial that used honey dressings to treat postoperative wounds with a reduction of hospital stay duration as a result (Robson et al 2012). Finally, in Royal Surrey County Hospital maxillofacial unit, honey dressings are used to cure infected wound that were resistant to antibiotics with a successfully results (Visavadia et al 2008).

In other skin infections, honey has demonstrated antibacterial activity against pathogens obtained from diabetic foot infections in *in vitro* studies (Abd El-Malek et al 2017), including resistant strains (Mohammadzamani et al 2020). In different

randomised controlled clinical trials, honey has eradicated MRSA from chronic venous ulcers (Gethin and Cowman 2008), has shown a similar antibiotic effect in prevention of catheter-associated infections (Johnson et al 2005) and honey combination with acyclovir has improved the herpes simplex gingivostomatitis symptomatology compared with the current treatment with acyclovir in children (Abdel-Naby Awad and Hamad 2018).

Related with gastrointestinal infections, honey has a bacteriostatic and bactericidal effect against gastrointestinal pathogens in *in vitro* studies (Alnaqdy et al 2005, Lin et al 2009, Lin et al 2010, Mohammed et al 2019). In animal models, has been observed an antityphoid effect of honey administrated by oral route, healing the typhoid fever symptoms with negative blood cultures after honey treatment (Hannan et al 2015). Finally, in a controlled clinical trial honey administration shortens the diarrhoea duration in bacterial gastroenteritis (Haffejee and Moosa 1985).

In ophthalmological infections, *in vitro* studies have shown antibacterial effect against blepharitis pathogens (Frame et al 2018), being honey an option to treat blepharitis, being safe and tolerate for patients without major adverse events (Craig et al 2017). In animal models, honey has a good antibacterial activity against *Pseudomonas* keratitis, being this activity as good as the antibiotic conventional treatment (Punitan et al 2019). In humans, honey has exhibited prophylactic effect against ophthalmitis after ocular surgery in humans (Cernak et al 2012).

Bacteria that causes respiratory tract infections have been susceptible to honey in *in vitro* study (El-Kased 2016). In animal models, intravenous honey administration has reduced the bacterial load in mice infected by *K. pneumoniae* resistant strains (Qamar et al 2018).

Finally, other infections in which honey has been studied in random clinical trials are in the gynaecology area, with antibacterial effect in vulvovaginal candidiasis (Banaeian et al 2017), and in otorhinolaryngology, being effective to treat chronic rhinosinusitis (Lee et al 2017).

JUSTIFICATION

TB disease is an important cause of morbimortality around the world. According the World Health Organization (WHO), affects around 10 million people each year and around million and a half per year dies for TB infection (WHO 2017b, WHO 2018, WHO 2019). This problem is aggravated by the spread of the TB resistance against the different treatment options, being the most common cases RR and MDR. In fact, it is estimated that 3.4% of TB new cases and 18% of TB cases previously treated are RR or MDR in 2018, causing more than 200,000 deaths as a result of the low treatment success in RR and MDR TB cases (WHO 2019).

This problem is mainly important in Africa and Asia, which the most TB cases are concentred (WHO 2019). For example, in Morocco the incidence of TB is considerable, with 99 cases per 100,000 population, 8.2 death by TB per 100,000 population and with an incidence of RR and MDR of 1.5 cases per 100,000 population (WHO 2019). In fact, a considerable number of TB cases diagnosed in Morocco have exhibit mutations that confers resistance to the different antituberculous drugs used (Chaoui et al 2009, Chaoui et al 2018, Ennassiri et al 2017, Ennassiri et al 2018, Oudghiri et al 2018). This RR and MDR cases in Morocco are a problem because treatment success in RR and MDR cases remains low also, with a 56% of success (El Hamdouni et al 2019, WHO 2019). In fact, according to El Hamdouni, “one of the most serious obstacles for TB control efforts is the low rate of treatment success among drug resistant TB patients as this might lead to the development of more resistance and the transmission of these more resistant strains to other persons“ (El Hamdouni et al 2019).

In addition of this panorama, the TB cases are more incident in persons living in urban areas (Tachfouti et al 2013), with low socioeconomic status (El Hamdouni et al 2019, Lönnroth et al 2009) and in young adults, between 14 and 44 years, (*Ministrére de la santé* 2015). All these health determinants and factors have a socioeconomical impact in Morocco, with a significant effect on national economies, by direct loss of productivity of the patients in working age and by altering investment in human and physical capital (Adeyi et al 2007).

In front of this situation, alternative therapeutic strategies are urgently needed. One of the possible alternatives is honey, a natural product with different compounds that exhibit antibacterial activity in different targets (Casteels et al 1990, Cokcetin et al 2016, Cushnie and Lamb 2005, Guo et al 2012, Kwakman and Zaat 2012), including antituberculosis effect (Dai et al 2010, Songsiang et al 2009). An interesting point of honey use as a possible treatment option is the SMIF hypothesis, supported by other researchers (Eteraf-Oskouei and Najafi 2013) and that argues that honey has a numerous compound that interact and work in synergy with antibiotic effect. This honey characteristic is crucial because, in the best of our knowledge, there is no data of bacteria developing resistance to honey and because bacteria are unlikely to acquire resistance if they are treated with compounds with different therapeutic target (Silver and Bostian 1993).

On the other hand, honey has demonstrated antibacterial activity *in vitro* studies (Cooper et al 1999, Lin et al 2009), including resistant strains (Mohammadzamani et al 2020), and *in vivo* studies, including in animal models (Hannan et al 2015, Medeiros et al 2016), cases report (Sylvester-Hvid et al 2019), case series (Costeloe et al 2018) and clinical trials in humans (Gethin and Cowman 2008, Haffejee and Moosa 1985, Johnson et al 2005), with a good results healing infections.

For the reasons exposed, honey looks like an ideal candidate to treat resistant TB infection, and this experimental study in animals is presented as a preclinical study to evaluate the possible use of honey and/or its compounds to treat the RR and MDR TB cases.

HYPOTHESIS AND OBJECTIVES

The **hypothesis** of the present study is that "*the combined therapy with the current antibiotic treatment and honey could reduce the antibiotic resistance of M. tuberculosis*".

The **main objective** of the present study is to evaluate whether honey exerts a synergistic effect with antibacterial antibiotic treatments against a resistant strain of *M. tuberculosis*. Both *in vitro* and in vivo studies will be performed. The specific or **secondary objectives** are the following:

- Determine the minimum inhibitory concentration (MIC) of the honey sample and selected honey compounds against resistant cultures of *M. tuberculosis* (*in vitro*).
- Examine a possible synergic effect between honey and commonly used antibiotics against *M. tuberculosis* resistant strains (*in vitro*). In addition, examine a possible synergic effect between selected honey compounds and commonly used antibiotics against *M. tuberculosis* resistant strains (*in vitro*).
- Compare the dose-response relationship of honey and selected honey compounds administrated intraperitoneally at different concentrations in mice infected with resistant cultures of *M. tuberculosis* (*in vivo*). Compare the lung damage after current treatment of *M. tuberculosis* against lung damage after honey treatment and after selected honey compounds treatment in mice infected.
- Analyse the composition of the honey sample that will be used in this study.

METHODOLOGY

1. Honey samples and chemical analysis

In the present study it will be used a natural multifloral honey sample from Al Hoceima province, that belongs to the Tanger-Tétouan-Al Hoceima Moroccan region, because is the region with more TB incidence registered in the last years (*Ministrére de la santé* 2018, Piro 2019) (*figure 9*). The sample will be purchased from "*Cooperative Agricole Jazeerat Nahl pour la production de produits apicoles*" from Al Hoceima, will be sterilised by gamma radiation to eliminate the possible microorganisms and spores that could be present (Cernak et al 2012 and Cooper et al 2001) and will be stored in a dark place at room temperature (20°C) until its use (Abselami et al 2018).

It should be noted by previous studies that honey samples from this region have all the physicochemical characteristics within the range allowed by the Codex Alimentarius (Chakir et al 2011, Codex standard 2001, Terrab et al 2002), so it meets the minimum quality requirements for human consumption, being viable application in this study and in the possible use in future clinical practice.

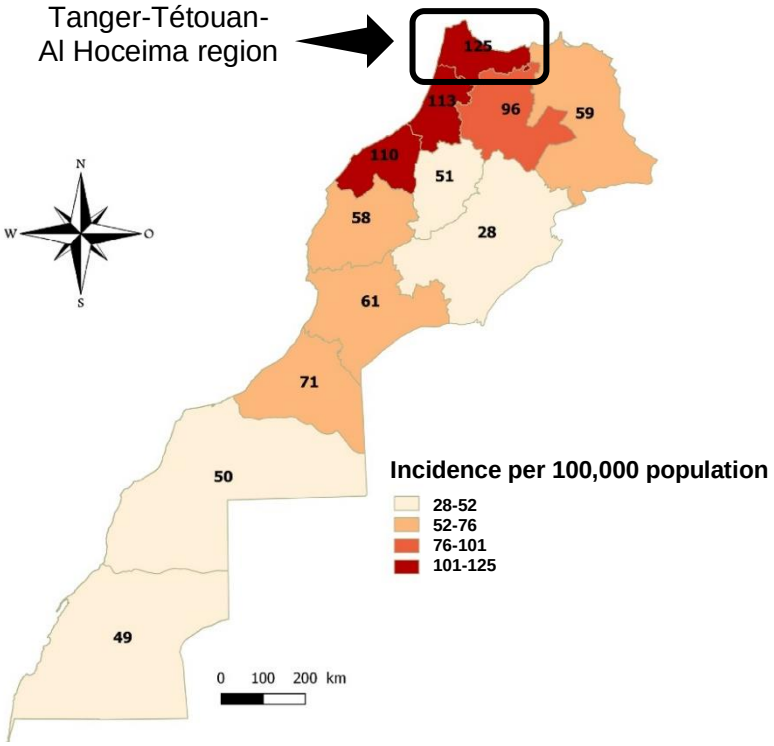


Figure 9. *The Morocco map with its respective TB incidences per regions, being marked the Tanger-Tétouan-Al Hoceima region.* ***Adapted*** *from Piro 2019.*

1.1. Honey analysis

In order to know if the honey sample used in the present work will have the components with honey antibacterial activity (methylglyoxal and phenolic compounds), and in what concentration they are in said sample, each of these components will be analyzed according to the following biochemical procedures.

The MGO component will be analyzed by HPLC analysis (Adams et al 2008) (*figure 10*). For it, 0.6g of honey will be dissolved in water (30% [w/v]). The honey solution (1.5mL) will be treated with 2% [w/v] o-phenyl-enediamine in 0.5M phosphate buffer (0.75 ml, pH 6.5) for 16 hours. Reactions will be performed in the dark at room temperature. Then, honey sample solution will be evaluated using the HPLC analysis, that this consisted of a Waters 515 HPLC Pump, Waters 2996 Photodiode Array Detector, a Rheodyne 7725i manual sample injector (5µL sample loop) and a Waters SymmetryShield RP18 5µm 3 x 250mm column. A gradient elution will be performed at 0.3 mL/min. Eluent A will be 0.075% acetic acid in water and eluent B will be 80% MeOH in water (final solution made up 0.075% acetic acid). Gradient steps will be (min, (% B)): 0 (10), 4 (10), 5 (42), 30 (55), 31 (100), 34 (100), 35 (10) and 40 (10). The system will be controlled using Waters Empower™ 2 Chromatography Software.

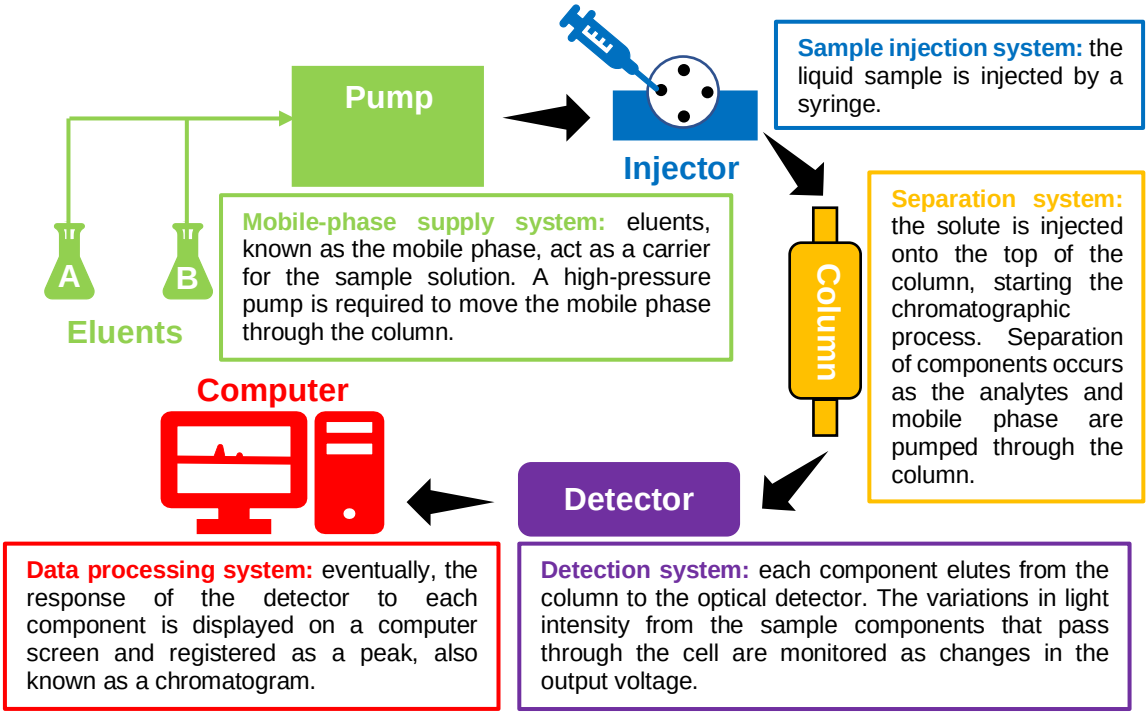


Figure 10. *Schematic diagram of high performance liquid chromatography (HPLC) system with the main parts.* ***Adapted*** *from Siouffi 2005 and Verma and Santoyo 2007.*

The chromatogram obtained from the HPLC will show different peaks, and with a commercial MGO as a reference it will determined if one of the obtained peaks from the sample corresponds to MGO peak, showing the presence or absence of MGO in the sample. At the same time, comparing the area under the peak obtained from the sample with the area under the peak from commercial MGO in a known concentration, being the both areas proportional with their concentration (Siouffi 2005), the MGO concentration will be calculated from the honey sample, and the results will be expressed in mg of MGO per kg of honey (mg/kg).

On the other hand, phenolic compounds such as caffeic acid, galangin, pinocembrin and quercetin will be analysed using gas chromatography-mass spectrometry (GC-MS) (Alotibi et al 2018, Hegazi and Abd El-Hady 2009) (*figure 11*). For it, 50g of the honey sample will be extracted with 250mL of ethyl acetate, and then it will be concentrated using rotary evaporator at 40°C. 5mg of the extract will be dissolved with 0.05ml pyridine and 0.1ml N,O-bis (trimethylsilyl) trifluoro-acetamide (Sigma) and then heated at 60°C for 30 minutes. Clean-up of the extract will be carried out by using solid phase purification membrane (Sartorius Syringe Filter 17576Q) and then it will be injected into the GC-MS.

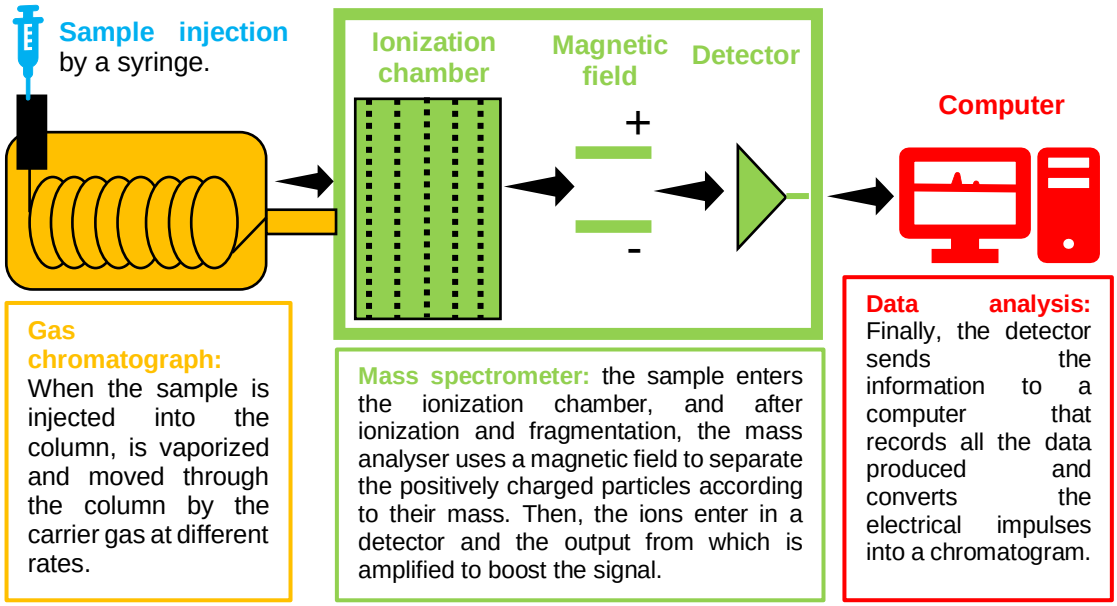


Figure 11. *Schematic diagram of a GC-MS with the main parts.* ***Adapted*** *from Kim et al 2006, WHO 2017a and Wu et al 2012.*

A Shimadzu GC-MS-2010 will be used and a DB-5 column, 30m x 0.32mm (internal diameter), will be utilized with helium as the carrier gas. The temperature will be adjusted from 40ºC to 260ºC at 5ºC/min. The mass spectra will be gathered in electron ionization mode at 70eV, with an ion source temperature of 150ºC. The scan repetition rate will be 0.5s. Then, peaks will be identified by computer search of user-generated reference libraries, incorporating mass spectra. The quantitative analysis will be the same as described previously in MGO identification and quantification and the results also will be expressed in mg/kg.

2. Microbiological procedures

M. tuberculosis belongs to a "Risk Group 3 microorganisms", and for this reason to it will be need a biosafety level (BSL) 3 laboratory (Richardson et al 2009, WHO 2004) (*figure 12*). Therefore, all microbiology procedures will be carried out in the cell culture laboratory with BSL-3 of the Severo Ochoa Molecular Biology Center of the Autonomous University of Madrid (Hernández and Mendaza 1987).

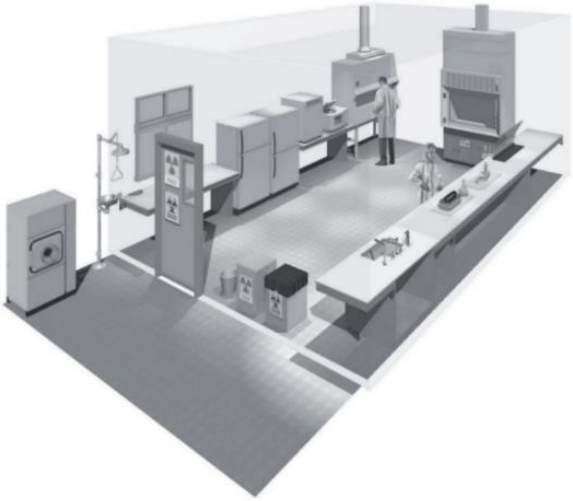


Figure 12. *A typical Biosafety Level 3 laboratory. The laboratory is separated from general traffic flow and accessed through an anteroom or an airlock. An autoclave is available within the facility for decontamination of wastes prior to disposal. A sink with hands-free operation is available. Inward directional airflow is established and all work with infectious materials is conducted within a biological safety cabinet.* ***From*** *WHO 2004.*

2.1. Bacterial strains and cultures

Resistant strain of *M. tuberculosis* H37Rv-RIF-R (ATCC® 35838™) will be purchased from American Type Culture Collection (Virginia, USA). *Mycobacterial* resistant cultures will be grown in Middlebrook agar 7H10 (Becton Dickinson) medium supplemented with albumin-dextrose-catalase (Difco Laboratories) and containing 0.05% Tween 80 and 20µg/mL kanamycin. The L-lysine monohydrochloride (Sigma) will be dissolved in distilled water and used at a concentration of 40mg/mL. Middlebrook agar plated will be placed in a 37°C crop oven and in aerobic conditions during 16h (Broset et al 2019).

2.2. Treatments preparation

In vitro studies, honey samples, the standard combination of antibiotics against TB, as well as the antimicrobial components (MGO, polyphenols) present in honey will be used.

The honey sample will be diluted in sterile distilled deionized water to obtain a 40% concentration (Qamar et al 2018). This dilution will be used to determine de MIC against *M. tuberculosis* H37Rv-RIF-R. After MIC determination (see below MIC determination section), 1mL of this dilution will be saturated in an 8mm diameter-filter paper (Badawy et al 2004) and will used for the synergism study (see below Synergism study). Moreover, for the standard combination of antibiotics against tuberculosis, they will be used 5µg/ml of isoniazid, 10µg/ml of streptomycin and rifampicin and 15µg/ml of ethambutol diluted in 5ml of sterile distilled water (Gay et al 1984). This solution of antibiotics will be saturated again in 8mm diameter-filter paper disc (CLSI 2017) and will used also for the synergism study. Finally, in a 40% aqueous solution, 200µM of MGO (MP Biochemicals, USA) will be saturated in 8mm disc (Mukherjeea et al 2011), and polyphenols including caffeic acid, galangin, pinocembrin and quercetin, per separate, will be dissolved in 5mg/mL of dimethyl-sulfoxide (DMSO). 10µl of the resultant solutions will be added in 8mm discs (Santas et al 2010). All these polyphenol compounds will be purchased from Sigma-Aldrich.

2.3. MIC determination

Defined as the lowest concentration of an antimicrobial substance that prevents visible growth of a microbe in a broth dilution susceptibility test (CLSI 2017), MIC is considered the gold standard for determining the susceptibility of organisms to antibiotics (Andrew 2002).

A 40% solution of the honey sample will be prepared with Mueller-Hinton broth and filter-sterilised with a 0.2µm filter (Sartorius) before serial dilution. Of the 12 columns in a microplate, the first column will be added with 40µl of honey, the second to the tenth with 40µl of Mueller-Hinton broth and the last two with a growth control (*M. tuberculosis* and Mueller-Hinton broth) and sterility check (plain Mueller-Hinton broth). For serial dilution 160µl of honey will be added into

the second column, which will be then sequentially transferred to the following wells up to the tenth well. After that, 80µl of inoculum will be added into each well except the last well, in which 80µl of plain Mueller-Hinton broth will be added instead so that the final concentrations of the honey will be 13.32%, 10.64%, 8.52%, 6.8%, 5.44%, 4.36%, 3.28%, 2.8%, 2.24% and 1.8% after inoculation (Lin et al 2009). The growth of the microorganisms in the microplates will be monitored at 37°C for 15 days using a microplate reader (BMG FLUOStar OPTIMA) and the results will be observed as the monitored growth curves (Lin et al 2010). The lowest concentration of honey needed to completely inhibit the growth of the isolate will be considered to be its MIC (Lin et al 2009).

Likewise, this procedure will be the repeated for selected honey compound (MGO, caffeic acid, galangin, pinocembrin and quercetin) to determine their MIC.

2.4. Synergism study

For the evaluation of the synergism between honey solution and standard antibiotics, the Ameri-Ziaei double antibiotic synergism test (AZDAST) (Ziaei-Darounkalaei et al 2016) will be used. This method will be carried out through the following steps (*figure 13*):

1. An adhesive to past paper discs on the floor of the petri dish will be made. The glue will contain the 1.5 times concentration molten cooled (48ºC) autoclaved Mueller-Hinton agar.

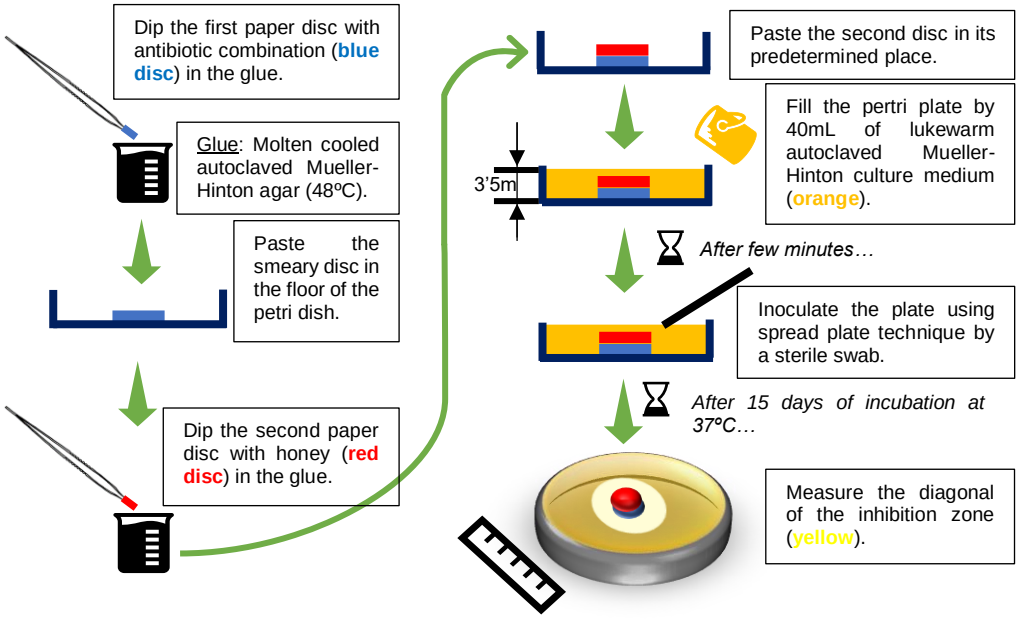


Figure 13. *AZDAST method representation.* ***Adapted*** *from Ziaei-Darounkalaei et al 2016.*

2. It will dip the first paper disc (A) with antibiotic combination in the glue.
3. The smeary A disc will be pasted in the floor of the petri dish.
4. Similarly, the second disc (B) with the honey will smeared and pasted in its predetermined places. Only combination site of dish has been shown in *figure 13*, but all the positions shown in the *figure 14* pattern must be pasted before to produce the following step.

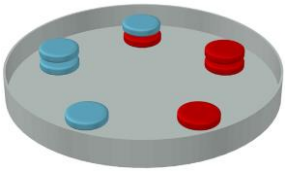


Figure 14. *Completed AZDAST petri plate before pouring agar. Top-center: combination position. Right and left: doubled treatments. Right and left: single treatments.* ***From*** *Ziaei-Darounkalaei et al 2016.*

5. The petri will be filled by 40mL of lukewarm (heat-out via 46ºC shaking water bath for 30min) autoclaved Mueller-Hinton culture medium. The agar depth should be 3.5mm.
6. The agar solidified will let within a few minutes.
7. The plate will be inoculated using spread plate technique by a sterile swab.
8. The plate will be incubated at 37ºC for 15 days.
9. The diagonal of the zone of inhibition will be measured in mm.

The AZDAST method will be made 6 times (n=6) and will be adapted with honey compounds per separate instead of honey. The interpretation of the results derived from this test are shown in the following table:

Table 3: interpretation guideline

Combination result from AZDAST petri plate	Equivalent definitions in slang	AZDAST interpretation
If the AB is greater than A and B and smaller or greater than AA and/or BB	1+1=3	Synergistic
If one of the A or B is equal to zero and AB is greater than A and B and smaller or greater than AA and/or BB	0+1=2	Potentiation (enhancement)
If the AB is smaller than A or B (or only smaller than greater one)	1+1=0	Antagonistic
If the AB is equal to AA and/or BB (which one is greater than A and B)	1+1=2	Additive
If the AB is equal to one of the A or B (equal to the greater one)	1+0=1	Not distinguishable

3. Experimental design with animals

A preclinical blinded, controlled and randomised experimental study will be made in Severo Ochoa Molecular Biology Center of the Autonomous University of Madrid (UAM). The total sample consist in 310 mice, will be infected by *M. tuberculosis* resistant strains and will be divided in different experimental groups to evaluate the efficacy of the different treatments (see below *Figure 16*).

3.1. Experimental procedures with animals

In this project, adult male and female mice (8 weeks old) of the strain Balb/c-OlaHsm (ENVIGO) will be used. All animals will be maintained in appropriate conditions of light and humidity, and with access to eat and water "*ad libitum*". The animals will be kept in the animal facilities of the Severo Ochoa Molecular Biology Center of the UAM, which has BSL-3 areas.

3.2. Sample size

Using the Granmo public software (http://www.imim.es/ofertadeserveis/software-public/granmo/) the sample size that will be used in the present study has been calculated. Accepting an α risk of 0.05 and a β risk of 0.2 in a two-sided test, 7.8 subjects (mice) will be necessary in each experimental group. A maximum number of mice that will be used in each experimental group will be 10. This number of mice by experimental group is similar that previous studies (Chang et al 2019).

3.3. Infection of mice with *M. tuberculosis*

Cultures will be carried out in liquid medium using the BACTEC system for *M. tuberculosis* (Siddiqi et al 2011). A sample of the microbial liquid cultures will be centrifuged, and the pellet will be resuspended in phosphate-buffered saline (PBS; Broset et al 2019, Gorski 2012). Then, mice will be infected by inhalation exposure system (Glas-Col) with 500µL PBS containing *M. tuberculosis* H37Rv-RIF-R, delivering approximately 10^3 bacilli per mouse (Wang et al 2019).

3.4. Pharmacological treatment

All the treatments will start 3 weeks after mice inoculation, coinciding with the maximum colony-forming unit (CFU) of *M. tuberculosis* in lungs (Cardona et al

1999). The different therapeutic options, exposed below, will be administrated once a day for 4 weeks (Williams et al 2009):

- Standard antibiotics treatment: animals will be treated with the standard antibiotic combination, including isoniazid (25mg/kg), rifampicin (10mg/kg), pyrazinamide (150mg/kg) and ethambutol (25mg/kg) diluted with water in a total of 0.2mL by gavage (Magden et al 2011, Rullas et al 2010, Williams et al 2009).
- Honey treatment: another set of animals will be treated intraperitoneally (i.p.) with 1ml of honey solution (Owoyele et al 2014).
- Treatment with honey polyphenols with antimicrobial effects: another set of mice will be treated with MGO and/or caffeic acid dissolved in saline solution (Pereira et al 2006), galangin, pinocembrin and/or quercertin dissolved in 1% DMSO in saline solution (Zha et al 2013, Gu et al 2017, Khan et al 2018). All these polyphenols will be purchased in Sigma-Aldrich. For each compound, mice will receive 0.25 mL i.p. (Khan et al 2018, Lee et al 2019).

3.5. Mice sacrifice

At different times of the follow-up, 5 animals from each experimental group will be sacrificed with intraperitoneal pentobarbital sodic overdose (Dutton et al 2019).

- The first sacrifices will be done just after the treatment finalization, that is 4 weeks after the treatment initiation and 7 weeks after the mice inoculation. The first sacrifices will be useful to evaluate the infection evolution under the different treatments.
- The following sacrifices will be done 4 weeks after the treatment finalization, comparing the possible remission or possible relapse under after abandoning the different treatments.

3.6. Analysis of mice infection and effectivity of treatment

For this analysis, the lungs will be aseptically removed after euthanasia with an overdose of sodium pentobarbital (90-100 mg/kg; i.p.).

To measure infection burden in lungs, the left lobes will be aseptically removed and will be homogenized (*figure 15*). The homogenates will be supplemented with 5% glycerol and the supernatant will be plated serial dilutions on appropriate media for the growth of *M. tuberculosis* (see section Bacterial strains and cultures). After 21 days of culture incubation at 37ºC in 5% CO_2 atmosphere, colonies will be counted using an automatic colony counter (aCOLyte-Supercount; Synoptics Ltd., Cambridge, United Kingdom) and will be confirmed by visual inspection to correct potential misreadings. The results will be represented as log_{10}CFU per lung (Cardona et al 2001, Rullas et al 2010).

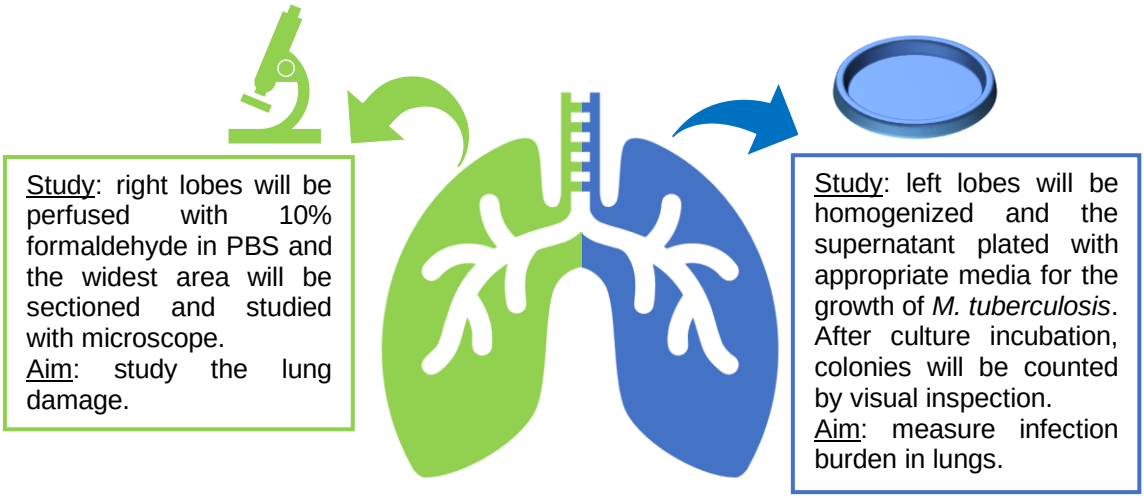


Figure 15. *Lungs distribution of every mice after euthanasia with the respective studies and aims for every portion.* ***Own elaboration.***

On the other hand, the right lobes of each of the two mice per experimental group will be perfused with fresh 10% formaldehyde in PBS (see *figure 15*). Tissues will be embedded in paraffin blocks and sections will be made across the widest area of each lobe, always keeping the same orientation in order to examine the same pulmonary fields in all mice. Sections, 5µm thick, separated 100µm one from each other (Cardona et al 2000), will be stained with haematoxylin-eosin and using the Ziehl-Neelsen technique (Cardona et all 1999) to determine the degree of involvement of the lungs by bacterial inoculation. Granulomas will be counted in every section with the aid of a 10x magnification objective, and their maximum diameter measured with a WHL 10x/20µm eyepiece (Olympus, Tokyo, Japan). Photomicrography will be performed using a BX5O/PM-20 System (Olympus) (Cardona et al 2000).

3.7. Experimental design summary

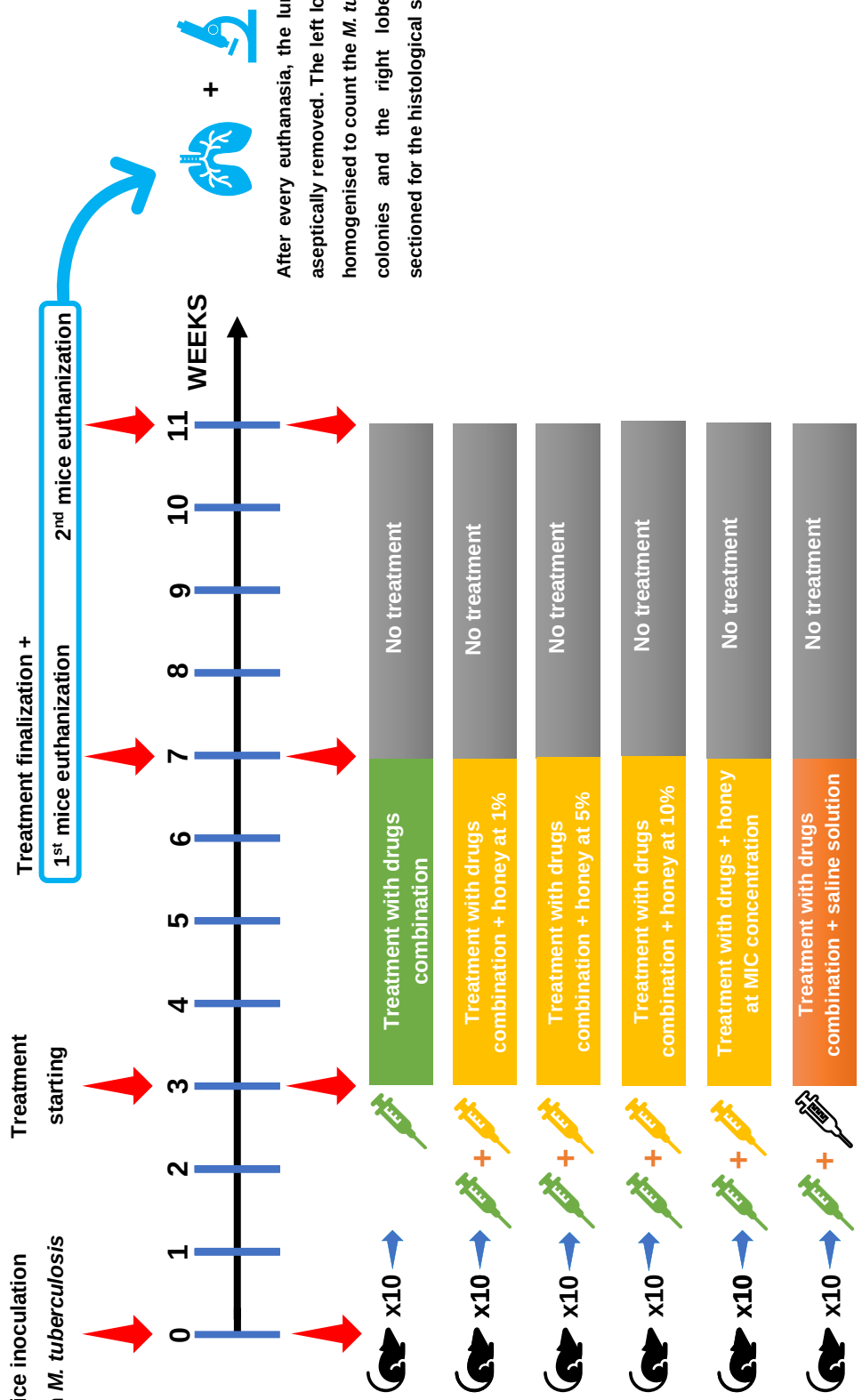


Figure 16. *Chronological summary of Task 4 with the different experimental groups with their corresponding treatments, the treatment starting and finalization and the euthanasia times-up. This figure it's applicable also for the Task 5, using honey compounds instead of honey, at the same concentrations, and DMSO in saline solution instead of saline solution in the last group.* ***Own elaboration.***

STATISTICAL ANALYSIS

In *in vitro* studies, the ***independent variables*** are the concentrations of the treatments used (honey solution, standard combination of antibiotics, antimicrobial components of honey; continuous quantitative variables), while the ***dependent variables*** are MIC and zone of inhibition measured in mm (AZDAST test; continuous quantitative variable).

On the other hand, in *in vivo* studies, the ***independent variables*** are the degree of bacteria inoculated to the mice by the inhalation method and the pharmacological treatments used with their respective doses (continuous quantitative variable), while the ***dependent variables*** are the number of bacterial colonies present in the lung tissue (expressed as $\log_{10}$CFU per lung, as a continuous quantitative variable), and the count and size of granulomas in lung tissue (discrete quantitative variable).

All *in vitro* and *in vivo* studies will be performed blindly, that is, researchers who apply pharmacological treatments will be different from those who evaluate the effects of such treatments. Likewise, all results will be expressed as mean ± standard deviation of the mean. Statistical comparisons between groups will be made by analysis of variance (ANOVA) in the case of the variables would have a symmetric distribution (i.e. normal distribution), with the correction of Welch (for different variances among groups). If the distribution of the variables are not symmetrical (i.e. non-normal distribution) the Kruskal-Wallis method will be applied. If the means are different statistically, a post hoc Tukey's test for multiple comparisons will be used in order to know what specific group is different. In contrast, if the variables distributions are not normal, for statistical comparisons between groups a Scheffe test will be used. Differences will be considered significant if $p<0.05$.

In one hand, randomizing and, in the other hand, being the duration so short, the covariables will be distributed randomly into the groups. Therefore, there is no confounding possibility.

LIMITATIONS

One of the major limitations of this project is that there are few previous studies that demonstrate the antimicrobial effect of the honey solution, and its synergy with standard treatments against tuberculosis. As described in the introduction section, most of these studies are *in vitro*, and there are no *in vivo* studies in this regard.

On the other hand, the low number of studies prior to the present research project makes it more necessary to demonstrate whether the honey solution, by itself or in combination with the standard antibiotic treatment against tuberculosis potentiates or not said antibiotic effect. Therefore, it is proposed to carry out *in vitro* studies to confirm existing results in the scientific literature, as well as new studies in animal models.

Finally, an experimental limitation is the little sample that will be used in this project, but being an experimental study with animals this sample is appropriated for the research.

ETHICAL AND LEGAL CONSIDERATIONS

The animal experimentation procedure will be presented to the Animal Experimentation Ethics Committee of the Severo Ochoa Molecular Biology Center. The experimental procedures will be adhered to the recommendations of the following laws and agreements for the care and use of laboratory animals:

- Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes.
- *Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia (BOE de 8 de febrero de 2013).*
- *Orden ECC/566/2015, de 20 de marzo, por la que se establecen los requisitos de capacitación que debe cumplir el personal que maneje animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia (BOE de 1 de abril de 2015).*
- *Ley 6/2013, de 11 de junio, de modificación de la Ley 32/2007, de 7 de noviembre, para el cuidado de los animales, en su explotación, transporte, experimentación y sacrificio (BOE de 12 de junio de 2013).*
- *Acuerdo de transparencia sobre el uso de animales en experimentación científica en España*. *Comisión COSCE de Estudio del Uso de Animales en Investigación Científica. 2016*

WORK PLAN

To accomplish the stated objectives of the project, the following tasks have been planned:

Task 1. Honey analysis study. In the first task, using the HPLC and GC-MS analysis described previously in the Methodology section, the main chemical compounds with antibacterial activity of the honey sample will be determined. The results obtained in this first task will be relevant, for the reason that will permit to know his composition, with which will be tested which of these compounds has a major inhibitory activity against de bacterial growth.

Task 2. Honey and honey compounds MIC determinations. This second task will be useful to investigate the susceptibility of *M. tuberculosis* to different honey concentrations detailed in MIC determination section. The most important aim of this task is to establish against *M. tuberculosis* resistant strains the MIC from the honey sample, displaying the optimum doses of this treatment. This MIC value will be used as the honey concentration to study the possible synergism with combined drugs treatment, as will be explained in Task 3, and also will allow to establish de different concentrations of honey that will be used in mice infected, as will be described in Task 4. All the procedures to determine the MIC will be repeated in triplicate (n=3) (Qamar et al 2018).

The MIC determination will be also carried for the different honey compounds (MGO, caffeic acid, galangin, pinocembrin and quercetin) and by the same reasons described for the MIC from the honey sample, the MIC of honey compounds will be essential to make the Task 3 and Task 5.

Task 3. *In vitro* study of the synergistic effect with the combination of honey or honey compounds and anti-tuberculosis drugs. The third task will evaluate the possible synergy of the honey sample with medications commonly used in TB infection, as described in the methodology. The results derived from this task will allow to determine if there is a synergistic effect of the honey solution on the

antimicrobial effect of standard antibiotics, in other words, if the honey solution potentiates these effects.

In this third task, the synergistic effect of the main antimicrobial components of honey (MGO, polyphenols) with the treatment of standard antibiotics also will be determined.

The experimental groups of this task are exposed in the following table (*Table 4*):

Table 4: petri dishes distribution

Groups	Petri dishes seeded with…	Nº samples (n)
1	TB + antibiotics + honey solution	6
2	TB + antibiotics + MGO	6
3	TB + antibiotics + caffeic acid	6
4	TB + antibiotics + galangin	6
5	TB + antibiotics + pinocembrin	6
6	TB + antibiotics + quercertin	6
7	TB + antibiotics + saline solution	6
8	TB + antibiotics + DMSO	6
		48

Task 4. Dose-response study of the combined treatment (antibiotics and honey) in mice infected with TB resistant strain. This fourth task pretends to determine the appropriate dose of combined treatment in TB lung infection. In these animals it will be determined the damage degree of the infection of the lungs and the remission with the treatments (bacteria recount, histological damage), and the evaluation of the honey compounds in the lung tissue, as has been described in the Methodology section. In this task also will be used the best bacteriostatic/bactericides doses observed in the task 2. The experimental groups will be the following ones (*Table 5* and *figure 16*):

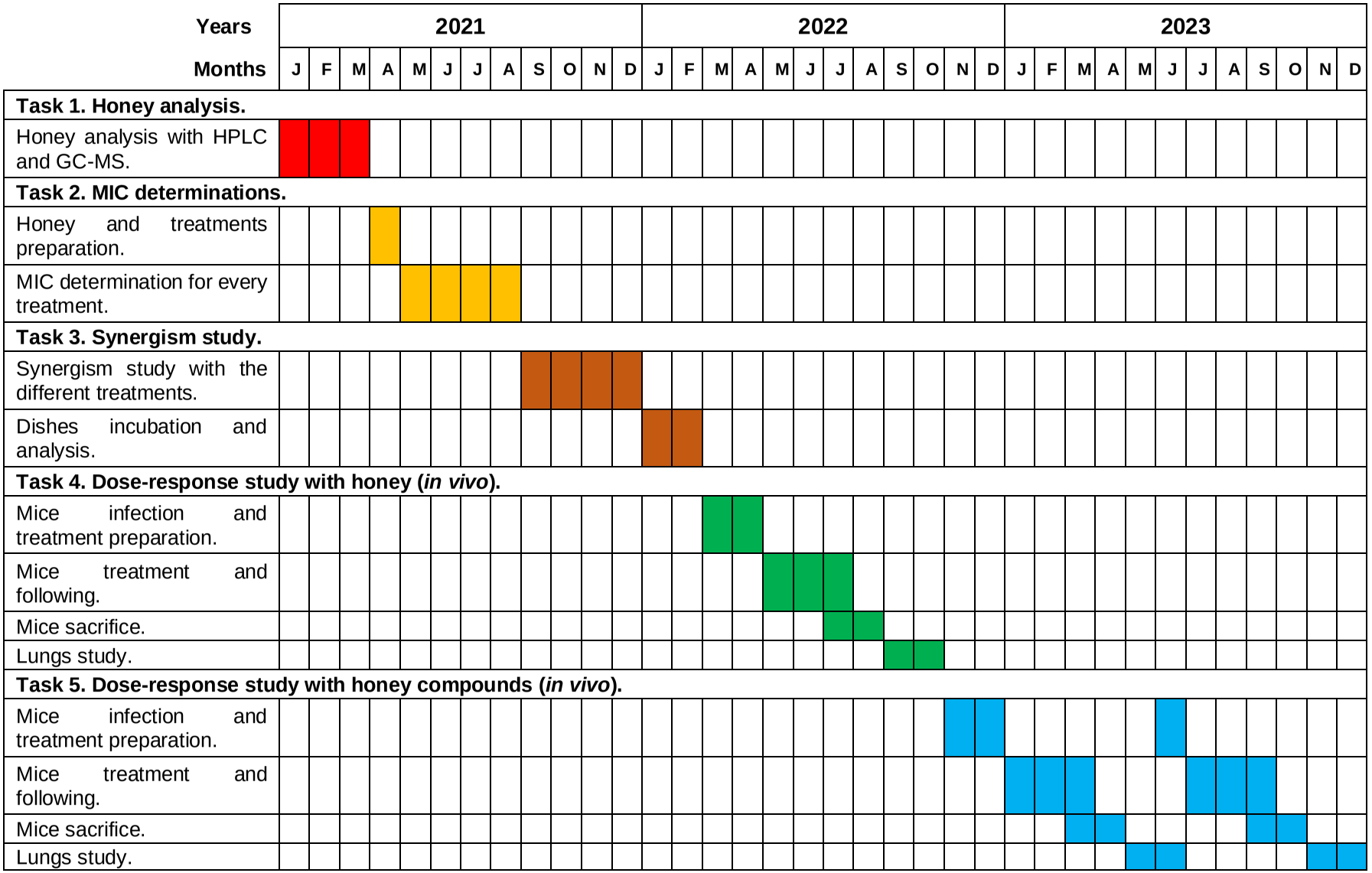
Table 5: mice distribution		
Groups	**Type of treatment**	**Nº samples (n)**
9	animals infected with TB and treated with antibiotics	10
10	animals infected with TB and treated with antibiotics + 1% honey solution	10
11	animals infected with TB and treated with antibiotics + 5% honey solution	10
12	animals infected with TB and treated with antibiotics + 10% honey solution	10
13	animals infected with TB and treated with antibiotics + honey solution at the effective concentration in the MIC test	10
14	animals infected with TB and treated with antibiotics + saline solution	10
		60

Task 5. Dose-response study of the combined treatment (antibiotics and selected honey components) in mice infected with tuberculosis resistant strain. This task is similar to that described in task 4 but using the selected components of honey that have anti-microbial effects. The experimental groups of this task will be previously infected by *M. tuberculosis* and will receive a combination of following treatments (*Table 6* and *figure 16*):

Table 6: mice distribution			
Groups	**Treatment**	**Concentration of the 2nd compound**	**Nº samples (n)**
15	antibiotics + MGO	1%	10
16		5%	10
17		10%	10
18		At the effective concentration in the MIC test	10
19	antibiotics + caffeic acid	1%	10
20		5%	10
21		10%	10
22		At the effective concentration in the MIC test	10
23	antibiotics + galangin	1%	10
24		5%	10
25		10%	10
26		At the effective concentration in the MIC test	10
27		1%	10

Table 6: mice distribution			
Groups	**Treatment**	**Concentration of the 2nd compound**	**Nº samples (n)**
28	antibiotics + pinocembrin	5%	10
29		10%	10
30		At the effective concentration in the MIC test	10
31	antibiotics + quercetin	1%	10
32		5%	10
33		10%	10
34		At the effective concentration in the MIC test	10
35	Antibiotics + DMSO in saline solution	1%	10
			250

CHRONOGRAM


Years
2021
2022
2023
Months
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
Task 1. Honey analysis.
Honey analysis with HPLC and GC-MS.
Task 2. MIC determinations.
Honey and treatments preparation.
MIC determination for every treatment.
Task 3. Synergism study.
Synergism study with the different treatments.
Dishes incubation and analysis.
Task 4. Dose-response study with honey (in vivo).
Mice infection and treatment preparation.
Mice treatment and following.
Mice sacrifice.
Lungs study.
Task 5. Dose-response study with honey compounds (in vivo).
Mice infection and treatment preparation.
Mice treatment and following.
Mice sacrifice.
Lungs study.

BUDGET

Table 7: budget	
Concepts	**Requested financial support**
STAFF	
Pre-doctoral contract for 3 years.	60,000 €
DURABLE EQUIPMENT	
CONSUMABLE	
Animals (purchase).	5,500 €
Animals (housing).	15,500 €
Honey purchase.	60 €
M. Tuberculosis strain (purchase).	3,000 €
Purchase of material and reagents to determine the components of the honey sample (HPLC, GC-MS).	12,000 €
Purchase of material and reagents for *in vitro* studies.	20,000 €
Purchase of antibiotics, and specific components of honey for *in vivo* studies.	3,000 €
Purchase of surgical material for tissue extraction.	1,000 €
Purchase of material and reagents for histological studies.	2,500 €
TRAVEL AND ALLOWANCES	
National and International Congress Attendance.	4,000 €
OTHER EXPENSES	
Scientific paper in open access.	2,500 €
Use of technical services.	25,000 €
SUBTOTAL	**154,060 €**
OVERHEAD COST (21%)	**32,353 €**
TOTAL REQUESTED	**186,413 €**

IMPACT

According the Global tuberculosis report (WHO 2019), millions of people continue to fall sick with TB every year with a 16% of mortality, affecting mainly the African, Asian and South American regions. In fact, 97% of the reported TB cases are from low and middle-income countries. This problem is aggravated with the drug-resistant TB crisis, representing 484,000 incident cases in 2018 and causing about 214,000 death the same year. The problem of this crisis is that, even with the current treatment, the success treatment it maintains low (WHO 2019).

In front situation, if the results of this preclinic study are favourable and it confirms the hypothesis proposed, the next step would be followed the clinical study phases to the human use of honey or honey compounds to benefit from its possible beneficial effects against RR and MDR TB infections. Therefore, the use of honey or honey compounds as a supplement treatment with the current antibiotic treatment could be applied in humans to reduce the morbimortality related with resistant strains in Morocco and around the world, improving the success treatment percentage and saving hundreds, or maybe thousands of lives every year. At the same time, this study could be considered as a pilot study and would be interesting to study if the honeys of different regions could have also this possible benefit effect against TB infections, with its health system consequences, with an increase of the treatment success against RR-TB and MDR-TB and therefore, with a decrease of the morbimortality. On the other hand, if the results are favourable, de socioeconomical impact could be amazing, with less young adult people infected and consequently more productivity of the people in working age (Adeyi et al 2007).

FEASIBILITY

This research project requires special protection facilities against pathogens, both for *in vivo* and *in vitro* studies. One of the research centers that have these facilities is the Severo Ochoa Molecular Biology Center of the Autonomous University of Madrid (UAM). For the execution of this project, the university institution will be contacted, and a scientific collaboration agreement will be established at the level of the research vice-rectors of both universities (UAM, UdG).

The research center has all the scientific devices described in the methodology of this project. To be able to use them, a budget item of 25,000 euros has been included.

The techniques described in the methodology section are standard techniques easily performed by a PhD student under the supervision of specialized technicians and by the members of the UdG research team (NEOMA Research Group). A budget item is requested for a predoctoral fellow.

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Annex 1: honey composition			
Comp.	**Examples**	**Measurement method**	**Changes in the concentration during storage**
Sugars (80%)	-Monosaccharides (75%): fructose (nd glucose (Kamal and Klein 2011). -Disaccharides (10-15%) (Kamal and Klein 2011).	Normal phase partition Liquid Chromatographic (LC) system (Kamal and Klein 2011).	-Sucrose concentration decreases 14% in honey stored at 4ºC, and in 79% in a room temperature (20ºC). -Fructose concentration increases 4% at 4ºC and 7% at 20ºC. -Glucose concentration increases 1.1% at 4ºC and 8.8% at 20ºC (Rybak-Chmielewska 2007).
Proteins (0.2-1.6%)	-Aminoacids: proline (the most abundant: 50-85%; a minimum value of 180mg/kh of proline is accepted as the limit value for pure honey), alanine, tyrosine… (Hermosín et al 2003). -Enzymes: like lisozyme and glucose oxidase (produce hydrogen peroxide). -Antimicrobial peptides (AMPs): apidaecin, abaecin, bee defensin-1 (or royalosin), cecropin and hemenoptecin.	Waters 2690 liquid chromatograph, fitted with a Novapack C18 column (4 mm, 300x3.9 mm) (Hermosín et al 2003).	-Aspartic acid, β-alanine and proline increases during the first 6 months of storage. -Proline concentration decreases after 6 months of storage. -Aspartic acid decreases after 12 months of storage. -Concentration of free amino acids decreases after 9 months of storage. -No significant differences are observed in 15 months storage (Iglesias et al 2006).
Organic acids (0.57%)	Gluconic acid (the predominant one, originated from glucose oxidase also), citric acid, among others (Mato et al 2006).	Rapid capillary zone electrophoresis (CZE) method with direct ultraviolet (UV) detection (Mato et al 2006).	-The first 15 months of storage, the free acidity remained practically constant, with a slight tendency to increase. -After 20 months, there is an increase in free acidity, because honey sugars and alcohols transform into acids by the action of honey yeasts (Cavia et al 2007).
Minerals (0.04-0.2%)	Potassium (30% of the total mineral content), magnesium, calcium, iron, phosphorous, sodium and manganese (Alqarni et al 2014).	Atomic absorption spectrophotometer (Model 3300, MS-DOS, PerkinElmer Inc., USA) (Alqarni et al 2014).	-Minerals are indestructible and are not subject to degradation by exposure to heat, light, oxidizing agents, extreme pH values or other factors (Fennema 2000.

Annex 1: honey composition			
Components	**Examples**	**Measurement method**	**Changes in the concentration during storage**
Vitamins (small accounts)	Vitamin B complex (B1, B2, B3, B5, B6, B8 and B9) and vitamin C (Leon et al 2013).	RP-HPLC equipment (Ciulu et al 2011, Leon et al 2013).	-Vitamin C is very vulnerable to chemical and enzymatic oxidation and has an accelerated rate of change due to various factors such as light, oxygen or heat (León-Ruiz et al 2013). -Hydrogen peroxide causes loss of vitamins with the oxidation of ascorbic acid Ciulu et al 2011).
Phenolic compounds	-Flavonoids (characterized by the presence of an x-phenyl-1,4-benzopyrone backbone, where x = 2, 3): chrysin, galangin, hesperetin, isorhamnetin, kaempferol, luteolin, myricetin, naringenin, pinobanskin, pinocembrin and tricetin (Ciulu et al 2016). -Non-flavonoids (phenolic acid; they act as antioxidants) (Ciulu et al 2016): ▪ Hydroxybenzoic acids: ellagic, gallic hydroxybenzoic, salicylic, syringic, and vanillic acid. ▪ Hydroxycinnamic acids: caffeic, coumaric and ferulic acid.	Shimadzu GC-MS-2010 (Alotibi et al 2018, Hegazi and Abd El-Hady 2009).	-Polyphenols can be easily oxidized to quinones, and perform a key role in the interaction with proteins. These interactions are intensified when honey are stored at high temperatures, by modifying the protein structure and size, leading to covalent bonds between proteins and quinones (Brudzynski et al 2013). -There is a decrease in the concentration of some phenolic compounds, like galangin, after pasteurization (Escriche et al 2014). -The flavonoid glycosides with a free hydroxyl in position 3 are sensible in the presence of slight oxidizing agents such us hydrogen peroxide (Truchado et al 2008).
Volatile compounds (>400)	Cis-rose, diketones, hexanal, heptanal, octane, 17-pentatriacontene sulphur compounds …	Gas chromatography-mass spectrometry (GCMS) (Radovic et al 2001).	-Some volatile compounds decrease across the time, like de 17-pentatriacontene, that disappear in 3 months (Moreira et al 2010). -Other ones increase with storage time, like octanal (Kaškoniene et al 2008).