Exploring bioactivities in *artemisia annua L.* extracts: extraction method and solvent screening

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Abstract

Artemisinin, the major active ingredient in *Artemisia annua L.*, has been used as an antimalarial ingredient and is gaining popularity for its antiviral characteristics. Furthermore, some recent articles revealed that this important molecule could be beneficial against the Sars-CoV-2 virus, brain tumours, covid-19, and other diseases.

Recently, there has been a push to enhance artemisinin extraction in terms of energy costs and solvent efficiency. Therefore, various innovative procedures, such as supercritical fluid extraction, pressurized solvent extraction, microwave-assisted extraction, and ultrasound-assisted extraction, are being studied and combined with several innovative solvents.

Most extraction solvents have significant toxicity, flammability, and limited selectivity, and large-scale use of these solvents has a negative impact on the environment. As a result, new extraction processes less harmful to the environment are being developed. For example, some green solvents, such as ionic liquids and deep eutectic solvents, have been proposed for artemisinin extraction. The concept of green chemistry seeks to eliminate or reduce harmful compounds in chemical applications.

This report will include an evaluation of alternate extraction techniques, considering artemisinin yield, in addition to examining the potentialities of green solvents after a preliminary study with ethanol 80/20. It is essential knowledge to select the method and solvents to prepare extracts from *Artemisia annua L.* that will be studied in terms of several bioactivities, including antimalarial.

Keywords:

Artemisinin, *Artemisia annua L.*, extraction methods, antimalarial, green solvents

Resumo

A artemisinina, o principal ingrediente ativo da *Artemisia annua L.*, tem sido usada como um ingrediente antimalárico e está ganhando popularidade por suas características antivirais. Além disso, alguns artigos recentes revelaram que esta importante molécula pode ser benéfica contra o vírus Sars-CoV-2, tumores cerebrais, covid-19 e outras doenças.

Recentemente, houve um impulso para melhorar a extração de artemisinina em termos de custos de energia e eficiência de solventes. Portanto, vários procedimentos inovadores, como extração de fluido supercrítico, extração de solvente pressurizado, extração assistida por microondas e extração assistida por ultra-som, estão sendo estudados e combinados com vários solventes inovadores.

A maioria dos solventes de extração tem toxicidade significativa, inflamabilidade e seletividade limitada, e o uso em larga escala desses solventes tem um impacto negativo no meio ambiente. Como resultado, novos processos de extração menos prejudiciais ao meio ambiente estão sendo desenvolvidos. Por exemplo, alguns solventes verdes, como líquidos iônicos e solventes eutéticos profundos, foram propostos para a extração de artemisinina. O conceito de química verde visa eliminar ou reduzir compostos nocivos em aplicações químicas.

Este relatório incluirá uma avaliação de técnicas alternativas de extração, considerando o rendimento de artemisinina, além de examinar as potencialidades dos solventes verdes após um estudo preliminar com etanol 80/20. É essencial o conhecimento para selecionar o método e solventes para preparar extratos de *Artemisia annua L.* que serão estudados em termos de várias bioatividades, incluindo antimalárico.

Palavras-chave:

Artemisinina, *Artemisia annua L.*, métodos de extração, antimalária, solventes verdes

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1. Scope and objectives

This study aims to extract biological molecules from natural matrices, namely artemisinin from *Artemisia annua L.*, which contain high quantities of this component. Artemisinin is used to treat malaria cases that are resistant to other treatments that have been used in the past to treat the disease. At this moment, the most common commercial sources of artemisinin are field-grown leaves and blooming tops of *Artemisia annua L.*, which vary seasonally and somatically. Today, the search for increased efficiency is driven by two trends: searching novel species with higher yields and the continued research and implementation of genetic improvement methods.

The most time-consuming step(s) for the sample under test during active plant component determination is sample preparation. Traditional solid-liquid (Soxhlet) extraction of the herb sample is undertaken in certain situations, which is time-consuming. As a result, improved extraction, such as ultrasound-assisted extraction, supercritical fluid extraction, pressurized solvent extraction, and microwave-assisted extraction, have been developed. Besides, several techniques to quantify artemisinin in the extracts have been documented. Still, most of them are insufficiently sensitive, provide unreliable findings, or are difficult to apply in everyday analyses, needing further developments.

The purpose of this study is to develop a method for obtaining extracts containing artemisinin that performs better on several bioactivities than those obtained using conventional extractive solvents, and thus are more likely to exhibit increased therapeutic activity. The new procedure and technique are expected to be significantly safer and more environmentally friendly (having lower toxicity and potential for biodegradability after use).

Therefore, the current project intends to provide a procedure that allows for the extraction of artemisinin in significantly less time and more efficiently than the extraction techniques currently in use for this compound. Extraction will be carried out over samples of dried *Artemisia annua L.* leaves by microwave-assisted extraction, using different solvents and varying other process variables. Some bioactivities will be tested, including antimalarial activity and the composition of artemisinin-based compounds measured.

2. Introduction

2.1. *Artemisia annua* L., artemisinin, and its derivates

The plant *Artemisia annua* L. (*A. annua*; sweet wormwood; Chinese wormwood) belongs to the Asteraceae family (formerly Compositae). It is a Chinese native that was first discovered in the steppes of Chahar and Suiyuan Provinces. It is also found and/or farmed in East Africa, the United States, Russia, India, Brazil, and a few other countries (Li, Ying; Wu, 2003; Sushil & Suchi, 2005). This plant has been also used in traditional Chinese medicine to treat of various diseases since ancient times. *A. annua* has been officially recognized as a medicinal plant and listed in *Chinese Pharmacopeia.* Its ethnopharmacology study points out that this species was recommended to treat bone steaming and fever, tuberculosis, lice, wounds, scabies, dysentery, acute convulsions, haemorrhoids, pain and swelling on tooth, pus, nasal polyps, eyesight improving, haemostasis and analgesic activities (Feng et al., 2020).

Additionally, this species is known for producing artemisinin, a sesquiterpene lactone compound, which is applied to treat of malaria (Lyu et al., 2021). Artemisinin is sesquiterpene lactone compound, isolated for the first time in 1971 from the Chinese medicinal herb *Artemisia annua* (Meshnick, 2002). Artemisinin and its derivatives artemether, artesunate, dihydroartemisinin and arteether (Dhingra et al., 1999; Lapkin et al., 2006), including the most recent addition to the family of these compounds, artemisone, is the precursor of relevant compounds (Haynes et al., 2006; Tu, 2016). This family of chemicals (**Figure 1**) is important in malaria treatment because of its quick activity against the most common Plasmodium falciparum malaria and associated brain complications.

Artemisinin is exclusive compound from *A. annua*, which it has been looked for in other Artemisia species multiple times without success (Brown, 2010; Klayman et al., 1984). The artemisinin concentration of *A. annua* varies greatly, ranging from 0.01% to 1% (mass percentage relative to the dry plant) depending on the variety, and even reaching 1.4 percent in certain cultivated strains.

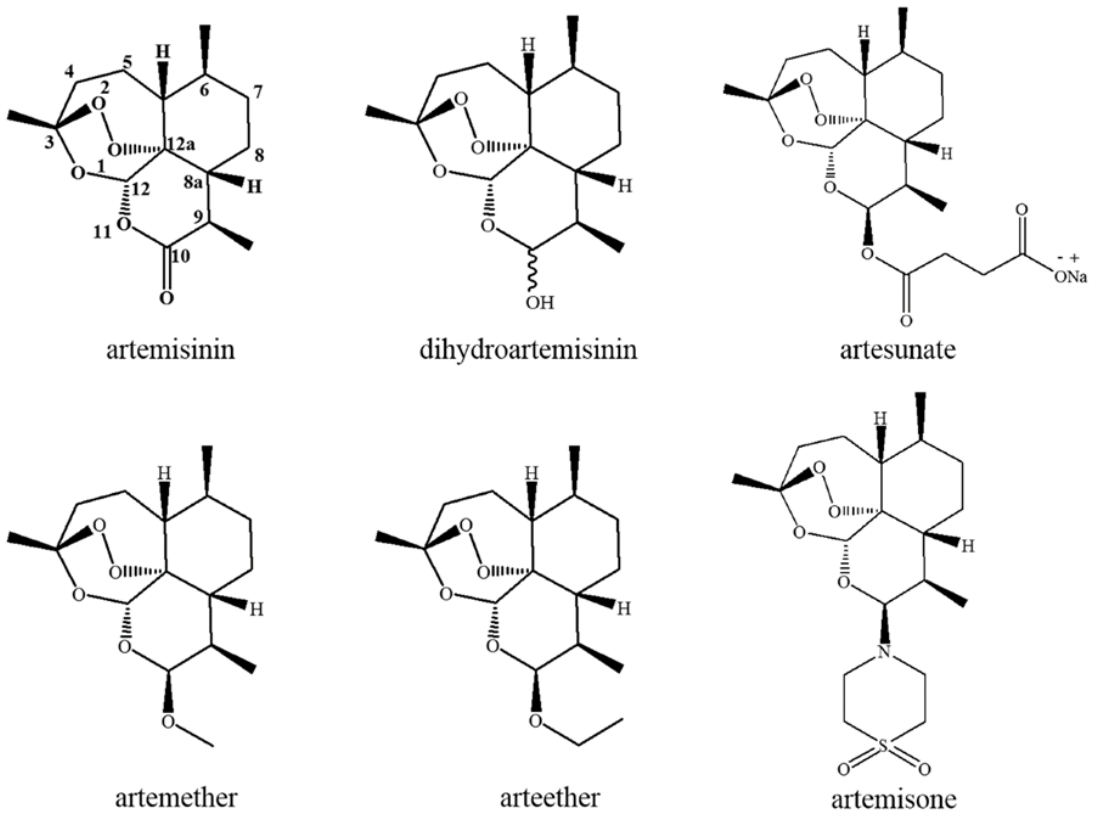


Figure 1 : Artemisinin derivatives using in malaria treatment.

In addition to its antimalarial activity, A annua shows anti-parasitic activity against other parasites. Its extracts have been shown to be effective against Toxoplasma gondii, Leishmania and Acanthamoeba infection (de Oliveira et al., 2009; Derda et al., 2016; Islamuddin et al., 2014).

Essential oils from A annua have been investigated for their strong antimicrobial potential. The essential oil of the plant includes a variety of components with economic potential, such as camphor, artemisia alcohol, ketone, borneol, and many others (Willcox et al., 2004). The main isolated constituents from its leaves and seeds showed remarkable anti-microbial activities in gram-positive bacteria, gram-negative bacteria, and fungi (Bilia et al., 2014).

The anti-inflammatory properties of the artemisinin enriched extracts were evaluated with high success in an in vitro study on lipopolysaccharide (LPS)-activated nitric oxide (NO), prostaglandin E2 (PGE2), and pro-inflammatory cytokine (IL-1β, IL-6, and IL-10) production in RAW 264.7 macrophages (W. S. Kim et al., 2015). In addition, A annua reported a reduction in pain, stiffness, and functional limitation in clinical trials (Stebbings et al., 2016).

Crude extracts of A. annua is said to have one of the highest antioxidant capacities as adjudged by its ORAC (oxygen radical absorbance capacity) value (Zheng and Wang, 2001) which is a property directly related to the total phenolic content of a plant (Ferreira et al., 2010).

It has been reported that artemisinin and its derivatives have antitumoral properties through cancer cell growth cycle arrest, promoting apoptosis, and inhibiting the angiogenesis of tumour. *A. annua* showed anti-cancer activities against various cell cancer lines and in human studies (Feng et al., 2020b).

Finally, the lipid content of *A. annua* was recently studied, as well as the therapeutic effects of lipophilic extracts on the skin (Ornano et al., 2016). Although more than artemisinin chemicals of the plant are currently used commercially to make cosmetics and flavourings, there is substantial potential to expand the commercial value of *A. annua* production by more comprehensively using other biomolecules, i.e., extending the biorefinery idea (Lynd et al., 1999).

2.2. Malaria disease and treatment with artemisinin and derivates

Malaria is a severe disease that affects more than 200 million people and causes a death rate between 0.3 and 2.2% globally and more than 11% in tropical climates (White et al., 2014; WHO, 2021). The disease is caused by a parasite protozoon belonging to the group of Plasmodium species, which is transmitted to humans by an infected Anopheles mosquito. The Plasmodium life cycle (**Figure 2**) has two stages: sexual in mosquitos and asexual in vertebrates´ host. After bite of infected mosquito, Plasmodium sporozoites reach to the host liver and invade the hepatocytes, forming merozoites and beginning the blood infection stage where lead to the rupture of erythrocytes (Talapko et al., 2019).

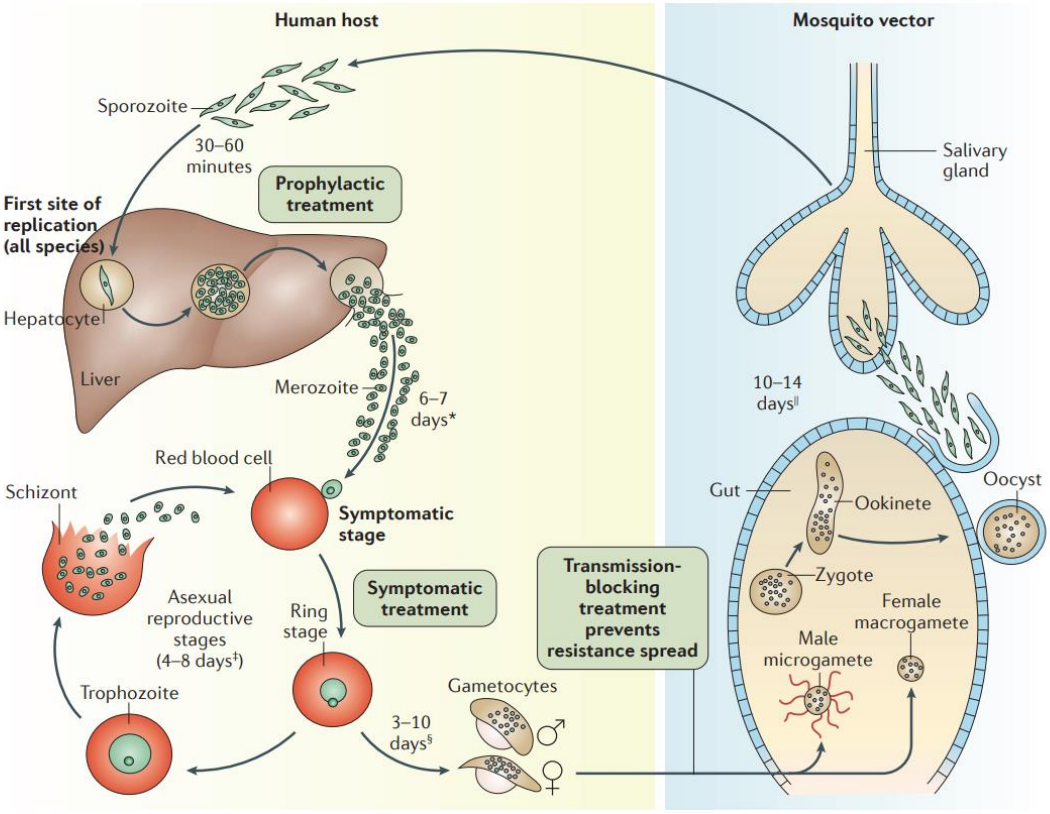


Figure 2 : Plasmodium life cycle modified from Phillips and collaborators (Phillips et al., 2017).

One of the most major medical achievements of the twentieth century was the discovery and use of artemisinin-based components, which changed the global treatment of malaria (Ramutton et al., 2012). **Figure 2** compiles the chemical structures of such compounds.

Because artemisinin's structure differs from that of any other known antimalarial, it is likely to have a unique mode of action. Its pharmacodynamic and pharmacokinetic characteristics, as well as its strong capacity for *Plasmodium* elimination, have raised many expectations for this new class of antimalarial drugs(Li et al., 2018). Synthetic chemists were the first to discover its mechanism (scheme presented in **Figure 3**), demonstrating that the endoperoxide bridge was required for antimalarial action (Brossi et al., 1988; Meshnick, 2002).

During 1991, Meshnick and colleagues demonstrated that artemisinin interacts with intra-parasitic heme, implying that intraparasitic heme or iron might activate artemisinin as harmful free radicals within the parasite (Meshnick et al., 1991). The malaria parasite is high in heme-iron, which is produced through the proteolysis of host cells' hemoglobin (Rosenthal & Meshnick, 1996). This may explain why artemisinin is poisonous to parasites only.

Artemisinin's selective toxicity might be owing to the production of covalent adducts with parasite components, which is mediated by free radical intermediates. Hemitis is a significant alkylation target. In parasite cultures treated with therapeutic amounts of artemisinin derivatives, artemisinin and hematoma adducts have been discovered (Hong et al., 1994). When artemisinin is incubated in a cell-free solution, it forms covalent bonds with the heme, and the same artemisinin adducts appear to develop in parasites treated with artemisinin.

The structure of an artemisinin-porphyrin adducts, as well as an artemisinin-induced porphyrin cycle degradation product, has been determined (Anne Robert & Bernard, 1997). Endoperoxides that are active react with porphyrins whereas inactive endoperoxides do not, suggesting that this interaction is vital in parasite eradication (Cazelles et al., 2002). As a result, artemisinin derivatives operate as both an activator and a target.

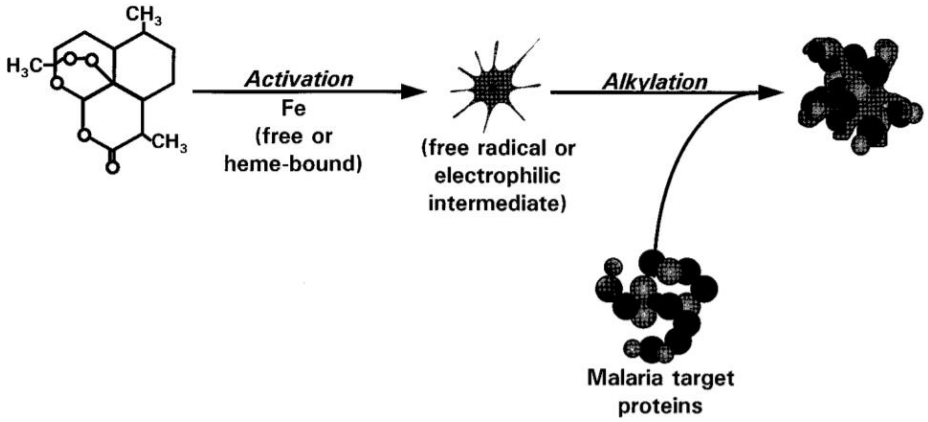


Figure 3 : Mode of action of artemisinin and derivates (Meshnick et al., 1996).

2.3. Challenges in artemisinin recovery

Artemisinin is insoluble in water and oil, but it is soluble in a variety of aprotic solvents such as chloroform, acetone, and alcohols. The parenteral preparation is challenging to compound due to the solubility issues. With a melting temperature of 156-157°C, and just a crystalline form has been found. It is exceptionally stable for an endoperoxide, since it can only decompose at temperatures of 190°C or above (Li & Zhou, 2010).

Solvents are often classified as polar or nonpolar. "Like Dissolves Like" is a common rule of thumb. This means that polar solvents dissolve ionic and ionizing covalent molecules, whereas nonpolar solvents dissolve nonpolar covalent compounds. This is a somewhat basic viewpoint since it disregards numerous solvent-solute interactions, yet it is a good rule of thumb. The structure of artemisinin, a sesquiterpene lactone endoperoxide molecule, implies that it is weakly polar and hence should be relatively soluble in a medium polarity solvent (Nti-Gyabaah et al., 2010).

Thus, traditional artemisinin extraction methods involve the use of hydrocarbons such as hexane or petroleum ether. These solvents are highly volatile, flammable, and not friendly for biochemical applications. In addition, this process gives a low yield due to low solubility of artemisinin in hexane, its decomposition in hot conditions, and losses during purification steps. Therefore, searching for new solvents with high artemisinin solubility and selectivity seems necessary while establishing a green and sustainable approaches.

2.4. Green solvents

Green chemistry principles are a collection of criteria for assessing a chemical reaction's or process's environmental cost and efficiency (Anastas, P. T.; Warner, 1998; Tang et al., 2008). These themes include waste minimization, the avoidance of toxic solvents and reagents, the elimination of unnecessary operations, and energy conservation. Their application has been particularly effective in the pharmaceutical business, since pharmaceutical operations usually include many stages, each of which may contain dangerous compounds and generate waste.

Thus, some alternative extraction methods such as ionic liquids, deep eutectic solvents, or hydrotropes have emerged for the extraction of artemisinin and its derivates.

2.4.1. Ionic Liquids

Organic solvents, which have numerous key problems such as high volatility, flammability, and toxicity, have created a lot of interest and rising demand for ionic liquids (IL) as an alternative. Non-flammability, thermal stability, low vapor pressure, and, most importantly, exceptional tunability and synthetic flexibility are some of the qualities of interest in ILs. These solvents have long been known as "designer" solvents that are mostly seen as environmentally friendly (Pacheco-Fernández & Pino, 2019).

2.4.2. Deep Eutectic Solvents

Nonetheless, their "green" character has been widely questioned in recent years due to their low biocompatibility and biodegradability (Espino et al., 2016; Paiva et al., 2014). Deep Eutectic Solvents (DES) have been progressively developing as a green alternative to IL (Florindo et al., 2019). DES, which are considered sometimes a subclass of IL, are made by combining chemicals in the solid state to generate a combination having a melting point much lower than that calculated assuming ideal solution behavior.

This is owing to the formation of intermolecular hydrogen bonds between the hydrogen bond acceptor (HBA) and the hydrogen bond donor (HBD). DES and IL share several physicochemical features (high viscosity, low volatility, non-inflammability, chemical and thermal stability) (Mbous et al., 2017).

2.4.3. Hydrotropes

To further respect the principles of green chemistry, a promising class of compounds for this purpose are hydrotropes. Hydrotropes are a class of compounds that improve the solubility of hydrophobic compounds in aqueous solution (Subbarao et al., 2012a). They can stabilize aqueous solutions and adjust their viscosity (Srinivas et al., 1997; Subbarao et al., 2012a). Hydrotropics are amphiphilic substances characterized mainly by a hydrophilic functional group (anionic group) attached to another group, usually a hydrophobic aromatic cycle (cationic group), in which the interaction is limited to dispersion contributions (Friberg et al., 2004). Despite their amphiphilic structure, these compounds do not function as surfactants because the hydrophobic fraction is very small. Classes of hydrotropics may be anionic, cationic or non-ionic amphiphiles.

2.4.3.1. Hydrotropes involved in improving water solubility

Due to a large number of additives, oversized in aqueous solubility, various lipophilic compounds were attributed. Now, the use of concentrated aqueous solutions of sodium citrate, sodium acetate, sodium benzoate, sodium salicylate, sodium ascorbate, urea and niacinamide has been attributed to a large number of low water soluble drugs to accelerate their aqueous solubility (Tripathi et al., 2023a). **Figure 4** shows the main factors for effective drug solubilization.

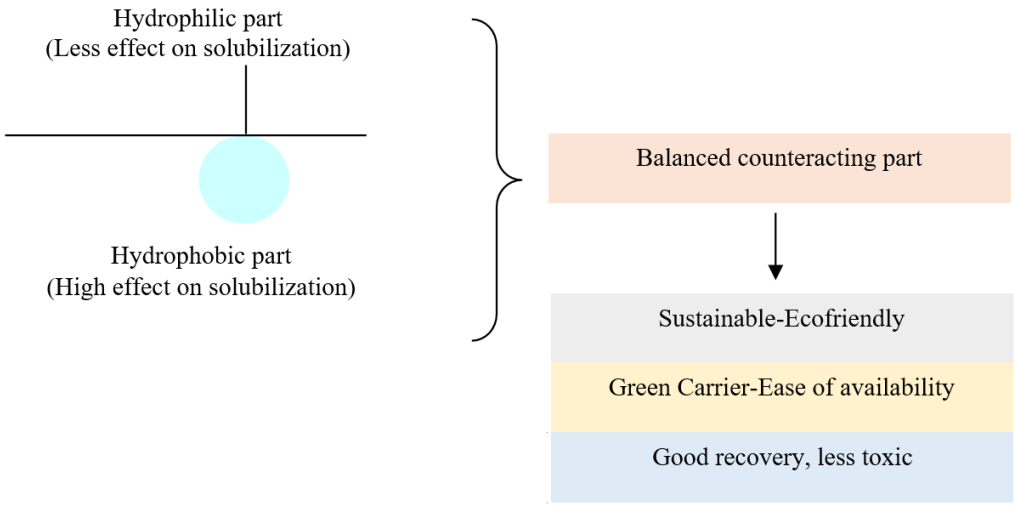


Figure 4 : Important key factors for effective solubilization by hydrotropes (Tripathi et al., 2023b).

2.4.3.2.Hydrotropy mechanisms

Various theories have been advanced and experimental studies have been conducted to explain the mechanisms behind hydrotropy. The evolution of hydrotropic mechanisms, from the point of view of various researchers, has been the subject of extensive studies which can be summarized in three main approaches (Balasubramanian et al., 1989; Gaikar & Sharma, 1993). Mainly, we know that hydrotropics and solutes interact strongly, which leads to the formation of hydrotropic-solute complexes (Da Silva et al., 1999).

The following approach indicates that hydrotropics modify the structure of the solvent by inserting themselves into the structure of liquid water; therefore, they are known as "structure maker" or "structure breakers" (Kim et al., 2010). The final mechanism is the most widely accepted, whereby hydrotropics have the ability to associate and form aggregates to act as micelles above a particular concentration (Subbarao et al., 2012a). Hydrotropic agents include a wide range of molecules, suggesting that more than one mechanism could describe hydrotropy.

2.4.3.3. Hydrotropic structure

Due to the presence of hydrophilic and hydrophobic parts in the structure, hydrotropics are often compared to surfactants. The predominant characteristic that distinguishes the hydrotropics from the surfactants is the short hydrophobic part of the hydrotropics, which is too small to induce the formation of micelles observed with surfactants (Nagarajan, Wah Heng, Galanakis, Nagasundara Ramanan, Raghunandan, et al., 2016).

As a promoter of solubility, hydrotropics are also known as surfactants, curing agents or co-solvents. Hydrotropia is the phenomenon that occurs when hydrotropic salt initiates the solubility of poorly soluble or water-insoluble solutes (Nagarajan, Wah Heng, Galanakis, Nagasundara Ramanan, Eshwaraiah Raghunandan, et al., 2016). **Figure 5** shows the mechanism of hydrotropics.

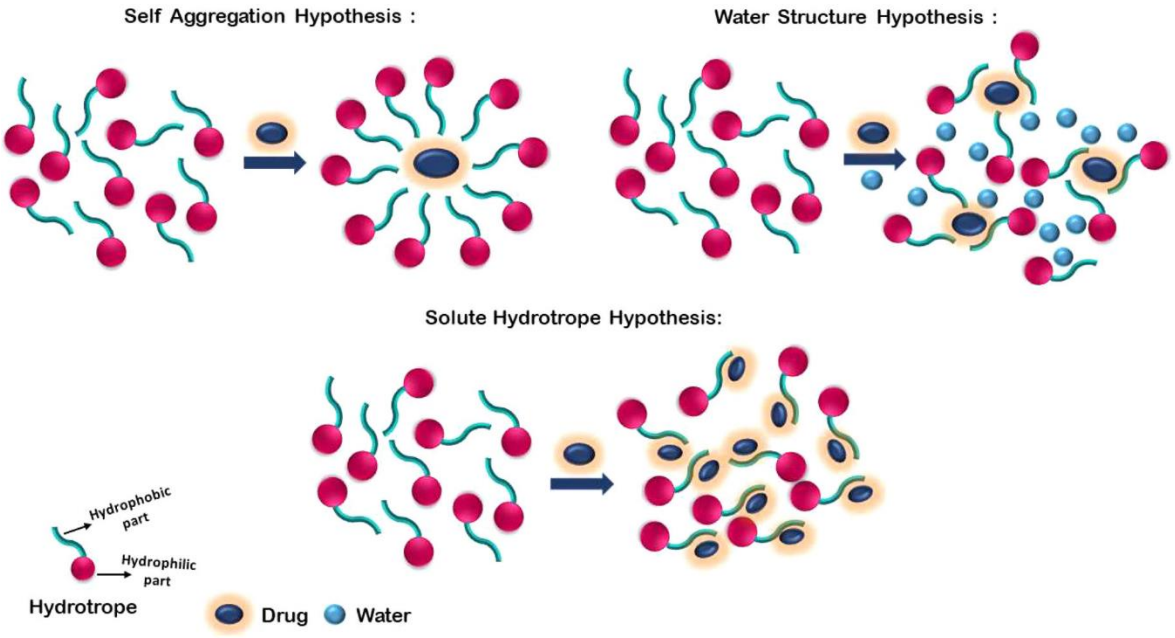


Figure 5 : Mechanisms of hydrotropic compounds solubilization (Paul et al., 2021).

2.4.3.4. Interaction of hydrotrope and plant matrix

As a surface-active molecule, hydrotropics first adsorb to the surface of the cellulosic cell walls of the plant matrix (Mishra & Gaikar, 2004). Reducing surface forces at the interface improves the wettability of the cell wall and then facilitates the penetration of hydrotropic molecules into the cellulosic structure of plant matter. The penetration of hydrotropics into the cell wall and membrane structure can induce molecular disorganization and change membrane permeability by dissolving some cell wall components. Hydrotropics are believed to be able to disrupt fluid lamellar structures, which are reminiscent of cellular membrane structures (Srinivas et al., 1997).

By inducing a change in the molecular organization of the cell membrane, a hydrotropic changes the permeability of the membrane and allows the desired phytochemical to be easily accessible by the hydrotropic solvent. More hydrotropic molecules are available to disrupt the cell matrix to a greater extent at higher concentrations of hydrotropic, and thus the permeability of the biomatrix is increased (Raman & Gaikar, 2002).

In addition to the mechanism of penetration of the hydrotropic, there are several other characteristics that greatly influence the rate of extraction of phytochemistry: first, the affinity of hydrotropic molecules to reduce the surface tension of plant cell walls by surface-active forces (Kumar et al., 2014). Another factor would be the ability to associate with the hydrophobic halves of the molecule at a given concentration.

The solubilization of a water solute will be inhibited mainly due to the presence of a hydrogen bond in the solute. The interaction of the hydrotropics with the sulphonyl and carbonyl groups, in this case, acts as a hydrogen acceptor and, finally, greatly increases the hydrotropic-solute aggregation. In contrast, hydrotropics of the amino or hydroxyl group are considered a potential hydrogen donor (Narang & Mahato, 2010).

In most scenarios, the incorporation of aromatic sulphonates, such as cumene sodium sulfonate, provides better solubility than aliphatic hydrotropics, such as urea (Kadam et al., 2012). In addition to the hydrogen bond, there are other aspects that could contribute to the extraction rate, such as the intermolecular interaction and the π-π piling of the hydrotropic with the cell wall of the plant (Narang & Mahato, 2010). The nature of the solute, such as its size and the presence of hydrophilic groups, such as –OH, $–NH_2$ and –CHO, also significantly improves the solubility of the compound in hydrotropical solvents (Sadvilkar et al., 1995).

2.4.3.5. Comparison of hydrotropic extraction with other extraction techniques

As a universal green solvent, the lack of driving forces to solubilize fewer non-polar compounds has limited the use of water for extraction or requires partitioning with a bio-based solvent such as ethanol (Gu & Jérôme, 2013; Singh et al., 2015). Due to the polarity of the solvent, the applicability of this method requires mainly assisted extraction techniques such as soxhlet and ultrasound to improve solubility.

Since ethanol is a volatile medium, a large amount of solvent is often required for extraction (Sadvilkar et al., 1995), while the products obtained were of low purity and crystalline quality. Supercritical fluid is a green solvent widely accepted for application. However, the introduction of an expensive instrument and the inability to solubilize heavy-duty compounds make this solvent unattractive (Azmir et al., 2013; Singh et al., 2015). In most cases, ionic liquids (ILs) are considered green solvents because of their extraordinary characteristics, including high conductivity and thermal stability (Harde et al., 2014).

However, there is another perception that these criteria are considered insufficient to classify this solvent as green because of the level of toxicity imposed by these solvents (Gu & Jérôme, 2013). Based on comparable aspects, hydrotropical solvents are compared to available green solvents, as shown in **Table 1**. To our knowledge, most extractions using hydrotropic solvents were carried out using the conventional agitation, agitation and homogenisation method. Many assisted extraction techniques can be applied to improve the solubility of compounds in hydrotropical solvents. **Table 2** shows the possible techniques.

In addition, MAE offers several advantages such as much less extraction time, much less temperature, improved product purity, and increased efficiency. The beneficial effects of the MAE are due to its unique heating system. The direct interaction of microwaves with the free water molecules present in the glands and vascular systems, causes a considerable increase in the uninternal pressure inside the plant cell. It is due to the evaporation of the internal moisture which leads to the subsequent rupture of the plant tissue and the release of the active compounds in the organic solvent. Therefore, MAE is an interesting alternative to conventional extraction methods, especially in the case of extractions of thermolabile compounds (Dhobi et al., 2009).

The hydrotropic-microwave assisted extraction is affected by several process parameters such as microwave power, time, solid liquid ratio, hydrotropic concentration and agitation speed. Process optimization is a necessity in order to determine the most influential variable and determine the optimal process parameters. When many factors and interactions influence the desired response, Response Surface Methodology (RSM) is an effective tool for optimizing the process (Nuttawan Yoswathana, 2013).

Table 1 : Comparison of hydrotropic solvent with available green solvents applied for extraction of hydrophobic phytochemicals.

Solvent	Example	Solvent Polar	Eco-Friendly	Non-Toxic	Flammable	Reusability	Operating Cost	Selectivity	Refs.
Hydro-tropic solvent	NaCS, NaPTS, NaSal, U, NaNBBS, NaBMGS,NaXS	Amphi-philic (both anion and cation)	Yes	Yes	No	Yes	Economical	High selectivity (hydrophilic and hydrophobic compound)	(Hodgdon & Kaler, 2007; Subbarao et al., 2012b)
Water	H_20	Very polar	Yes	Yes	No	Yes	Most economical	Non-selective for the hydrophobic compound	(Gu & Jérôme, 2013)
Bio-based solvent	Ethanol	Polar	Yes	Yes	No	Yes	Economical	Low selectivity for hydrophobic compound	(Gu & Jérôme, 2013; Sultana et al., 2009)
Super-critical fluid	Carbon dioxide	Non-Polar	Yes	Yes	No	Yes	Very Expensive	High selectivity (hydrophilic and hydrophobic compound)	(Azmir et al., 2013; Singh et al., 2015)
Ionic liquid	Imid-azolium cations (paired with various organic and inorganic anions	Amphi-philic (both anion and cation)	Yes	No	No	Yes	Very Expensive	High selectivity (hydrophilic and hydrophobic compound)	(Gu & Jérôme, 2013; Harde et al., 2014)

NaCS: Sodium cumenesulfonate, NaNBBS: Sodium n-butyl benzene sulfonate,NaPTS: Sodium p-toluene sulfonate, NaBMGS: Sodium butyl monoglycol sulfate, NaXS: Sodium xylene sulfonate, NaSal: Sodium salicylate, U: Urea.

Table 2 : Principle, advantages and disadvantages of different assisted methods of extraction.

Method	Principle	Advantage	Disadvantage	Refs.
Soxlet extraction	The plant material is refluxated with a hydrotropic solvent. After refluxation, the extract is concentrated by evaporation of the solvent.	Continuous contact with fresh solvent.	- Longer extraction time. - Evaporation of water leaves. hydrotropic salt with the extract. Therefore, an additional step is required to separate the hydrotropic salt from the extract. - Product recovery because precipitation is not applicable because the final product is concentrated. - Thermal degradation during extraction only at the boiling point of the solvent.	(M. Prado et al., 2014; Singh et al., 2015)
Ultrasonic-assisted extraction (UAE)	Sound waves at a frequency greater than human auditory range disturb the plant matrix. The solvent enters the solid matrix and releases the extract.	Simple and shorter extraction time, lower liquid-to-solid ratio. The product can be recovered as a precipitate.	- Heat: thermal degradation of phytochemicals. - Modification of phyto-chemical bioactivity.	(Misra et al., 2020; Singh et al., 2015)
Microwave assisited extraction (MAE)	The humidity of the cell is evaporated by the heat of the microwave. The pressure formed in the cell changes the porosity of the cell and the migration of hydrotropic solvents into the cell.	Simple and shorter extraction time, lower liquid-to-solid ratio. The product can be recovered as a precipitate.	- Heat: thermal degradation of phytochemicals. - Modification of phyto-chemical bioactivity.	(M. Prado et al., 2014; Misra et al., 2020)
Pressurized Liquid Extraction (PLE)	Uses a high pressure (50-200°C) hydrotropic solvent to replace water or organic solvent for extraction.	Selective extraction can be adjusted by changing physical parameters. High efficiency.	- Heat: thermal degradation of phytochemicals. - High energy consumption. - Product recovery because precipitation is not applicable because the final product is concentrated. - Alteration of phytochemical bioactivity.	(Misra et al., 2020; Strati & Oreopoulou, 2014)

2.5.Extraction techniques

Traditional methods for recovering bioactive chemicals from plant materials included maceration, which relied on the leaching of molecules from solid material (Naviglio et al., 2019). Maceration improves mass transfer and solubility of compounds by combining solvents with heat and/or agitation. Rising public acknowledgment of the value of natural substances, as well as increased public awareness of sustainable development and environmental preservation, have fueled intensive research in recent decades to porpose new extraction techniques.

'Green' extraction techniques, such as pressurized liquid extraction, supercritical fluid extraction, ultrasound-assisted extraction, deep eutectic solvent-assisted extraction, cold plasma-assisted extraction, microwave-assisted extraction, enzyme-assisted extraction, and electrical technologies, require less time, energy, and solvent and thus align with sustainable development strategies. These innovative extraction technologies were created to address constraints associated with traditional extraction methods (Garcia-Vaquero et al., 2020).

In the following sections some of these methods are briefly reviewed to show their usefulness in the area of biomolecules extraction from biomass.

2.5.1. Ultrasound-assisted extraction

The ultrasound extraction method uses ultrasonic vibration to help the dissolution of compounds present, for instance, in plant materials. The mechanical waves that are conveyed through the liquid in solid-liquid extractions using this approach will disorganize the particles longitudinally while the sound will do sequential compressions and distensions (F. Chemat et al., 2011). The liquid will be displaced and moved away by these distensions, resulting in cavitation bubbles. The bubbles will reach critical size at the maximum distention and will collapse in the following compression stage. The energy released by this implosion is enormous (Esclapez et al., 2011), as are the mechanical consequences at the solid-liquid interface, which will damage the plant's cell walls, allowing solvent penetration and enhancing extraction efficiency (Toma et al., 2001).

To summarize, the ultrasonic extraction approach takes less time and is potentially more efficient than conventional methods (Barba et al., 2016). However, preliminary research is required to get a satisfactory result with this approach, in order to determine the best power, frequency, temperature, solvent, particle size, and solvent to sample ratio (Carciochi et al., 2014). Chemat and collaborators concluded that the advantage of employing ultrasound for the extraction of the antimalarial drug artemisinin is substantiated by ten times quicker extraction rates for ultrasound compared to heat maceration (S. Chemat et al., 2017).

2.5.2. Supercritical fluid extraction

Due to its capacity to extract useful compounds from herbs with high yield and superior quality, supercritical fluid extraction (SFE) is one of the extraction processes used in herbal processing (Bernardo-Gil et al., 2011). Its advantages include the ability to execute extractions at near-ambient temperatures, which prevents the material of interest from becoming thermally degraded (Azmin et al., 2016). The main disadvantage is the cost associated to the compression stage. SFE is also being investigated as a potential alternative to traditional extraction techniques (Bernardo-Gil et al., 2011).

Carbon dioxide (CO_2) is the most well-known and commonly utilized of the available SFE solvents (Díaz-Maroto et al., 2002). Non-toxicity, non-flammability, absence of solvent residue in the finished product, and lack of reactivity with extraction materials and equipment are all characteristics of CO_2.

Because of these characteristics, it is extensively employed in a variety of extraction techniques. Furthermore, this gas is less costly than other typical solvents, lowering the extraction process's cost. One of the most significant advantages of CO_2 in SFE processes is the decrease of environmental and health risks associated with organic solvents (Geng et al., 2007; Schulz & Martinelli, 1991). Only one fluid phase occurs in the SFE environment, and its characteristics are similar to those of an intermediate phase between gas and liquid.

Kohler et al. (1997) found that Artemisinin from the aerial parts of *A. annua* can be quantitatively extracted in less than 20 min with a supercritical fluid mixture made of carbon dioxide and 3% ethanol (v/v) at 15.15 MPa and 50°C (Kohler et al., 1997).

2.5.3. Microwave-assisted extraction

Microwaves are electromagnetic waves with a frequency ranging from 0.3 to 300 GHz (Camel, 2001). Microwave-assisted extraction (MAE) is a method of extracting active compounds from plants using a liquid solvent, usually water. alcohol, or an alcoholic solution. The increased extraction in MAE is due to alterations in the vegetable cell structure generated by electromagnetic radiation (Veggi et al., 2013). In traditional extractions, however, the phytochemicals are mass transferred from the inside (herbal particle) to the outside (solvent), while the heat is transferred from the outside (heat source) to the inside (herbal particle) (Kokolakis & Golfinopoulos, 2013).

Through molecular interactions with the electromagnetic field and conversions of electromagnetic energy to thermal energy, microwave energy is given directly to the herbal particle (Thostenson & Chou, 1999). As a result, MAE usually yields a quick extraction time and a high extraction yield, due to a combination of two transport phenomena: heat and mass gradients operating in the same direction (Kokolakis & Golfinopoulos, 2013).

(Hao et al., 2002) determined that microwave assisted extraction of artemisinin from *A. annua L* is superior to the Soxhlet technique, supercritical CO_2 extraction, and regular stirring extraction. MAE takes substantially less time than other procedures and achieves greater extraction rates than other approaches. Supercritical CO_2 extraction yields the lightest extractive color but the slowest extraction rate (Hao et al., 2002).

2.5.4. Pressurized liquid extraction

This method, also known as accelerated solvent extraction, employs liquid solvents at high temperatures and pressures below the solvent's critical point, resulting in increased solubility and mass transfer than when the temperature and pressure are normal (Pronyk & Mazza, 2009). Higher temperatures lower the solvent's viscosity and surface tension, allowing for more diffusion mass transfer and penetration of the solute-matrix linkages, which improves extraction efficiency (Ramos et al., 2002).

There are two techniques to employ pressured liquid extraction. The first method is static, in which the solvent must be pressurized in a closed system extractor (batch system). The product leaves through a valve after extraction, and a fresh solvent is introduced for the next extraction cycle. This mode is the most popular since it is the most efficient due to the solvent's strong capacity to penetrate the pores of plant material.

The other method is dynamic, which means the solvent is continually added. Carabias-Martinez et al. (2005), and Nieto et al. (2010) employed a wide range of solvents to extract chemicals from leaf vegetables.

Sixt and Strube (2017) concluded that pressurized hot water shows a high productivity and yields of extracting artemisinin from *Artemisia annua L.* (Sixt & Strube, 2017).

Summarizing, microwave-assisted extraction provides a number of benefits, including shorter time, less solvent, greater extraction rate, and better products at a cheaper cost (Ganzler et al., 1986). The Soxhlet process takes many hours, sometimes even more than 20 hours, whereas microwave-assisted extraction takes only a few minutes (Paré et al., 1991). Microwave-assisted extraction is easier and less expensive than supercritical fluid extraction, and it may be employed on a wider range of materials with less restrictions on extractant polarity (Klayman, 1985).

3. Materials and methods

3.1. Plant material

Artemisia annua L. was grown in a greenhouse (Switzerland). *A. annua*'s leaves were collected and dried in collaboration with the company Media Plant. The dried plant parts were crushed to fine powder using an electric grinder. Exposure to direct sunlight was avoided to prevent the loss of active components.

3.2. Extraction

As a standard procedure for the extraction of antioxidant compounds and determination of bioactivities, a hydroethanolic extraction was performed. Four g of milled sample was weighed accurately into an 80 mL of centrifuge tube. A total amount of 80 mL of ethanol-water mixture at 80:20 (v/v) was added to the tube. The tube was transferred to the microwave at 50°C, 200W for 10 min. At the end of the microwave treatment, all samples were filtered through all Whatmann No. 4 paper and ethanol was pressure separated in a rotating evaporator. The solution was evaporated, frozen at -80°C for 24 hours and freeze-dried. The samples were then weighed and stored out of the light in a refrigerator at 4°C before being analysed and used in bioactivity tests.

As an alternative to organic solvents, urea was used as hydrotrope extractant. 0.8, 2, and 4 g of ground sample were accurately weighed in an 80 ml centrifuge tube. A total of 80 mL of urea or water (0M, 1.8M and 3.6 M) was added to the tube. The tubes were microwaved at 50°C, 200 W for 10 min. At the end of the microwave treatment, all samples were filtered through all Whatmann No. 4 paper. The extracts were storage in the freeze (-20ºC) until be used to test the bioactivities.

Table 3 : Concentration of different extraction solvents and Artemisia annua dry weight.

Ethanol		
Code	**Concentration (v/v)**	**Dry weight (g)**
EtOH	80/20	4
Urea		
Run	**Concentration (mol/L)**	**Dry weight (%)**
Low	0	1
Mid-Low	0	5
Mid	1.8	2.5
Mid-High	3.6	1
High	3.6	5

All extractions were carried out at least in duplicate. **Table 3** shows the different combinations of extractions performed.

The extraction yield was used to determine the solvent efficiency in extracting compounds from the plant material. The extraction yield (presented in %) was calculated as shown in the following equation:

Extraction Yield (%) = (Extracted solids (g)) / (Initial dry material (g)) × 100

3.3. Bioactivities

Different *Artemisia annua* bioactivities were determined for the hydroethanolic extract as a preliminary assay to understand the potential of the plant. The following assay were tested:

Antioxidant activity by TBARs method:

TBARs is a colorimetric assay in which lipid peroxidation produces malondialdehyde (MDA) as secondary breakdown product and reacts with the thiobarbituric acid (TBA) to form MDA-TBA complex with the production of a pink pigment (Ndhlala et al., 2010). The inhibition of lipid peroxidation in porcine (Sus scrofa) brain homogenates in the presence of antioxidant is detected by measuring the absorbance of MDA-TBA complex at 532 nm (**Figure 6**).

The porcine brains were purchased from a local slaughterhouse, dissected and dissolved in Tris-HCl buffer (20mM, pH 7.4) in a proportion of 1:2 (w/v) brain tissue homogenate wich was centrifuged at 3000g for 10min. Each dilution of the sample solutions (0.2mL) was pipetted into test tubes, adding also 0.1mL of $FeSO_4$ (10μM), 0.1mL of ascorbic acid (0.1mM) and 0.1mL of the supernatant of the brain tissue homogenate. Two controls were also preformed, one with the extraction solvent and another with Tris-HCl buffer (20mM, pH 7.4) and adding the abovementioned reagents (without the extract). The tubes were incubated at 37°C for 1h.

Trichloroacetic acid (0.5mL, 28%, w/v) was added to stop reaction, together with 0.38mL of thiobarbituric acid (TBA, 2%, w/v). The mixture was then incubated at 80°C for 20min. The mixtures were centrifuged at 3000gr for 10min to eliminate the precipitated protein. The absorbances of the supernatant samples were measured at 532nm. The percentage of inhibition was calculated through this equation:

$$\text{Inhibition ration (\%)} = [(A - B) / A] \times 100\%.$$

(A-absorbance of the control and B- absorbance of the compound solution).

TBARs measurements were performed once on triplicate samples. The results were expressed in IC_{50} values (μg/mL), (extract concentration providing 50% of antioxidant activity).

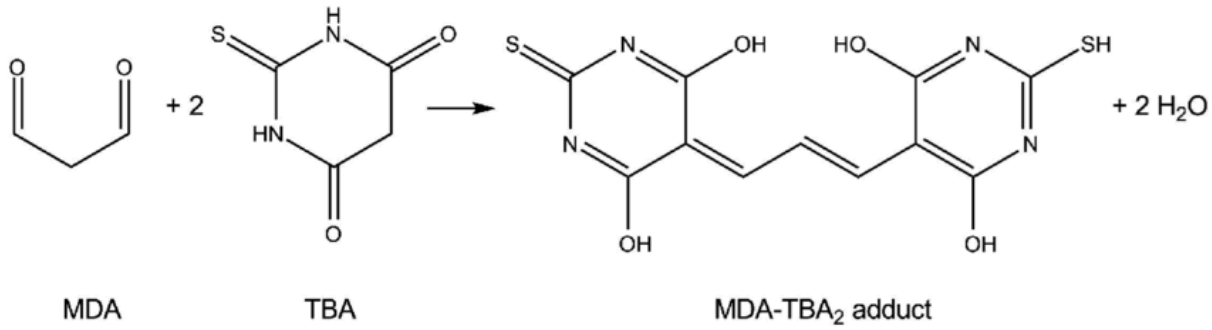


Figure 6 : Colourimetric reaction between the malondialdehyde (MDA) and thiobarbituric acid (TBA).

Antibacterial activity

Food contaminants (bacteria): The extracts were tested against five Gram-negative bacteria, namely, *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC BAA-2146), *Morganella morganii* (ATCC 25830), *Proteus mirabilis* (ATCC 25933) and *Pseudomonas aeruginosa* (ATCC 9027) and three Gram-positive bacteria, namely *Enterococcus faecalis* (ATCC 49533), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923).

All these microorganisms were purchase at Frilabo, Porto, Portugal. The bacteria were incubated at 37ºC an appropriate fresh medium, for 24h before analysis to maintain the exponential growth phase.

Clinical bacteria: The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Morganella morganii and* three Gram-positive bacteria (*Enterococcus faecalis, Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus (MRSA*), were tested.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC)

The MIC determinations on all bacteria were conducted using colorimetric assay according to Pires et al, 2018. The samples were first of all dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20mg/mL for the stock solution. 100µL of the stock solution (20mg/mL) was added in the first well of the 96-well microplate in duplicate. with 90µL of Tryptic Soy Broth (TSB).

In the remaining wells 90µL of TSB medium were added. Then the samples were serially diluted to obtain the concentration ranges (10 to 0.03125mg/mL).

To finish, 10µL of inoculum (standardized at 1.5×10^6 Colony Forming Unit (CFU)/mL) was added at all the well assuring the presence of 1.5×10^5 CFU. Two negative controls were prepared, one with TSB and another one with the extract. Two positive controls were prepared with TSB and each inoculum and another with medium, antibiotics, and bacteria. Ampicillin and Steptomicin were used for all bacteria tested and Meticilin was also used for *Staphylococcus aureus.*

The microplates were incubated at 37°C for 24h. The MIC of samples was detected following addition (40µl) of 0.2mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth determinate by change the coloration from yellow to pink if the microorganisms are viable.

For the determination of MBC, 10µL of liquid from each well that showed no change in color was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37°C for 24h. The lowest concentration that yielded no growth determine the MBC. MBC was defined as the lowest concentration required to kill bacteria.

Antifungal activity

The antifungal activity was performed according to described by Heleno et al., 2013. *Aspergillus fumigatus* (ATCC 204305), *Aspergillus brasiliensis* (ATCC 16404) were used. The organisms were obtained from Frilabo, Porto, Portugal. The micromycetes were maintained on malt agar and the cultures stored at 4°C and were further placed in new medium and incubated at 25ºC for 72h.

In order to investigate the antifungal activity, the fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100µL per well.

The samples were first dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO) and 95% of autoclaved distilled water to give a final concentration of 20mg/ mL for the stock solution. Afterwards, 90µL of this concentration was added in the first well (96-well microplate) in duplicate with 100µL of Malt Extract Broth (MEB).

In the remaining wells 90µL of medium MEB were placed. Then the samples were serially diluted obtain the concentration ranges (10 to 0.03125mg/mL). Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microplate.

The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentration (MFC) was determined by serial sub cultivation of a 2µL of tested compounds dissolved in medium and inoculated for 72h, into microplates containing 100µL of MEB per well and further incubation 72h at 26°C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial fungicide ketoconazole (Frilabo, Porto, Portugal), was used as positive control.

Cytotoxic potential with human tumor cell lines

The following human tumor cell lines were used: AGS (gastric adenocarcinoma), CaCo (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), NCI-H460 (lung carcinoma). Non-tumor cell lines were also tested: Vero (African green monkey kidney) and PLP2 (primary pig liver culture). All of them maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2mM), penicillin (100U/mL) and streptomycin (100mg/mL), with the exception of Vero, maintained in DMEM medium supplemented with fetal bovine serum (10%), glutamine and antibiotics. The culture flasks were incubated in an incubator at 37ºC and with 5% CO_2, under a humid atmosphere. The cells were used only when they had 70 to 80% confluence.

A known mass of each of the extracts (8 mg) was dissolved in H_2O (1mL), in order to obtain the stock solutions with a concentration of 8mg/mL. From which successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8mg/mL). Each of the extract concentrations (10µL) were incubated with the cell suspension (190µL) of the cell lines tested in 96-well microplates for 72 hours. The microplates were incubated at 37ºC and with 5% CO_2, in a humid atmosphere, after checking the adherence of the cells. All cell lines are tested at a concentration of 10,000 cells/well, except for Vero in which a density of 19,000 cells/well was used.

After the incubation period, the cells were corrected: TCA (10% w/v; 100µL) was previously cooled and plates were incubated for 1 hour at 4ºC, washed with water and, after drying, a SRB solution (0.057%, m/v; 100µL) was added, left to stand at room temperature for 30 minutes. To remove non-adhered SRB, plates were washed three times with a solution of acetic acid (1% v/v) and placed to dry. Finally, an adhered SRB was solubilized with Tris (10mM, 200µL) and the absorbance at a wavelength of 540nm was read in the Biotek ELX800 microplate reader. The results are expressed in terms of the concentration of extract with the ability to inhibit cell growth by 50% - GI_{50}. As a positive control of the use of an ellipticine.

Anti-inflammatory activity

The extracts were dissolved in H_2O in order to obtain a final concentration of 8mg/mL. From which successive dilutions were carried out, obtaining the concentrations to be tested (0.125 - 8mg/mL). The RAW 264.7 mouse macrophage cell line, obtained from DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, was grown in DMEM medium, supplemented with heat-inactivated (SFB) fetal serum (10%), glutamine and antibiotics, and kept in an incubator at 37ºC, with 5% CO_2 and under a humid atmosphere. Cells were detached with a cell scraper.

An aliquot of the cell suspension of macrophages (300µL) with a cell density of 5x105cells/mL and with a proportion of dead cells below 5% according to the Trypan blue exclusion test, was placed in each well. The microplate was incubated for 24 hours in the incubator with the conditions previously indicated in order to allow an adequate adherence and multiplication of the cells. After that period, the cells were treated with different concentrations of extract (15µL, 0.125 - 8mg/mL) and incubated for one hour, with the range of concentrations tested being 6.25 - 400µg/mL.

Stimulation was performed with the addition of 30µL of the liposaccharide solution - LPS (1mL/mL) and incubated for an additional 24 hours. Dexamethasone (50mM) was used as a positive control and samples in the absence of LPS were used as a negative control. Quantification of nitric oxide was performed using a Griess reagent system kit (nitrophenamide, ethylenediamine and nitrite solutions) and through the nitrite calibration curve (100mM sodium nitrite at 1.6mM) prepared in a 96-well plate. The nitric oxide produced was determined by reading absorbances at 540nm (ELX800 Biotek microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA) and by comparison with the standard calibration line.

The results were calculated through the graphical representation of the percentage of inhibition of nitric oxide production versus the sample concentration and expressed in relation to the concentration of each of the extracts that causes the 50% inhibition of nitric oxide production - IC_{50}.

3.4. Urea extracts bioactivities

Urea extracts were tested for antioxidant activity through TBARS assay. In addition, the cytotoxicity was evaluated, for both urea and hydroethanolic extracts, following the next protocol:

The human fibroblasts HFF-1 (ATCC; SCRC-1041™) were used to evaluate the cytotoxicity of the samples using the Sulforhodamine B colourimetric assay (SRB). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10%) and 1% penicillin/streptomycin. The extracts were redissolved in the different extraction solvents and tested at 400 µg/mL. Control cells were incubated with the solvents. Each sample (10 µL) was incubated with a cell suspension (90 µL - 10,000 cells/well) in a 96-well microplate for 48 hours. After the incubation period, an ice-cold solution of 50 µL trichloroacetic acid (10% w/v) was added to the cells.

The plates were incubated for 1 hour at 4ºC, washed with water and dried. SRB solution (100 µL; 0.057%, m v) was then added and incubated at room temperature for 30 minutes. To remove the excess of SRB, plates were washed three times with an acetic acid solution (1% v/v) and left to dry. Finally, the SRB was solubilised with Tris (10 mM, 200 µL), and the absorbance was measured at 540 nm (SPECTROstar Nano Multi-Detection Microplate Reader; BMG Labtech, Ortenberg, Germany). Results were expressed as percentage of cell viability after incubation with 400 µg/ml of the different extracts. Triton was used as a positive control.

3.5. Statistical analysis

All the assays were carried out at least in duplicate with three different repetition each. The statistical differences were obtained through one-way analysis of variance (ANOVA) followed by followed by Tukey's HSD or Dunnett post hoc test, to perform multiple comparisons between different urea extracts or between these extracts and the hydroethanol extract, respectively. These treatments were carried out using PRISM 9; version 9.4.1.

To understand the behavior of hydrotropes such urea in extraction processes of bioactive compound, a screening non-orthogonal design was performed employing advance extraction technologies as mentioned in the previous section. Since our group has just started to explore extraction protocols using hydrotropes, it is necessary to first explore and understand responses, controlling as much as possible general factor involved.

The first approach considered 2 factors (urea concentration and solid-liquid ratio) at 5 different levels, following a factorial design-type adapted to non-orthogonal distribution of testing point shown in the table section 2A) in the **Table 6**.

The responses analyzed (antioxidant effect and cell viability) were monitored in terms of their IC_{50} values and viability, respectively, and were modeled and computed with the non-linear curve model: Weibull type 2 4-parameter logistic (4PL), using the R studio "DRC" package. Weibull type modelling was selected from 5 different models applied. For general data wrangling, data visualization and statistical analysis different software were used at convenience, packages such as scipy, numpy, pandas, matplotlib, pingouin and seaborn were used in python 3.8.12, packages including tidyverse, tidyr, ggplot2, drc, patchwork, ggpubr, broom, AICmodavg, ggcorrplot, DoE.base, SixSigma, rgl and rsm were used in R studio 2022.07.1, along with DesignExpert 13.0.12 (Stat-Ease Inc, Minneapolis, MN, USA) for quick visualizations and statistical diagnostics.

4. Results and discussion

4.1. Hydroethanolic extract

The hydroethanolic extract obtained from *Artemisia annua* was evaluated for its antibacterial activity against Gram+ and Gram- bacteria. **Table 4** includes the results for antibacterial activity expressed as MIC and MBC concentrations as well as a positive control of a well-known antibiotic, ampicillin.

Table 4 : Antimicrobial activity of A annua extract. Ampicillin were used as control for bacterial growth, ketoconazole for fungus growth. The results were given as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fun gicidal concentration (MFC; all in mg/mL).

			Positive control Ampicillin (10mg/mL)	
Antibacterial activity (mg/mL)	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative				
Escherichia coli	5	> 10	> 0,15	< 0,15
Klebsiella pneumoniae	10	> 10	10	> 10
Morganella morganii	5	> 10	> 10	> 10
Proteus mirabilis	5	> 10	< 0,15	< 0,15
Pseudomonas aeruginosa	5	> 10	> 10	> 10
Gram-positive				
Enterococcus faecalis	10	> 10	< 0,15	< 0,15
Listeria monocytogenes	10	> 10	< 0,15	< 0,15
Staphylococcus aureus	5	10	< 0,15	< 0,15
Antifungal activity (mg/mL)	**MIC**	**MFC**	**Ketoconazole (1mg/mL)**	
			MIC	**MFC**
Aspergillus fumigatus	2,5	>10	0,5	1
Aspergillus brasiliensis	2,5	>10	0,06	1,25

The extracts showed inhibitory and bactericidal concentrations ranging from 5 to 10 mg/mL. *Staphylococcus aureus* was the strain showing the highest sensitivity against the hydroethanolic extract with a MIC of 5 and MBC of 10 mg/mL. The extract was also effective to inhibit the growth of *Escherichia coli, Morganella morganii, Proteus mirabilis,* and *Pseudomonas aeruginosa,* nevertheless did not show bactericidal activity at the maximum concentration tested (10 mg/mL). In this sense, *A. annua* hydroethanolic extract was more effective than ampicillin to inhibit the growth of *Morganella morganii* and *Pseudomonas aeruginosa*. Therefore, the extract seems to be very active against Gram negative bacteria strain.

Hexane fractions from *A. annua in vitro* plants, which contain mainly artemisinin and its precursor showed a strong inhibitory effect against these kind of bacteria (Appalasamy et al., 2014). In addition, in other research work, ethanol extracts of this plant presented an effective inhibition growth against *S. aureu, B sutillis,* and *B atropoeus,* while the used solvent did not present activity(Ikram et al., 2015).

Regarding its antifungal activity, the extract showed antifungal activity against the tested micromycetes, being able to inhibit fungal growth at 2.5 mg/mL and having fungicidal capacity at concentrations above the 10 mg/mL.

In P. Gautam et al. studies show the antifungal activity of artemisinin against A. fumigatus, a human pathogenic filamentous fungus. Artemisinin was able to inhibit the growth of A. fumigatus after a 24-hour incubation, but with a higher IC_{50} value of 125 μg/ml (~0.4 μM) (Gautam et al., 2011).

The results for antioxidant, anti-inflammatory, and cytotoxic activities are shown in the **Table 5**.

Simply put, inflammation is the immune system's first protective response to a threat. A healthy immune response with optimal expression and resolution of the inflammatory process is the key to good health, but an active and persistent immune system with chronic inflammation can lead to many problematic conditions that may require therapeutic interventions (Lawrence & Gilroy, 2007; Serhan et al., 2007).

The extract has exhibited strong anti-inflammatory potential against RAW 264.7 of 37,8 ± 6,7 μg/mL. Artemisinin has been reported to inhibit proinflammatory cytokine. It established potent anti-inflammatory effects in animal models of sepsis induced by CpGODN, LPS, heat-killed E. Coli 35218 or live E. Coli in RAW 264.7 cells (Wang et al., 2006).

The antioxidant activity was determined by thiobarbituric acid reactive substances (TBARS) assay. The results were given as IC_{50}, which indicates the extract concentration required to prevent by 50% the malondialdehyde formation. Therefore, lower IC_{50} values correspond to higher antioxidant activity of extract. *Artemisia annua* extracts showing a potent activity with a low IC_{50} of 10.25 ± 0.95 μg/mL.

In addition, the values for cytotoxic activity were expressed as GI_{50}, which indicates the extract concentration (in μg/mL) required to inhibit cell growth by 50%. Ethanolic extract was found to be very effective against all four tumour cell lines. For inhibition of growth of human gastric adenocarcinoma (AGS), the best results were 21.1± 1.3 μg/mL. For the CaCo (colorectal adenocarcinoma) was 54 ±7. For breast adenocarcinoma (MCF-7) 54.1 ± 4.1 μg/mL and non-small cell lung cancer (NCI-H460), the extract obtained from GI_{50}, 61.4 ± 5.3 μg/mL.

However, the extract was also effective against two different normal cell lines tested: the VERO (African Green Monkey Renal Epithelial Cell Line) and PLP2 (porcine liver cell culture). The extract showed a GI_{50} of 50 ± 2.3 and 19,5 ± 2,3 μg/mL, respectively. Therefore, the hydroethanolic extract was cytotoxic against normal cells in a lower or similar concentrations of other bioactivities.

Table 5 : Antioxidant, cytotoxic, and anti-inflammatory activity of A. annua hydroethanolic extract.

Antioxidant activity (µg/mL)	**IC_{50}**	**Positive control Trolox (µg/mL)**
TBARs	10,25 ± 0,95	9,1 ± 0,3
Cytotoxicity activity (µg/mL)	**GI_{50}**	**Ellipticine (µg/mL)**
AGS	21,1 ± 1,3	1,21 ± 0,03
CaCo	54,0 ± 7,0	1,21 ± 0,02
MCF-7	54,1 ± 4,1	1,02 ± 0,02
NCI-H460	61,4 ± 5,3	1,01 ± 0,01
PLP2	19,5 ± 2,3	1,40 ± 0,10
VERO	50,0 ± 2,3	1,41 ± 0,06
Anti-inflammatory activity (µg/mL)	**IC_{50}**	**Dexametasome (µg/mL)**
RAW 264.7	37,8 ± 6,7	6,3 ± 0,4

4.2. Urea extracts

Urea was selected as extraction solvent in order to benefit from its hydrotropic action and to obtain artemisinin-enriched bioactive extracts. Additionally, to compare with the bioactivities obtained with the hydroethanolic extract, the yield of the different extractions, the antioxidant, and cytotoxicity bioactivities were determined.

Regarding the extraction yield, **figure 7A** shows that the hydroethanolic extract obtained a low yield (10.3% ± 0.2), similar to that obtained by the Mid-Low and High extractions (16.6%± 1.1 and 17.2% ±2.6, respectively). **Figure 7B** shows that these two extractions produced the worst result for the urea extraction, being the best, this obtained by the Mid-High extraction (68.9 ± 5.1). The two extracts with the lowes t yields were characterised by a high solid-to-liquid ratio (S-L ratio) of 5%, while the best performer had the highest urea concentration and the lowest S-L ratio (**Table 3**).

Therefore, the results could indicate that the urea concentration should be increased for better yields, and the S-L ratio should be reduced. However, these results were not considered for further modelling because they showed an above-normal variation. This high variation may be due to the difficulty separating urea from the extract and removing the water used during the extraction process. Thus, future work will be necessary to improve the drying process and the separation of hydrotrope and extract to decrease the variability shown in this assay.

Regarding cytotoxicity, the hydroethanolic and urea extracts were tested in a human fibroblast cell line (HFF-1). The urea extracts presented low cytotoxicity, so it was impossible to calculate their IC_{50} down to their maximum concentration tested (400 µg/mL). Therefore, the maximum concentration tested was used to compare the extracts among them (**Figure 8**). The hydroethanolic extract showed high cytotoxicity values with a viability percentage of 29.6% compared to the urea extracts (**Figure 8A**). Likewise, the Mid-High and High urea extracts had the highest viability significantly and were, therefore, the least cytotoxic (**Figure 8B**; 75.5 and 73.3%, respectively).

Finally, concerning antioxidant activity, urea extracts had a significantly lower IC_{50} than ethanol extracts being less antioxidants (**Figure 9A**). Among them, the Low extract presented the highest antioxidant activity with an IC_{50} of 36.1 ± 0.4 µg/mL

Although the IC_{50} obtained for urea extracts is lower than ethanol, the cytotoxicity of these extracts is also much lower. Therefore, as the effective antioxidant concentration is much lower than that which produces cytotoxicity, this extract could be used for some applications.

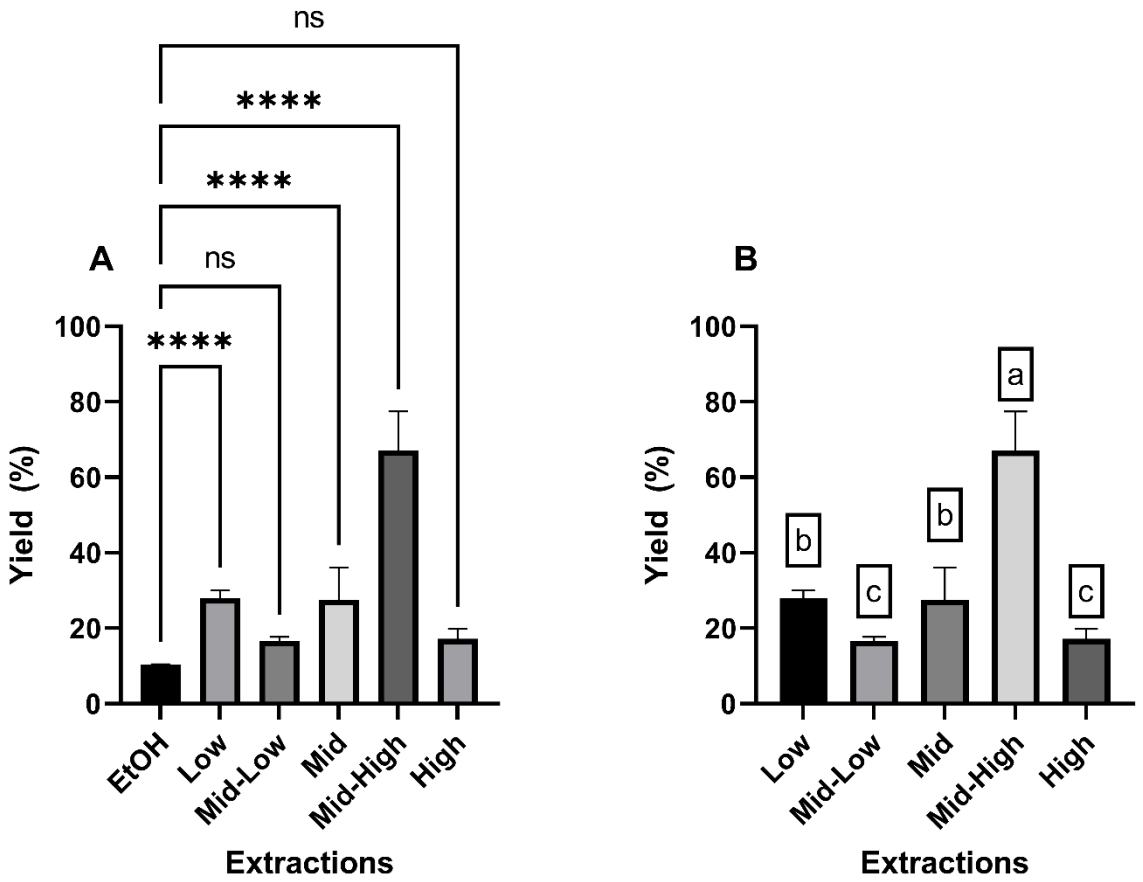


Figure 7 : Yield percentages for different extractions performed. Statistical differences were assessed by one-way ANOVA, followed by A Dunnett's test against EtOH extract as control (**** p-value < 0.0001; ns: non-significant differences) or B Tukey's HSD post hoc test (α = 0.05)**.** Different letters indicate significant differences among extractions.

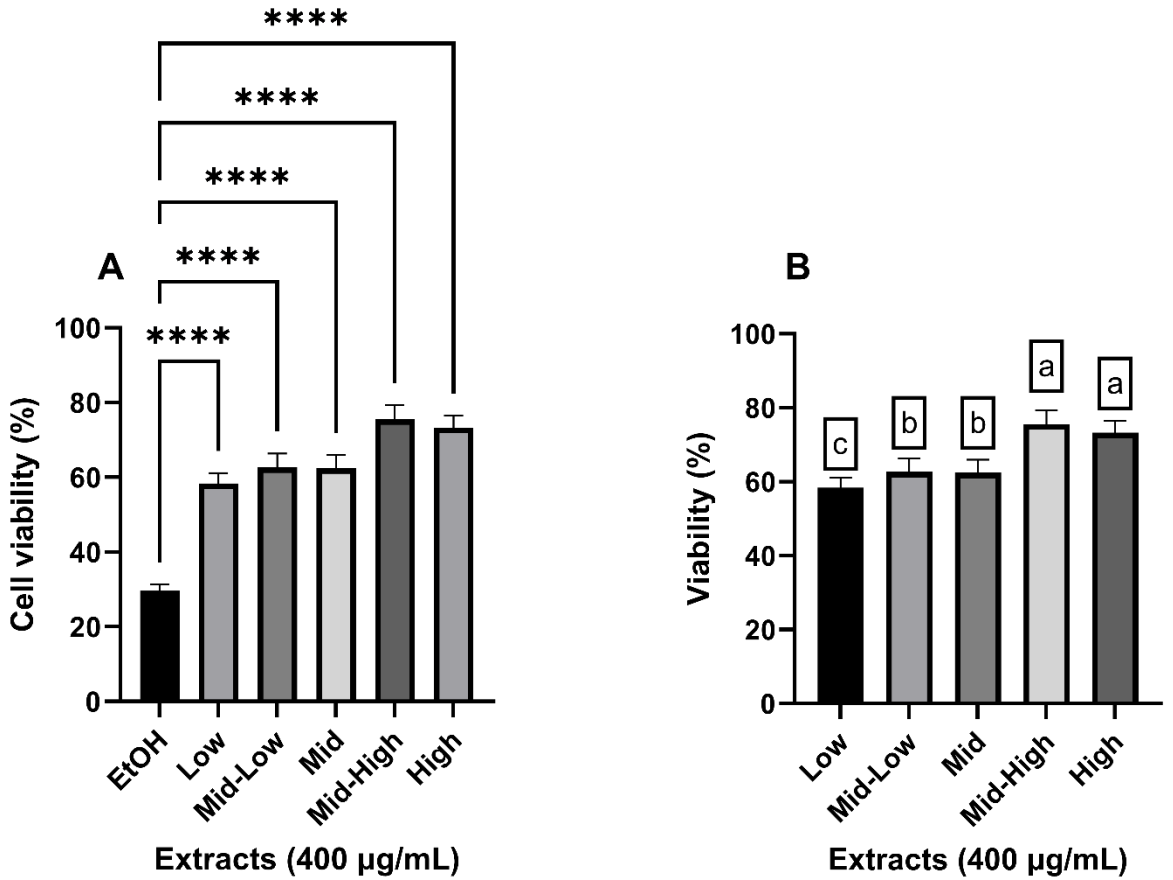


Figure 8 : Cell viability in HFF-1 cells for different extractions performed. Statistical differences were assessed by one-way ANOVA, followed by A Dunnett's test against EtOH extract as control (**** p-value < 0.0001; ns: non-significant differences) or B Tukey's HSD post hoc test (α = 0.05). Different letters indicate significant differences among extractions.

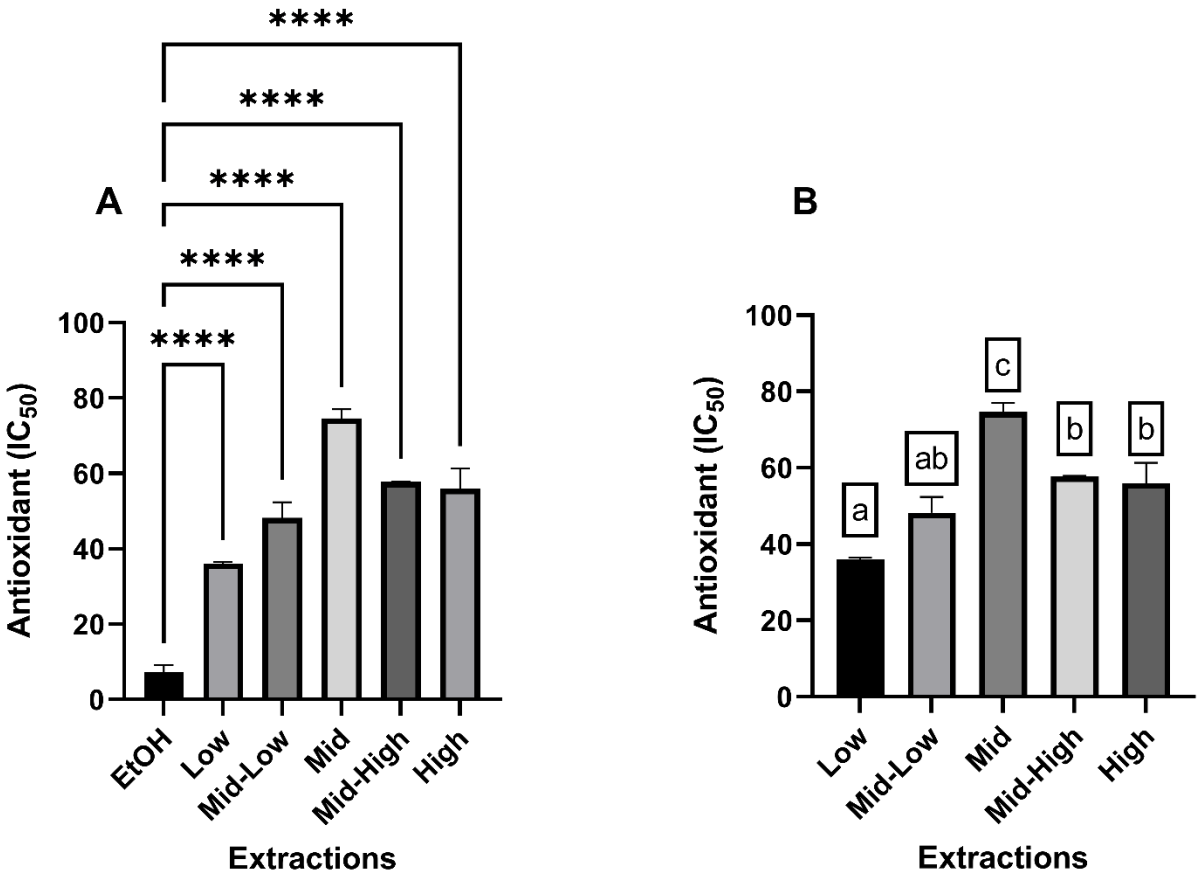


Figure 9 : Antioxidant activity (IC_{50}) for different extractions performed. Statistical differences were assessed by one-way ANOVA, followed by A Dunnett's test against EtOH extract as control (**** p-value < 0.0001; ns: non-significant differences) or B Tukey's HSD post hoc test (α = 0.05). Different letters indicate significant differences among extractions.

4.3. Impact of urea extracts factors

As starting point in the exploration of hydrotropes extraction assisted by microwave technology, the effect of the binary solvent composed by urea and water at different molarities (0 – 3.6 M) was tested along with distinct amounts of solid to liquid ratios (1 – 5 %), monitoring the antioxidant effect of the recovered extracts as well as their cellular viability as shown in **Table 6** – B1), afterwards, in the section B2 (**Table 6**), a quick overview of the dispersion statistics is presented with a particular emphasis on the coefficient of variation (CV), since the design applied does not follow an orthogonal dispersion, it is convenient to understand the variation of each level response prior to the development of statistical tests and modeling, obtaining fair CV values considering the usage of biological tests with the higher range of 7.6 and variation coefficient of 9.6 for the high level of the antioxidant test, while the higher C.V for viability was 4.57 for the low level.

Afterwards, both responses were treated separated as shown in **Figure 10**, where section A1 to 5 belongs to antioxidant response, while in **Figure 11** section C1 to 5 was assigned to viability response. Subsections were treated and represented in the same manner for ease of explanation, displaying in subsections 1 (A and B) a 3D representation of the obtained responses (blue point for above, and orange for below surface collected responses) as well as the surface model representation of the trend points and underneath a contour plot which display the same trend but in a 2-dimension plot.

The subsection 2 and 3 (A and B), exhibit the overall factor behavior for each response starting for the urea concentration and followed by the solid to liquid ratio and at their right side in subsection 4 of both responses, a graphical representation of modeled predicted responses were confronted to the actual values to consider the precision of the model generated. Finally, in subsections 5, numerical data of the modeling is presented showing data of the ANOVA for quadratic model (aliased) and their fit statistics.

4.5 Factors modelling

With the gathered responses and the description provided for the **Table 6** before, the focus on the modeling should started with the ANOVA tables presented, highlighting first that this model is aliased, and therefore, incomplete for 100% reliable predictions, although, with such grade of information presented, a great overview is provided, for instance in both responses only urea concentration proved to be highly significant (0.0013 and 0.0002 for antioxidant and viability responses), also with high significant values for the urea concentration quadratic effect, which is clearly visualized on the 3D representations, were antioxidant effect displayed concave inflexion, while viability presented a convex inflexion.

For screening analysis like this kind, we would expected inverse inflexion in both responses, generally, a convex inflexion is wanted for a minimizing target such as antioxidant IC_{50} values and concave inflexion is wanted for a maximizing target such as viability, even though, analyzing the fit statistics for both responses in their subsections 5, a proper fitting of the model can be spotted at the adjusted and predicted R^2 values, hence, the proper trend of investigation has been settle, pointing towards to the lowest concentrations of urea for antioxidant response, or extending the domains explored so far, which would definitely benefit as well the viability response according to the ascending trend spotted in this screening analysis, and as a confirmation of the good fitting of the points, subsections 4 exhibit as well a proper linear distribution between the predicted and actual points.

Therefore, extending the design on higher concentrations of urea will provide better navigation towards to an efficient optimization, while testing higher percentage of solid to liquid ratio might provide an even larger and perhaps significant response.

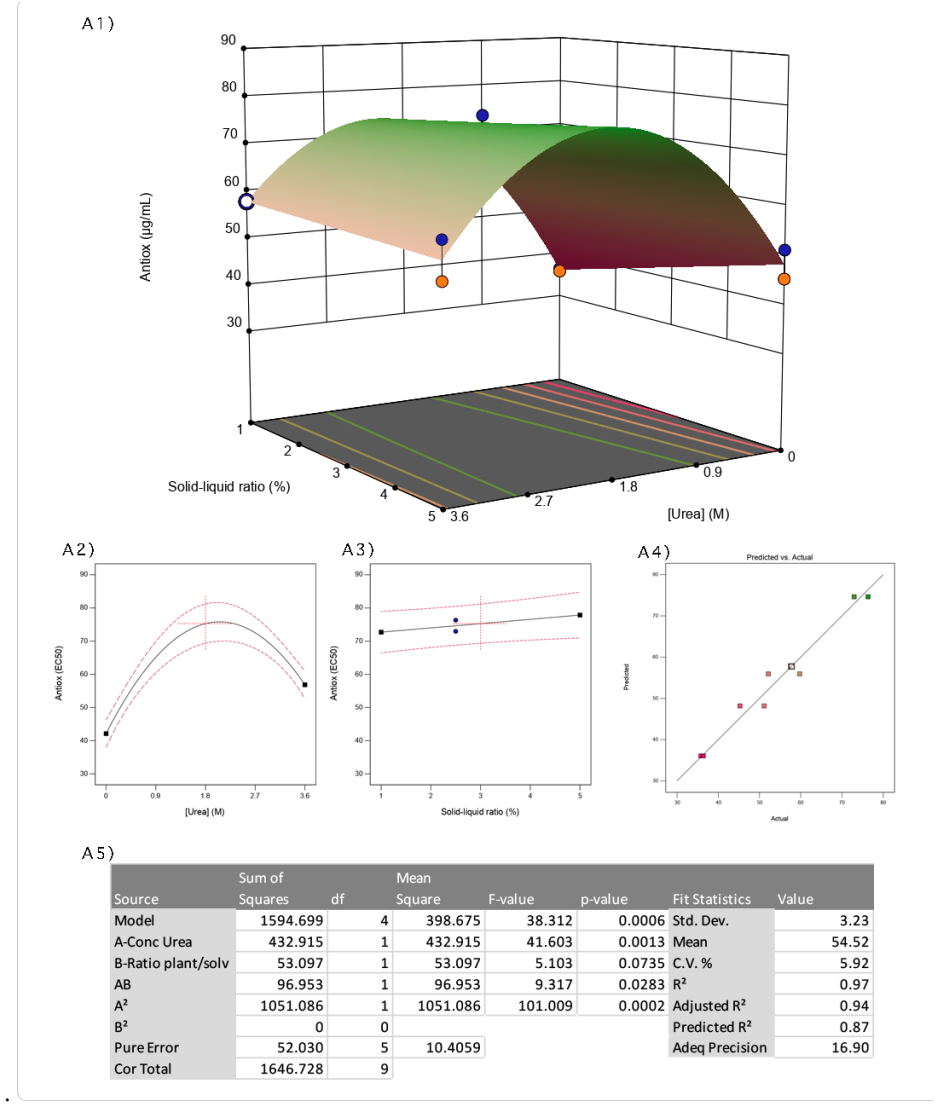
Table 6 : Summary of non-orthogonal design (B1) and calculated responses (B2).

B 1)

Design point	[Urea]	Solid-liquid ratio	Antiox	Viability
Low	0	1	35.78	56.50
Low	0	1	36.37	60.27
Mid-Low	0	5	51.13	63.50
Mid-Low	0	5	45.25	61.98
Mid	1.8	2.5	76.32	64.36
Mid	1.8	2.5	72.95	60.56
Mid-High	3.6	1	57.68	76.42
Mid-High	3.6	1	57.82	74.60
High	3.6	5	52.14	74.37
High	3.6	5	59.74	72.21

B 2)

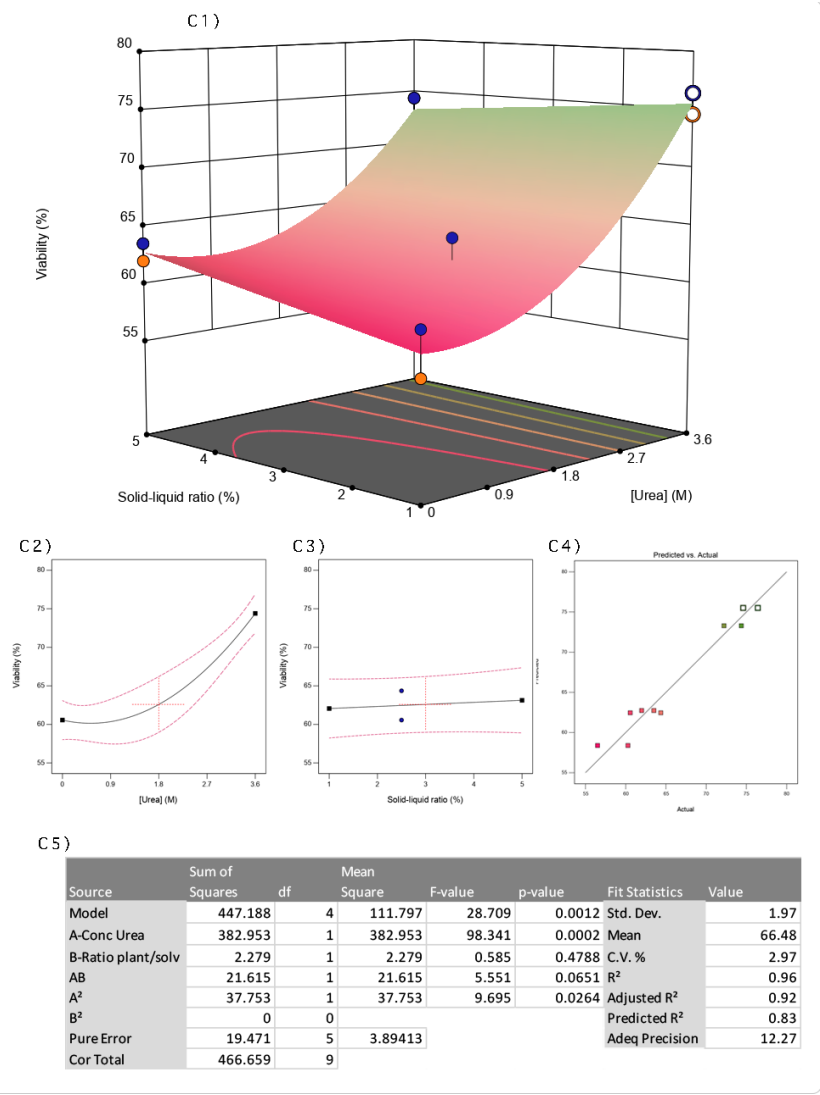
Response	Design point	Lower	Upper	Difference	Average	Stdv	CoefVar
Antioxidant assay	Low	35.78	36.37	0.60	36.08	0.42	1.17
	Mid-Low	45.25	51.13	5.87	48.19	4.15	8.62
	Mid	72.95	76.32	3.37	74.63	2.38	3.20
	Mid-High	57.68	57.82	0.13	57.75	0.09	0.16
	High	52.14	59.74	7.60	55.94	5.37	9.60
Viability assay	Low	56.50	60.27	3.77	58.38	2.67	4.57
	Mid-Low	61.98	63.50	1.53	62.74	1.08	1.72
	Mid	60.56	64.36	3.80	62.46	2.69	4.30
	Mid-High	74.60	76.42	1.82	75.51	1.29	1.71
	High	72.21	74.37	2.16	73.29	1.53	2.09



A5)

Source	Sum of Squares	df	Mean Square	F-value	p-value	Fit Statistics	Value
Model	1594.699	4	398.675	38.312	0.0006	Std. Dev.	3.23
A-Conc Urea	432.915	1	432.915	41.603	0.0013	Mean	54.52
B-Ratio plant/solv	53.097	1	53.097	5.103	0.0735	C.V. %	5.92
AB	96.953	1	96.953	9.317	0.0283	R^2	0.97
A^2	1051.086	1	1051.086	101.009	0.0002	Adjusted R^2	0.94
B^2	0	0				Predicted R^2	0.87
Pure Error	52.030	5	10.4059			Adeq Precision	16.90
Cor Total	1646.728	9					

Figure 10 : Screening analysis of factors and responses. Section A1 to A5 displays the data modeling using an aliased polynomial approach for the antioxidant response. A1 represent the 3D final modeling with testing points (blue above and orange below surface) and contour plots. A2 and A3 shows the single factorial effect trend. A4 test the linearity of the response of the predicted values generated by the quadratic model vs the actual experimental values, while A5 presents numerical data of the ANOVA and fit statistics of the model**.**



Source	Sum of Squares	df	Mean Square	F-value	p-value	Fit Statistics	Value
Model	447.188	4	111.797	28.709	0.0012	Std. Dev.	1.97
A-Conc Urea	382.953	1	382.953	98.341	0.0002	Mean	66.48
B-Ratio plant/solv	2.279	1	2.279	0.585	0.4788	C.V. %	2.97
AB	21.615	1	21.615	5.551	0.0651	R^2	0.96
A^2	37.753	1	37.753	9.695	0.0264	Adjusted R^2	0.92
B^2	0	0				Predicted R^2	0.83
Pure Error	19.471	5	3.89413			Adeq Precision	12.27
Cor Total	466.659	9					

Figure 11 : Screening analysis of factors and responses. Section C1 to C5 displays the data modeling using an aliased polynomial approach for the antioxidant response. C1 represent the 3D final modeling with testing points (blue above and orange below surface) and contour plots. C2 and C3 shows the single factorial effect trend. C4 test the linearity of the response of the predicted values generated by the quadratic model vs the actual experimental values, while C5 presents numerical data of the ANOVA and fit statistics of the model**.**

5. Conclusions and future work

Artemisia annua L. is a plant with a high potential for bioactivities beneficial to human health. However, its hydroethanolic extract has proven highly cytotoxic against three non-cancerous cell lines. Otherwise, the hydrotropic extraction with urea seems to be a great alternative to obtain bioactive compounds by reducing their cytotoxicity and having the same amount or maybe better than hydroethanolic extract.

For future work, we are implementing a methodology to identify and quantify the bioactive compounds present in the extracts to correlate them with the obtained bioactivities. In addition, we will select those extracts with the highest bioactivities and the highest concentration of artemisinin to perform an anti-malaria assay and test the true potential of these extracts.

We are planning to extract with another hydrotropic solvent such as Sodium Salicylate and we also would like to try to mix Urea and Sodium Salicylate together and see what we can obtain.

Finally, if all results are positive, we will proceed to the optimisation of the extraction based on how the initial factors that we have studied correlate with the final bioactivities.

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