Recovery of added value molecules from bio-residues: *Aesculus hippocastanum* L. as a source of saponins and sterols

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Abstract

It is estimated that about 80% of the world population living in developing countries depends on herbal treatments as primary health and traditional medicine source. Nowadays, even in the most developed countries, plants continue to be of great importance both as sources of nutrients and bioactive compounds. These compounds can exert beneficial functions in our body and are often incorporated into nutraceutical/pharmaceutical formulations. However, and regarding the use of wild species, it is essential to consider that they are a finite natural resource, and their excessive harvest can have negative impacts on ecosystems. In this way and promoting concepts that are currently fundamental in developing technologies and products, namely the circular economy and sustainability, bio-residues have been identified as an excellent source of interesting bioactive compounds.

Aesculus hippocastanum L., commonly known as horse-chestnut, is a large tree native to southeastern Europe and widely used as an ornamental and landscape plant in parks and gardens worldwide. Regarding its fruits/seeds, most of them end up in landfills, making these plants a source of bio-residues with no economic value. Horse chestnut seeds are not edible due to the bitter taste conferred by the bioactive compounds present, namely pentacyclic triterpene saponins. Saponins are compounds of high interest since they have multiple bioactivities, being recognized for their anti-inflammatory, hypocholesterolemic, immunomodulatory and even antitumor potential.

Although there is an increasingly widespread tendency to adopt environmentally-friend extraction methodologies to obtain interesting compounds, the fact is that conventional technologies are still widely used. Frequently because of their practicality and lower cost, as well as because extraction yields are sometimes lower with green methodologies.

According to the abovementioned, the present work aimed to investigate the real difference between conventional techniques (Soxhlet) and more innovative and greener techniques (ultrasound-assisted extraction) for extracting saponins. With this, it is intended to implement the extraction these valuable compounds, which is not performed in our laboratory and is of great interest given their bioactive potential. In addition, since the unsaponifiable fraction of chestnuts seeds can be a source of phytosterols, the chemical characterization of this fraction was also the subject of study.

A. hippocastanum seeds (pulp, bark, and skin) were extracted by Soxhlet and ultrasound-assisted extraction (UAE), and to optimise the extraction of saponins from the seed samples the response surface methodology (RSM) was used. The saponins profile of the samples were initially

characterised by ultrafast liquid chromatography coupled to a photodiode array (UFLC-PDA), using aescin as standard. Then, and after obtaining the optimal extraction points by RSM, the bioactivity of the *A. hippocastanum* pulp, skin and bark was evaluated.

It was possible to detect saponins, namely aescin, on the samples obtained from both methods, and it was possible to identify the different aescin isoforms all along. For the optimisation, time, solvent/solid ratio, and concentration of solvents (ethanol and methanol) were tested in Soxhlet extraction; for extraction by UAE, time, ultrasonic power, and concentration of solvents (ethanol, and methanol) were tested. The polynomial models were fitted to the experimental data, statistically validated, and used to determine the optimal extraction conditions. Generally, the extraction of the target compounds was greatly affected by all the independent variables.

After obtaining the optimal points for the extraction of saponins, both Soxhlet and UAE were performed for the pulp, skin, and bark of *A. hippocastanum*. The bioactivity of these extracts was evaluated, namely the antimicrobial activity against human pathogens, cytotoxicity in human tumor cells and non-tumor cells, and anti-inflammatory potential. Regarding the antimicrobial properties, gram-positive bacteria, and *Aspergillus brasiliensis* seemed to be more sensitive pathogens to this type of extracts. However, since no relationship between the type of extraction or solvents was observed, this could be due to possible synergistic effects between saponins and other bioactive compounds. Concerning the cytotoxic and anti-inflammatory activities, generally, the most promising values were obtained for the bark. These results can prove the synergistic effects previously inferred.

Overall, this work was based on the recovery of added-value molecules from bio-residues currently without any application or economic value, promoting the recycling of materials and sustainable development, in addition to being a comprehensive study of the methodologies for extracting bioactive compounds of interest. The results of this study could be exploited by companies interested particularly in saponin-rich natural ingredients, such as the pharmaceutical, cosmetic, and food industries.

Keywords:

Horse chestnut; Saponins; RSM; Analytical techniques; Eco-friendly and emerging methodologies; Bioactive potential.

Resumo

Estima-se que cerca de 80% da população mundial que vive nos países em desenvolvimento depende de tratamentos baseados em plantas como fonte primária de cuidados de saúde e medicina tradicional. Atualmente, mesmo nos países mais desenvolvidos, as plantas continuam a ser de elevada importância, tanto como fonte de nutrientes como de compostos bioativos. Estes compostos podem exercer funções benéficas no nosso organismo e, por isso, são muitas vezes incorporados em formulações nutracêuticas/farmacêuticas. Contudo, e no que diz respeito à utilização de espécies selvagens, importa considerar que estas são um recurso natural finito, e a sua colheita excessiva pode ter impactos negativos nos ecossistemas. Deste modo, e promovendo conceitos que são fundamentais no desenvolvimento de tecnologias e produtos, nomeadamente os conceitos de economia circular e sustentabilidade, os bio-resíduos têm vindo a ser identificados como uma excelente fonte de compostos bioativos de interesse.

Aesculus hippocastanum L., vulgarmente conhecido como castanheiro-da-Índia, é uma árvore robusta nativa do sudeste da Europa e amplamente utilizada como planta ornamental e paisagística em parques e jardins de todo o mundo. Embora os frutos e sementes desta árvore possam também ser utilizados para fins ornamentais, a sua maioria acaba em aterros, fazendo destas plantas uma fonte de bio-resíduos sem valor económico. As suas sementes não são comestíveis devido ao sabor amargo conferido pelos compostos bioativos presentes, nomeadamente as saponinas triterpénicas pentacíclicas. As saponinas são compostos de elevado interesse uma vez que possuem múltiplas bioatividades; são reconhecidas pelo seu potencial anti-inflamatório, hipocolesterolémico, imunomodulador e mesmo antitumoral.

Embora exista uma tendência em adotar metodologias de extração ecológicas para a obtenção de compostos de interesse, o facto é que as tecnologias convencionais ainda são amplamente utilizadas. Muitas vezes pela sua praticidade e baixo custo, bem como pelos rendimentos de extração que são muitas vezes inferiores quando se utilizam metodologias mais verdes.

Assim, o presente trabalho teve como objetivo investigar a diferença entre a utilização de técnicas convencionais (Soxhlet) e técnicas mais inovadoras e ecológicas (extração assistida por ultrassons) para extração de saponinas. Desta forma, pretende-se implementar a extração destes compostos a nível laboratorial, uma vez que é de elevado interesse dado seu potencial bioativo. Além disso, como a fração insaponificável das sementes de castanha-da-índia pode ser uma fonte de fitoesteróis, estes compostos foram também alvo de estudo.

As sementes de *A. hippocastanum* (polpa, pele e casca) foram extraídas por Soxhlet e através de extração assistida por ultrassons, e para otimizar a extração de saponinas das amostras foi utilizada a metodologia de superfície de resposta (RSM). O perfil de saponinas das amostras foi inicialmente caracterizado por cromatografia líquida ultra-rápida acoplada a um detetor de díodos (UFLC-PDA), utilizando a aescina como padrão. Em seguida, e após a obtenção dos pontos ótimos de extração por RSM, a bioatividade da polpa, pele e casca de *A. hippocastanum* foi avaliada. Foi possível identificar saponinas, nomeadamente aescina, nas amostras obtidas por ambos os processos, sendo possível identificar as diferentes isoformas de aescina. Para otimizar a extração das moléculas alvo, foram testados o tempo, a relação solvente/sólido e a concentração dos solventes (etanol e metanol) na extração por Soxhlet; para extração assistida por ultrassons, o tempo, a potência ultrassónica e a concentração de solventes (etanol e metanol) foram testados. Os modelos polinomiais foram ajustados aos dados experimentais, validados estatisticamente e usados para determinar as condições ótimas de extração. No geral, a extração de saponinas foi afetada por todas as variáveis independentes.

Após a obtenção dos pontos ótimos para a extração das saponinas, foram efetuados os dois tipos de extração em estudo para a polpa, pele e casca de *A. hippocastanum*. A bioatividade destes extratos foi avaliada, nomeadamente a atividade antimicrobiana, a citotoxicidade em células tumorais humanas e em células não tumorais, e o potencial anti-inflamatório. Em relação às propriedades antimicrobianas, as bactérias gram-positivas testadas e o fungo *Aspergillus brasiliensis* foram os patógenos mais sensíveis a este tipo de extratos. No entanto, uma vez que não foi observada relação entre o tipo de extração ou solventes, tal pode ser devido a possíveis efeitos sinérgicos entre saponinas e outros compostos bioativos. Em relação à atividade citotóxica e ao potencial anti-inflamatório, os valores mais promissores foram obtidos para os extratos da casca da castanha da Índia.

No geral, este trabalho baseou-se na recuperação de moléculas de valor acrescentado a partir de bio-resíduos atualmente sem aplicação ou valor económico, promovendo a reciclagem de materiais e o desenvolvimento sustentável, além de ser um estudo abrangente das metodologias de extração de compostos bioativos de interesse. Os resultados deste estudo podem ser explorados por empresas interessadas em ingredientes naturais ricos essencialmente em saponinas, como as indústrias farmacêutica, cosmética e alimentar.

Palavras-chave:

Castanha-da-Índia; Saponinas; RSM; Técnicas analíticas; Metodologias "verdes" e emergentes; Potencial bioativo.

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List of abbreviations

ANOVA	Analysis of variance
AGS	Gastric adenocarcinoma
CaCo-2	Colorectal adenocarcinoma
CIMO	Centro de Investigação de Montanha
$FeSO_4$	Iron(II) sulfate
GC	Gas Chromatography
GI_{50}	Extract concentration that inhibits the cell growth by 50%
H_2O	Water
IC_{50}	Extract concentration that causes 50% inhibition of nitric oxide production
INT	Iodonitrotetrazolium
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LOD	Limit of detection
LOQ	Limit of quantification
MBC	Minimum bactericidal concentration
MCF-7	Breast adenocarcinoma
MFC	Minimal fungicidal concentration
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NED	N- (1-naphthyl) ethylenediamine hydrochloride
NO	Nitric oxide
PBS	Phosphate buffered saline
PLP2	Porcine liver cell culture
RSM	Response surface methodology
SD	Standard deviation
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
UAE	Ultrasound-assisted extraction

UFLC-PDA	Ultrafast liquid chromatography coupled to a photodiode array detector
UV	Ultraviolet
WHO	World Health Organization

I Introduction

According to WHO (World Health Organization), about 80% of the population living in developing countries uses only conventional medicines to treat various diseases or as a health-promoting agent (Neergheen-Bhujun, 2013). For the preparation of herbal extracts, used for various purposes, it is possible to use all parts of the plant, such as seeds, roots, leaves, fruits, skin, owers, or even the entire plant. These plants (or parts), rich in bioactive compounds, could be used as medicinal agents due to their direct or indirect therapeutic effects. These compounds present in plants raw material will act mainly in a synergistic way, preventing some diseases, promoting the well-being, or even contributing (as co-adjuvants) to some disease's treatment (Neergheen-Bhujun, 2013), (Shaw et al., 2012).

Aesculus hippocastanum L., commonly known as horse chestnut, is a large tree native to Southeastern Europe belonging to the Sapindaceae family, widely used as an ornamental and landscape plant parks and gardens due to its beauty and hardiness. Trees grow up to 30 m and a thickness of 1 m, with a short stem having a rounded crown (Shaffique et al., 2019).

A. hippocastanum L., (pulp, bark and skin) is listed as a medicinal plant in pharmacopoeias in many countries, including Portugal. Saponins stand out as one of the main bioactive compounds present in the seeds of horse chestnuts. These molecules are responsible for the bitter taste of the seeds, which is why they are considered inedible. One of the most well-known saponins present in this species is aescin, a complex mixture of more than 30 triterpene glycosides. Among the triterpene glycosides that make up aescin, β-aescin, a mixture of two glycosides (aescin Ia and aescin Ib), prevails. Although mainly present in seeds, aescin may be found in small amounts in leaves, bark, and pericarp of immature fruits (Štajner et al., 2014).

Saponins are natural surfactant molecules, and their extracts have high interfacial activity. The hydrophobic glycine and hydrophilic sugar residues that establish its heterogeneous amphiphilic composition are the basis of this natural surfactant. The interfacial behaviour of saponins allows their use in several formulations, including for food application (Böttcher & Drusch, 2016). It is important to highlight that saponin-rich extracts can have several interfacial properties, even when they have the exact botanical origin (Pagureva et al., 2016). Besides these properties, saponins are also known for their bioactive potential, namely, immunostimulant, hypocholesterolemic, antitumor, anti-inflammatory, antibacterial, antiviral, antifungal, and antiparasitic activities (Sparg et al., 2004).

The unsaponifiable fraction of *A. hippocastanum* is rich in sterols. Sterols, namely phytosterols, are constituents of plant cell membranes, found in high quantities in vegetable oils and fats, cereals and cereal products, vegetables, fruits, and berries. More than 200 types of phytosterols have been found in different plant species (Lagarda et al., 2006; Schuler et al., 1991). These molecules are composed of 28 or 29 carbon atoms in the main structure. They resemble structural cholesterol (four-ring steroid nucleus, 3-hydroxyl group, and sometimes a 5, 6-double bond) and functional cholesterol (stabilisation of phospholipid bilayers in cell membranes). There is a side chain composed of 9 to 10 carbon atoms in most phytosterols, while cholesterol has a side chain consisting of 8 carbon atoms (Lagarda et al., 2006). Sterols are also considered to provide several health benefits, including anti-cancer activity (Shahzad et al., 2017), anti-inflammatory potential (Shaffique et al., 2019), antioxidant and neuroprotective properties (Rui et al., 2017), and cardiovascular protection (Orem et al., 2017).

Given the therapeutic potential associated with plants and their availability, often seasonal, and possible impacts of their harvest on the environment and its ecosystems, researchers feel the need to decrease their massive use. This fact, associated with the high amounts of bio-residues generated in plant cultivation or further in the industrial sectors of diverse areas, led to the growing interest in bio-residues as a source of bioactive compounds. In this way, it is possible to combine concepts such as valorisation of natural resources, sustainability and circular economy, reducing the amount of bio-residues generated by the various manufacturers and the costs associated with their disposal (Cherubin et al., 2018).

The present work is based on these principles, insofar as it intended to extract bioactive molecules, particularly saponins, from bio-residues generated worldwide, given the wide ornamental use of horse chestnut. As above mentioned, *A. hippocastanum* is often found as an ornamental tree in urban communities and grassland fields, generating large amounts of residues (fruits/seeds). However, nowadays, these end up, in the great majority, in landfills, without any use or economic value (Kapusta et al., 2007a).

1.1 Natural matrices and bio-residues as a source of bioactive compounds

Plants have played a nutritional and therapeutic role for humans for many years (Akinyemi et al., 2018). The growing concern and awareness of the need for a balanced diet, the problems associated with possible imbalances, and the advances in technology have changed the notions of food, health, and agriculture (Food and others, 2017). Plants contain a variety of active substances, comprising primary and secondary metabolites. They form the basis of many health formulations, such as nutraceuticals, medicines, or cosmetics (Seremet et al., 2018). Nowadays, it is also considered

that agricultural residues may constitute biomass that can be reused to obtain compounds of interest, adding value to this "waste", and reducing the impact of its disposal. Several natural and derivative materials are included, such as woody and herbaceous plants, wood waste, energy crops, agricultural and industrial waste, paper waste, municipal solid residues, grass, food production residues, animal residues, algae, aquatic plants, etc. Every year, the amount of these residues continues to reach very high levels (Mythili et al., 2013).

The interest in the horse chestnut residues, namely in the tree bark, buds, or seeds, arises due to bioactive compounds present in this matrix (Baraldi et al., 2007). Some references report that horse chestnut has traditionally been used for animal feed, as a valuable source of nutrients for deer, and consumed as part of the diet by some native American people (*Chestnuts Worldwide*, 2021). However, in multiple randomised clinical trials, horse chestnut has been studied to demonstrate that the tree has medicinal properties, presenting itself as a plant of medical/clinical interest (Sirtori, 2001). Given the surface-active properties, *A. hippocastanum* extracts are commonly used in the cosmetic and pharmaceutical sectors, with the most active constituents being Oleane-type Saponins (Kapusta et al., 2007b). Nevertheless, it is essential to highlight that certain molecules, namely saponins, are considered toxic, so their consumption is not advised. Therefore, this plant is attractive from the point of view of the extraction of bioactive compounds of interest (Altemimi et al., 2017).

1.2 Botanical and chemical characterization of *Aesculus hippocastanum* L.

There are twelve species in the genus *Aesculus*, which are divided into four subgenera: *Hippocastanum*, *Pavia*, *Calothyrsus*, and *Macrothyrsus*. These twelve species are also divided into five sections based on bud viscidity, fruit exocarp ornamentation, ower colour, and petal morphology. There are two Eurasian species commonly used in medicine: *A. hippocastanum* (common horse chestnut) and *A. chinensis* var. *chinensis* (Chinese horse chestnut) (Zhang et al., 2010). **Figure 1** illustrates the *A. hippocastanum* plant, showing its fruits and seeds.

Figure 1. *Aesculus hippocastanum* L. plant, with evidence in its fruits and seeds (source: istockphoto).

1.2.1 Botanical characterization of *Aesculus hippocastanum* L.

Aesculus hippocastanum L. is a plant found in southeast Europe's forests, belonging to the Sapindaceae family, and it is usually referred to as 'Kastana and horse chestnut (Shaffique et al., 2019). The tree is best grown separately in full sunshine, although it also grows well in the shade. Although it is not a winter-hardy tree species, this genus can withstand winter temperatures below -20 ºC, demonstrating that this tree species also tolerates low temperatures in winter (Jarzębski et al., 2019). This is a large tree growing to about 30 meters and is renowned for its beauty. There are 3-7 leaflets on the leaves, each of which is 13-30 cm long. At the base of the petals, the owers are generally white with a yellow to pink blotch. A green, spiky capsule containing one nut-like seed called conkers or horse chestnut seed usually develops 1-5 fruits on each panicle (Shaffique et al., 2019). The common name horse chestnut may have originated from the traditional Turkish and Greek use of seeds for horses to treat overexertion or coughing (Zhang et al., 2010). **Table 1** shows the taxonomic classification of *A. hippocastanum*.

Table 1. Taxonomic classification of *Aesculus hippocastanum* L. (Vieu & Vieu, 2014).

Kingdom	Plantae
Division	Spermatophyta
Sub-division	Magnoliophytina (Angiospermae)
Class	Magnoliopsida
Subclass	Rosidae
Order	Sapindales
Family	Sapindaceae
Genera	*Aesculus*
Species	*A. hippocastanum*

A. hippocastanum has been used in folk medicine over the years. It has been used to treat coronary artery disease and treat chronic venous insufficiencies, such as pain, heaviness, and tension in the legs (Busia, 2016). The bark and leaves of horse chestnut have been employed as an astringent to treat diarrhoea and haemorrhoids (Wichtl, 2004). Because of its pharmacological profile, this plant is often used in Portugal for the same purposes, namely: i) constricting properties in the congestive states of the venous system, haemorrhoids, varicose veins, phlebitis, haemoptysis; ii) action on endometrial vessels; and iii) anticoagulants, coagulants (Botanical Garden, 2021). There are different ways of using this plant in Portugal: i) bark decoction or infusion is used internally to fight uterine and hemorrhoidal haemorrhages, chronic bronchitis, digestive system inflammation, arthritis, neuralgia and rheumatism; ii) the external use of this infusion is used to treat eczema, wounds and burns; iii) alcoholic infusion of dried owers is used against rheumatic pain, neuralgia and arthritis; iv) seed oil is often used for human consumption and starch is sometimes used for human consumption

after washing with alkaline water; and v) seed is often used in cosmetics and pulp for soap processing (*Serralves Foundation*, 2021).

1.2.2 Chemical characterization of *Aesculus hippocastanum* L.

Aesculus hippocastanum L. seeds are natural products of a very complex chemical structure. Besides the well-known aescin, the seeds also contain coumarin glycosides, flavonoid derivatives (including glucosides of quercetin and kaempferol), starch, tannins, oil, cellulose, protein (adenine, adenosine, guanine, uric acid), sucrose, glucose, or proanthocyanidins (including leucoanthocyanidin and catechin). It is also possible to find salts of calcium, magnesium, copper, and manganese, methionine, B group vitamins, vitamin C, vitamin K, and provitamin D (Baraldi et al., 2007; (Kalil, 2009).

The horse chestnut seed is considered to be a recalcitrant seed. Starch has the highest incidence in the seed while fats, which play a significant role in the pharmaceutical industry, come second. Proteins has the lowest incidence in the horse chestnut seed, which, besides its structure, plays a role in the prevention of excessive drying up of the seeds (Mladenovic & Kurjakov, 2011). The oil consists of oleic, linoleic, palmitic, stearic, and linolenic acids. Sterols, triterpenes, aliphatic alcohols, vitamins, hydrocarbons, pigments, among other compounds are part of the unsaponifiable portion.

The seed pericarp contains hippocastanoside, a compound that differs from aescin essentially in the aglycone portion (Baraldi et al., 2007). **Figure 2** displays the general chemical characterisation of *A. hippocastanum*.

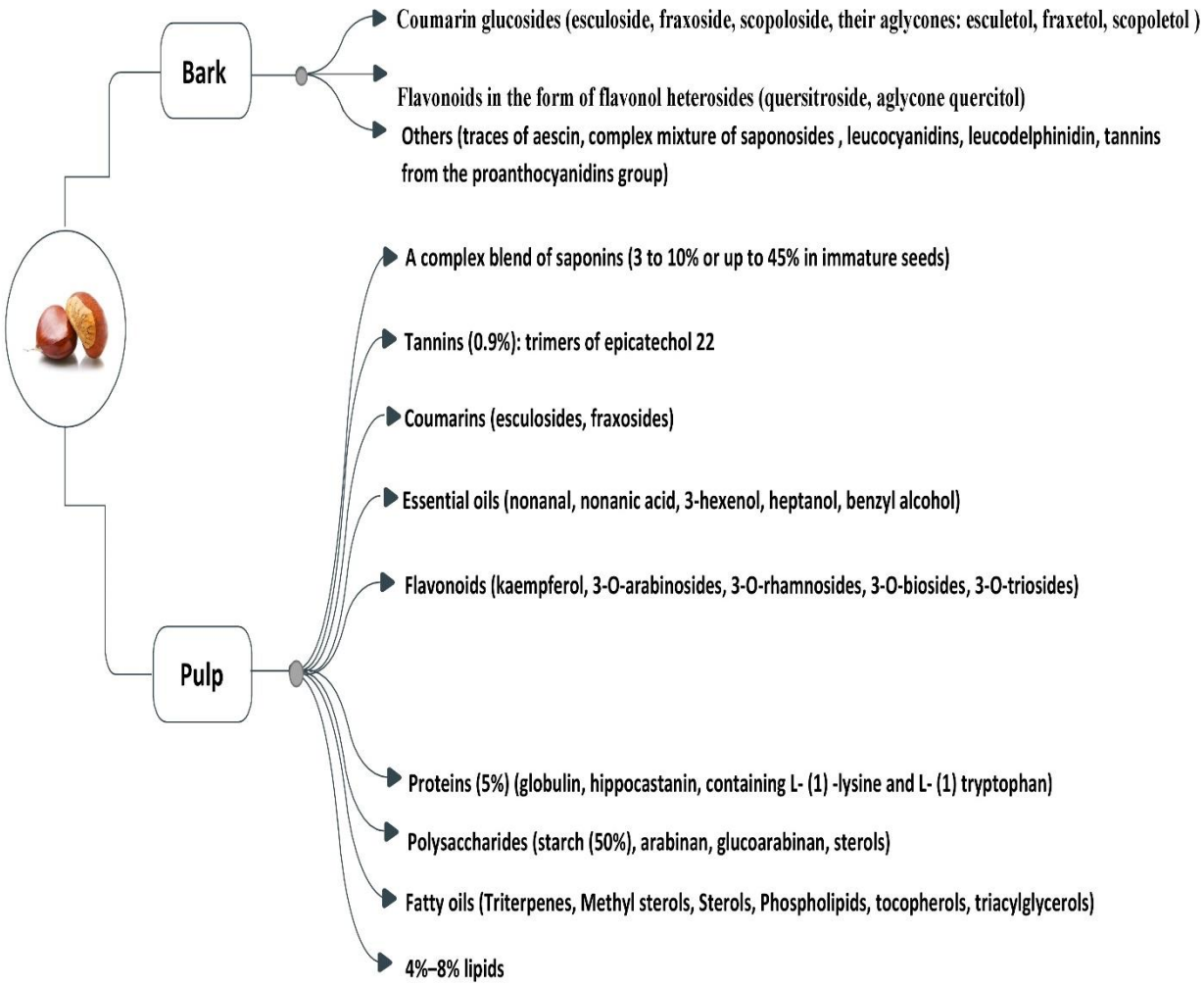


Figure 2. General chemical characterization of *Aesculus hippocastanum* L. (Vieu & Vieu, 2014).

1.3 Secondary metabolites from *Aesculus hippocastanum* L. with bioactive interest

Secondary plant metabolites are categorized into many classes based on their chemical structures. The secondary metabolites of the species under study were explored based on the present study's objectives, namely focusing on constituents with therapeutic effects, particularly saponins and sterols.

Due to restrictions imposed by the measures of the current pandemic and problems with the chromatographic equipment, it was not possible to achieve the proposed goals for the sterols analysis. However, the theoretical concepts referring to these molecules will be discussed in the following sections.

Each following section will begin by presenting the definition of the class of secondary plant metabolites, including their structure, botanical distribution and pharmacological applications, before moving on to representative examples of the molecules(Seremet et al., 2018).

1.3.1 Saponins chemical aspects and bioactive properties

As a result of increased interest and intense research activity in food micro-components of plant origin, our knowledge of saponins application in food has risen significantly in recent years (Guclu-Ustundag & Mazza, 2007). While historically regarded as anti-nutrients, due to recent findings suggesting a good range of bioactive properties, most likely due to their aglycones' structural variability, there is a growing interest in saponins (Francis et al., 2002). Therefore, a wide variety of applications in the pharmaceutical, food and cosmetic industries may be expected (Sparg et al., 2004). For example, horse chestnut saponins have been used as an astringent in the treatment of diarrhoea and haemorrhoids. The leaves and seeds have anti-inflammatory properties, while the bark and twigs have been used to cure dermatitis. The seed extracts are also utilized in a variety of cosmetics (such as shampoo, cream, and toothpaste) (Foca et al., 2011; Zhang et al., 2006).

Saponin aglycons (sapogenins) contain one or more hydroxyl groups, in addition to the carbonyl and carboxyl groups. Generally, the sugar components are attached at the sapogenins C-3 position. Moreover, saponins can be characterized according to the number of sugar chains, as monodesmosidic and bidesmosidic saponins. Monodesmosidic saponins are steroidal and triterpenoid saponins that have the sugar unit connected through an acetal bond at C3. On the other hand, bidesmosidic saponins, found among the triterpenoid saponins of the oleanane type (with bonds at C-3 and C-28) and steroidal saponins of the furostane type (with bonds at C-3 and C-26), have two sugar units attached (Guclu-Ustundag & Mazza, 2007; Yabe et al., 2003). Moreover, there are two forms of triterpenoid saponins: neutral and acidic. Neutral, when a typical sugar is attached to the sapogenin, and acidic, when the sugar moiety contains uronic acid or one or more carboxylic groups attached to the sapogenin (Lásztity et al., 1998).

The principal active component in horse chestnut is aescin (**Figure 3**), which is responsible for most of its therapeutic benefits (Kędzierski et al., 2016). Some researchers have already studied the presence of Saponins, namely aescin, in horse chestnuts. They analysed the variations in these secondary metabolites throughout time to evaluate when they could obtain the highest quantities of bioactive compounds. They had found that when horse chestnut was harvested 12 to 20 weeks after it bloomed, the level of aescin increased by up to 90%, considerably increasing the amount of saponins that could be removed. Aescin (also known as escin) contain two major groups of isomers, with different properties and activities, namely β-aescin, the main active compound of aescin, containing aescin Ia and aescin Ib; and α-aescin, containg isoaescin Ia and isoaescin Ib (Foca et al., 2011). Aescin derived from horse chestnut has been shown to have an anti-obesity effect (Sirtori, 2001). β-aescin is a natural inhibitor of leukemic cell growth that could be useful in the development

of new anti-leukemic medications. It has also been proven to be useful in the treatment and prevention of colon cancer (Foca et al., 2011).

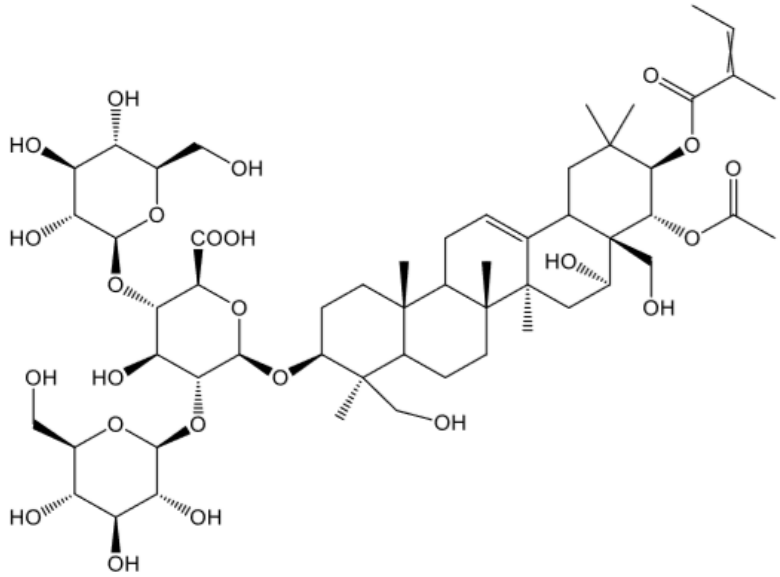


Figure 3. Chemical structure of the main component of aescin (Kędzierski et al., 2016).

Saponins are high molecular weight amphiphilic compounds consisting of a hydrophobic aglycone (triterpenoid or steroid), designated as sapogenin, linked to one or more hydrophilic sugar moieties through an ether or ester glycosidic linkage, at one or two glycosylation sites (Guclu-Ustundag & Mazza, 2007). A trisaccharide chain is connected to C3 of the aglycone and a -glucopyranosyl unit is attached to C4 of the glucuronic acid in horse chestnut saponins (Zhang et al., 2006). According to the chemical structure of the sapogenin, saponins can be classified into steroidal or triterpenoid saponins. There are two major forms of steroidal saponins: the spirostane (**Figure 4a**) and furostane (**Figure 4b**) (Lásztity et al., 1998).

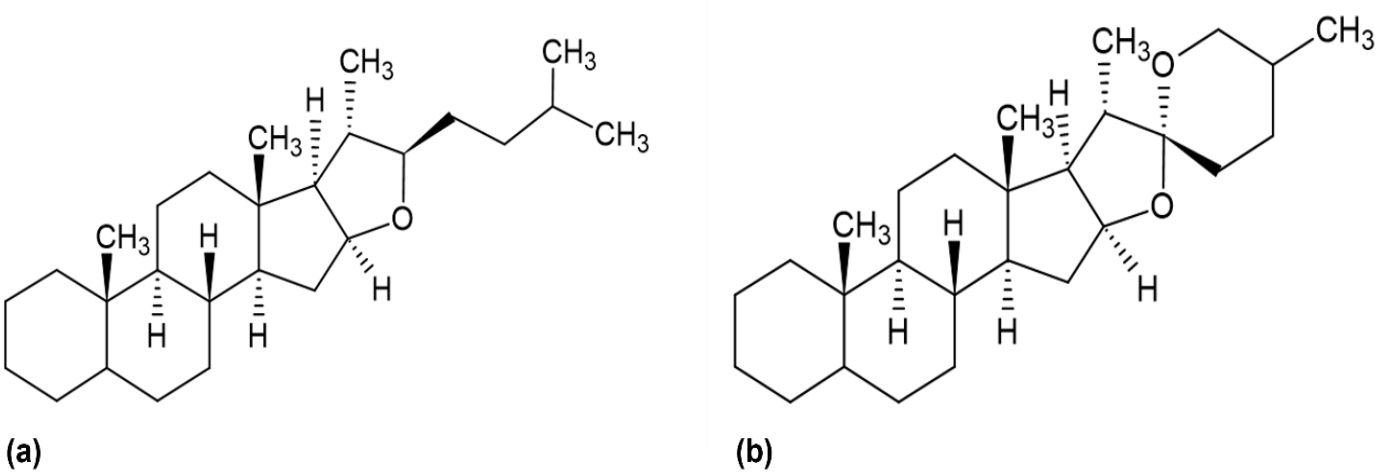


Figure 4. Chemical structure of a) spirostane and b) furostane (Lásztity et al., 1998).

Triterpenoid saponins (**Figure 5**) consist mainly of a C30 pentacyclic skeleton, commonly as oleanane (**Figure 5a**) and ursane (**Figure 5b**) structures, or C30 tetracyclic skeleton, as dammarane (**Figure 5c**) structures (Sparg et al., 2004), and are mainly found in the dicotyledonous (*e.g.* families Sapindaceae, Araliaceae, or Primulaceae) (Garai, 2016).

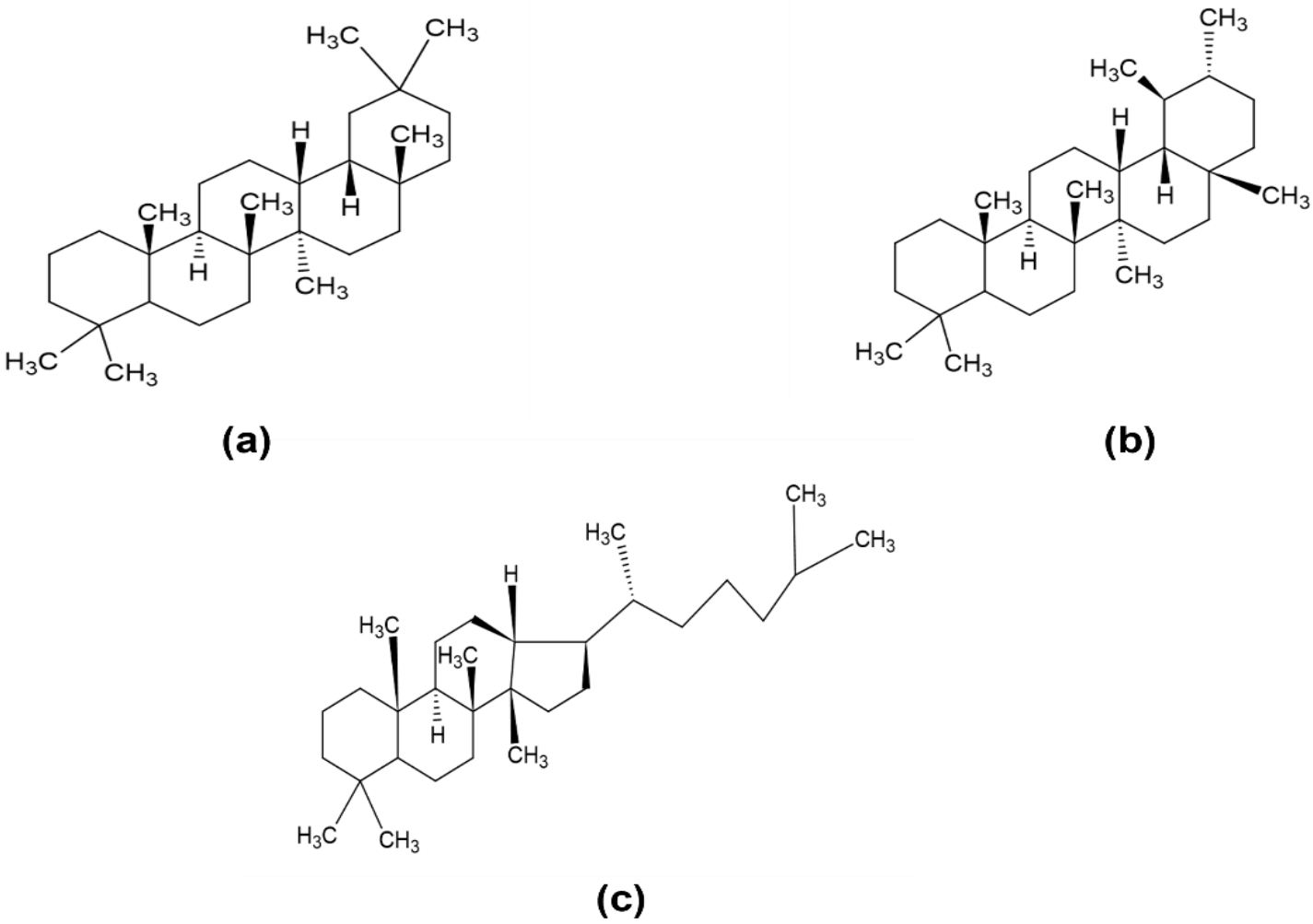


Figure 5. Chemical structures of a) oleanane, b) ursane, and c) dammarane (Sparg et al., 2004)

1.3.2 Sterols chemical aspects and bioactive properties

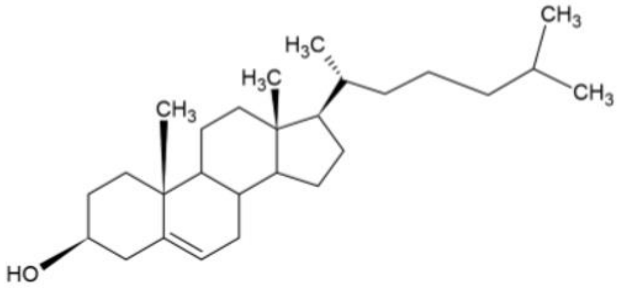
Due to their excellent cholesterol-lowering capacity, plant sterols have attracted growing attention. However, free plant sterols have specific characteristics of low solubility in oil, insolubility in water, high melting point, and low bioavailability, which restrict their food use (Jarzębski et al., 2019). Nevertheless, its application has been possible in drugs (production of therapeutic steroids), cosmetics (creams, lipstick), nutraceuticals (anti-cholesterol additives), and even as functional components in some foods (Fernandes & Cabral, 2007).

Sterols may be classified according to their source as phytosterols (plant origin), zoosterols (animal origin) and mycosterols (fungal origin) (Rysz et al., 2017). All sterol forms share a common

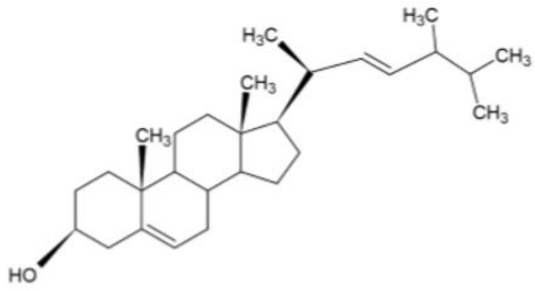
chemical backbone structure and vary in the number and position of double bonds or side carbon chain lengths. Based on the difference in the number of carbon-based methyl groups, plant sterols are further divided into 4,4-dimethyl sterols, 4α-monomethyl sterols, and 4-desmethyl sterols.

In nature, 4,4-dimethyl sterols and 4-monomethyl sterols are quantitatively minor, whereas 4-desmethyl sterols are quantitatively abundant (Brufau et al., 2008). These include campesterol, β-sitosterol, Δ5-svenasterol, stigmasterol, and brassicasterol (He et al., 2018**;** Phillips et al., 2002). β-Sitosterol and campesterol, have an ethyl and a methyl group at C-24, respectively, whereas stigmasterol is identical to β-sitosterol but with a double bond at C-22. These are represented in **Figure 6**. Plant sterols are structurally similar to cholesterol, with the exception of an extra methyl (campesterol) or ethyl (sitosterol) group at the C-24 position on the side chain (Igel et al., 2003). These phytosterols may be also divided into five different types: free sterols, esterified sterol, esters, sterol glycosides, and glycosides of acylated sterols (Moreau et al., 2002). The main bioactivities associated with plant sterols are lowering total blood cholesterol (TC) and low-density lipoprotein (LDL) by inhibiting intestinal cholesterol absorption (Plat & Mensink, 2005).

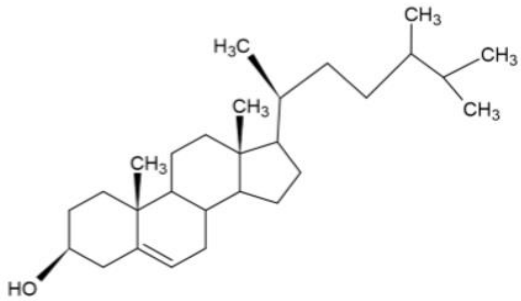
Regarding *A. hippocastanum*, the total sterol content has been reported around 8 g/kg of oil, with a prevalence of free sterols over esterified sterols (Zlatanov et al., 2013).



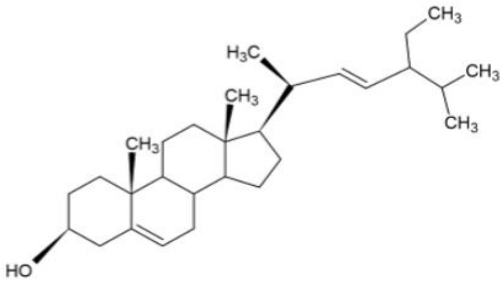
(a) Cholesterol.



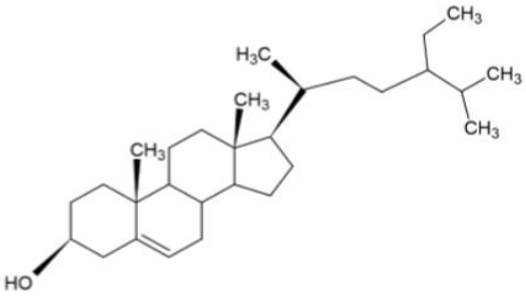
(b) Brassicasterol.



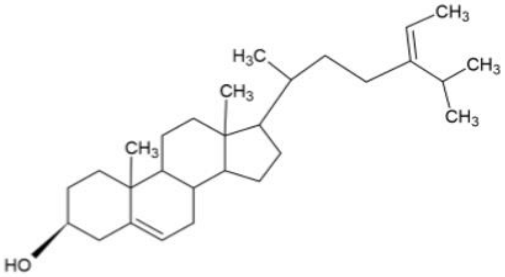
(c) Campesterol.



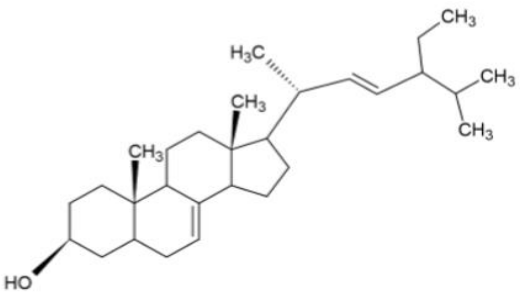
(d) Stigmasterol.



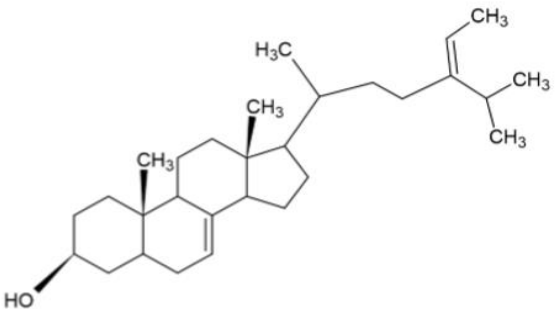
(e) β-Sitosterol.



(f) Δ^5-Avenasterol.



(g) Δ^7-Stigmasterol.



(h) Δ-Avenasterol.

Figure 6. Chemical structure of different sterols (Zlatanov et al., 2013).

1.4 Techniques for extracting saponins and sterols

The separation of one or more molecules from an organism, based on their chemical or physical properties, is defined as extraction. The extraction of certain substances could lead to the discovery of new drugs. If a molecule reveals promising results in a particular area, it could become the target for developing new formulations with specific benefits (Sasidharan et al., 2011).

The identification and characterisation of bioactive compounds is a significant challenge after plant extracts have been obtained. Most plant extracts occur as a mixture of various types of bioactive compounds or phytochemicals with different polarities. A simple, fast, and inexpensive technique is a phytochemical screening assay that gives the researcher a quick response to the different types of phytochemicals or secondary metabolites found in plants (Sasidharan et al., 2011).

1.4.1 Soxhlet extraction

Soxhlet is a classic solid-liquid extraction technique. Its main advantage is that the sample contacts a fresh portion of solvent continuously, which helps move the balance of transfer to the solvent. Moreover, after extraction, this method does not need filtration and is independent of the vegetable matrix. Compared to other methods, the most significant disadvantages of this process are the long time and the vast amount of solvent consumed, contributing not only to economic losses, but also to environmental issues. The risk of thermal degradation of the compounds is not negligible if the plant material contains thermolabile molecules, because samples are heated at high temperatures for a long period of time. The subsequent evaporation/concentration step is limited given the large amount of solvent used. In addition, this process is constrained in terms of solvent selectivity and is not easily automatable (Luque de Castro & Priego-Capote, 2010; Sarvin et al., 2018).

1.4.2 Ultrasound-assisted extraction

Ultrasound-assisted extraction is a process that is simple, productive, and almost inexpensive. The most important advantages are the improvement in extraction effectiveness and the acceleration of kinetics. Compared to some traditional techniques, it allows working at relatively low temperatures, preventing compound thermal degradation. This methodology is usually easy to implement and to apply. Like Soxhlet extraction, the extraction by sonication enables the use of a broad variety of solvents to obtain several natural compounds. However, the effect of the ultrasound extraction on the extraction efficiency and kinetics, is related to the matrix. The presence of a dispersed phase contributes to the attenuation of the ultrasonic waves and the active zones in the extractor remain close to the emitter of the ultrasound. During the operation, this method does not

allow the solvent to be renewed. The limiting step after extraction is the filtration and rinsing (Esclapez et al., 2011; Yoong Cheok et al., 2014).

1.4.3 Extraction with supercritical fluids

An alternative to the use of chemical solvents is the extraction with super critical fluids, such as carbon dioxide (CO_2). Super critical CO_2 has been used for the extraction and separation of high added value natural products. Near the critical point, the solvent power is sensitive to variations in temperature and pressure. It is straightforward to isolate the compounds using this technique simply by reducing the pressure. Besides, the transport properties of super critical fluids (*e.g.* viscosity and diffusivity) make it possible to penetrate more deeply into the solid plant matrix and thus, to extract more effectively and rapidly (Esclapez et al., 2011).

1.5 Chromatographic separation techniques

Many chromatographic separation techniques, such as thin-layer chromatography (TLC), column chromatography, ash chromatography, ultrafast liquid chromatography coupled to a photodiode array (UFLC-PDA), can be used in the isolation of these bioactive compounds to obtain pure compounds. Several methods of analysis have been developed to promote the structural determination of bioactive compounds, including TLC, UFLC-PDA, LC/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS), capillary electrophoresis, ion spray mass spectrometry (MS), gas chromatography/MS (GC/MS), and nuclear magnetic resonance (Jeong et al., 2012). MS provides highly specific details, such as accurate mass, isotope distribution patterns for elemental formula determination, and characteristic fragment ions for structural elucidation or identification by spectral matching to authentic compound data. In addition, the high sensitivity of MS allows detection and measurement of picomole to femtomole levels of many primary and secondary metabolites (Contrepois et al., 2015).

II Scope and objectives

Innovative and environmental-friendly extraction techniques aim to increase yield and sustainably achieve maximum compounds production. However, as we can see in **Figure 7**, even in recent times, conventional methods often appear as an alternative to "greener" technologies when it should be the other way around.

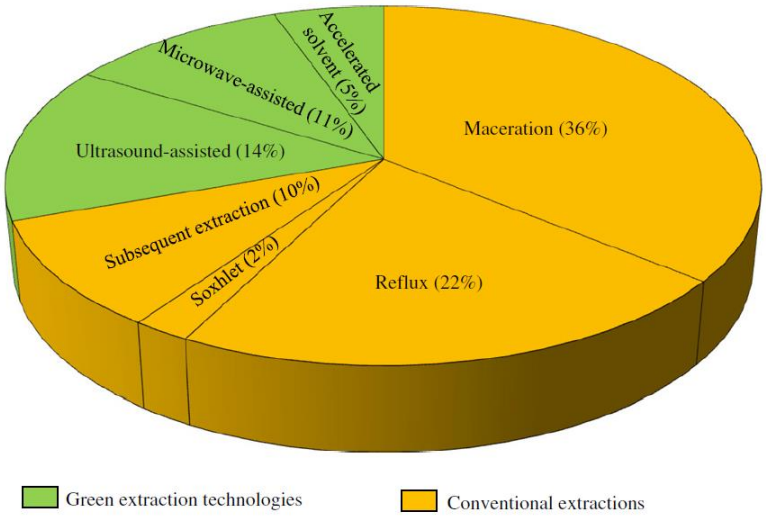


Figure 7. Extraction techniques employed in the extraction of saponins from plant materials (Yoong Cheok et al., 2014).

The present work intended to value the species *Aesculus hippocastanum* L., commonly known as horse chestnut, as a source of bioactive compounds, namely saponins. The fruits/seeds of this tree are not edible and currently do not present any economic advantage; they are bio-residues generated from ornamental plants, hence the increased interest of this project.

Although research is increasingly focused on adopting "green" methodologies for extracting bioactive compounds, the truth is that conventional technologies are still widely used. So, this work intends to understand the fundamental differences in terms of obtaining saponins, comparing traditional and innovative techniques.

Therefore, the specific objectives of this project were: i) optimization of saponin extraction from *A. hippocastanum* seeds using a conventional (Soxhlet) and a more innovative technique (ultrasound-assisted extraction), comparing the results obtained from each one; ii) evaluation of the bioactive properties of the most promising saponin-rich extracts obtained; iii) characterization of the unsaponifiable fraction of *A. hippocastanum*, regarding the sterol profile.

The response surface methodology (RSM) was used to optimize the extraction of saponins from the seed samples. RSM corresponds to a set of mathematical and statistical techniques useful for modelling and analysing problems in which the response is influenced by several variables. It is a statistically designed experimental protocol in which several factors are simultaneously varied to obtain the optimal extraction conditions for the desired compounds (Desai et al., 2008).

III Material and methods

3.1 Sampling of A. hippocastanum seeds

The fruits of *A. hippocastanum* L. (approximately 4 kg) were harvested in the orchard of the School of Agriculture of Bragança. Afterward, the fruits were peeled to conduct a more detailed study, dividing the seeds into bark, skin, and pulp (**Table 2**, **Figure 8)**. Finally, the samples were frozen, lyophilized, reduced to a fine dried powder (~20 mesh), and stored in a place protected from light and temperature for subsequent analyses.

Table 2. Harvested quantity of *Aesculus hippocastanum* L. fruits

Fruit part	Fresh fruits (g)	Lyophilized fruits (g)
Bark	58,18	49,22
Skin	146,32	138,92
Pulp	1844,33	1432,83

Figure 8. *A. hippocastanum* L. fruit.

3.2 Chemical characterization

3.2.1 Saponins extraction

To implement saponins extraction at the laboratory level, two different techniques were carried out and compared, namely extraction by Soxhlet (**Figure 9**) and ultrasound-assisted extraction

(UAE; **Figure 10**). The optimization was carried out using the horse chestnut pulp since there was a greater amount of this part of the sample.

The optimization protocol was performed using Design Expert 11 (Stat-Ease, Minneapolis, MN, USA), and relied on the Response Surface Methodology using Box-Behnken models for the design of experiments followed by an optimization of the maximization of the response.

Figure 9. Soxhlet apparatus used to extract saponins.

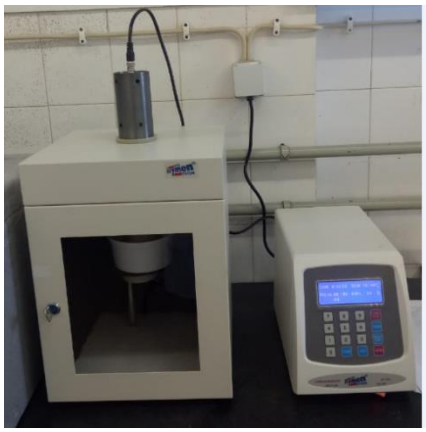


Figure 10. Ultrasound equipment used for UAE extraction.

Soxhlet extraction

The Soxhlet extraction of the powdered samples was performed according to **Table 3**. Different parameters were considered to elaborate the experimental design: solvent proportion (ethanol and methanol); solvent/solid ratio; and time of extraction. After extraction, the organic solvents were evaporated in a rotary evaporator (R-210 rotary evaporator, Büchi, Flawil, Switzerland)

under reduced pressure to remove the solvent. All the aqueous extracts were frozen and lyophilized for further analyses.

Table 3. Experimental design for Soxhlet extraction.

Ethanol / Methanol			
Run	**Solvent (%)**	**Time (min)**	**Solvent/solid ratio (%)**
1	50	375	27.5
2	50	720	50
3	100	375	50
4	100	375	5
5	100	30	27.5
6	50	30	5
7	50	375	27.5
8	0	720	27.5
9	50	375	27.5
10	50	720	5
11	50	30	50
12	0	375	5
13	50	375	27.5
14	100	720	27.5
15	0	30	27.5
16	0	375	50
17	50	375	27.5

Ultrasound-assisted extraction

The ultrasound-assisted extraction (UAE) of the powdered samples was performed according to **Table 4**, using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) comprising an ultrasound power in the range between 50 and 500 W at a frequency of 20 kHz, equipped with a digital timer.

Different parameters were considered to elaborate the experimental design: solvent proportion (ethanol and methanol); ultrasonic power; and time of extraction. Based on the literature, a ratio solvent/solid of 75% was used and maintained for all the extracts. After extraction, the resulting suspension was filtered through filter paper (Whatman No. 4), and the organic solvents were evaporated in a rotary evaporator (R-210 rotary evaporator, Büchi, Flawil, Switzerland; **Figure 11**)

under reduced pressure to remove the solvent. All the aqueous extracts were frozen and lyophilized for further analyses.

Table 4. Experimental design for UAE extraction.

Ethanol / Methanol			
Run	**Solvent (%)**	**Power (%)**	**Time (min)**
1	0	275	75
2	100	500	38.5
3	0	275	2
4	50	50	2
5	50	500	2
6	50	275	38.5
7	50	50	75
8	50	500	75
9	50	275	38.5
10	0	500	38.5
11	50	275	38.5
12	50	275	38.5
13	0	50	38.5
14	50	275	38.5
15	100	275	2
16	100	50	38.5
17	100	275	75

Figure 11. Rotary evaporator equipment.

After obtaining the dried extracts, these were dissolved in water, ethanol, or methanol according to the extraction procedure. The extracts obtained after extraction with pure solvents were dissolved in the corresponding solvent (1 mL). The extracts obtained by mixtures ethanol: water or methanol: water were dissolved in ethanol: water (1 mL; 20:80, v/v) and methanol: water (1 mL; 20:80, v/v), respectively. All the extracts were prepared in a concentration of 20 mg/mL, filtered through a 0.2 µm disposable LC nylon disk filter and transferred into an injection vial to be analyzed by UFLC-PDA.

3.2.2 Saponins chromatographic analysis

The saponins analysis was performed for all the samples understudy for the optimization design, using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan; **Figure 12**). The chromatographic separation was achieved using a Waters Spherisorb S3 ODS2- C18 (3 µm, 4.6 mm × 150 mm, Waters, Mil-ford, MA, USA) column thermostatted at 30 ºC. 0.1% formic acid in water and (B) acetonitrile were also used as eluents, with the following isocratic elution gradient: 20% B (5 min), 20% B to 25% B (5 min), 25–35% B (5 min), 35–60% B (10 min), 60% B (10 min), 60–25% B (10 min), 25–20% B (10 min), and re-equilibration of the column (10 min), using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 220 nm as preferred wavelength.

Figure 12. Equipment (UFLC-PDA) used for saponins analysis.

3.2.3 Sterols composition

The sterols composition was intended to be evaluated by GC-MS according to the NP EN ISO 12228 method (12228, 2001) (NP EN ISO 12228, 2001). Unfortunately, it was impossible to perform this analysis because of the pandemic restrictions and later due to system malfunction. However, an attempt was made to optimize the isolation and identification procedure for phytosterols in horse chestnuts.

The unsaponifiable fraction was isolated by separation on an aluminum oxide column (prepared with 10 g of aluminum oxide, mixed with 20 mL of ethanol). After decanting the aluminum oxide, the solvent was discarded to the top of the oxide layer. For the extraction of the unsaponifiable matter, briefly, 0.40 g of oil extracted by Soxhlet was saponified with 5 mL of ethanolic potassium hydroxide solution in a volumetric flask; the contents were boiled under reflux for fifteen minutes. After this period, heating was stopped, and 5 mL of ethanol was added to the volumetric flask. Then, an aliquot of 5 mL of the solution present in the flask was removed to add to the interior of the aluminum oxide column; the eluate was collected in a new flask until the solution reached the top of the aluminum oxide layer. The unsaponifiable matter was eluted first with 5 mL of ethanol and then with 30 mL of diethyl ether. Finally, the rotary evaporator was used to remove solvents. For the preparation of trimethylsilyl ethers, 2 mL of diethyl ether were added to the obtained extract, and after transfer to a *vial*, the solvent was evaporated under a nitrogen flow. The final extract was dissolved in the silylation reagent (*N,O*-Bis (trimethylsilyl) trifluoroacetamide (BSTFA with 1% TMCS); 200 µL). The *vials* with the extract dissolved in the silylation reagent were placed in the oven for 15min at 105 ºC. After this period, the *vials* were allowed to cool to room temperature, and the samples should be injected into the GC-MS.

3.3 Bioactivity evaluation

The bioactivity was evaluated in the methanolic and ethanolic extracts obtained both by Soxhlet and UAE. The extraction conditions were applied according to the optimal points given by the RSM design and were applied to the pulp, skin and bark of *A. hippocastanum* (**Figure 13**). For both Soxhlet and UAE extractions, the procedure followed was the previously described (section 3.2.1).

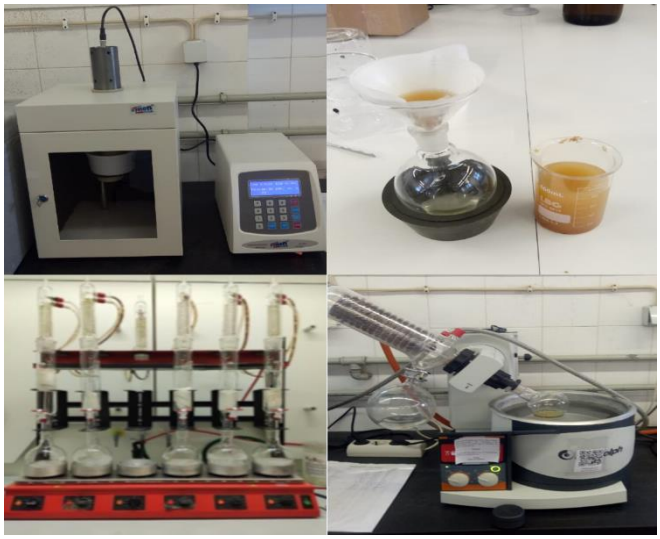


Figure 13. Methanolic/ethanolic extraction of *A. hippocastanum*.

Given the evidence of saponins bioactivity, the antimicrobial (antibacterial and antifungal) properties, the cytotoxic potential and the anti-inflammatory activity of the obtained extracts were evaluated.

3.3.1 Antimicrobial activity

The evaluation of the antimicrobial activity was assessed through the microdilution method (CLSI:MFDASTFBAG; 2015), applied to harmful bacteria and fungus strains. The microbial strains were clinical isolates donated from the Hospital Center of Trás-os-Montes e Alto Douro (Vila Real, Portugal). Five of these strains were Gram-negative bacteria – *Pseudomonas aeruginosa* (isolated from expectoration), *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Morganella morganii* (all isolated from urine), and four Gram-positive bacteria – *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal fluid), and methicillin-resistant *Staphylococcus aureus* (MRSA, isolated from expectoration). The fungi used to test the antifungal potential were *Aspergillus Brasiliensis* and *Aspergillus fumigatus*. The minimal inhibitory concentration (MIC) of the samples was calculated using the quick colorimetric assay with *p*-

iodonitrotetrazolium chloride (INT). MIC was defined as the lowest concentration that inhibits the visible bacterial growth determined by changing colour from yellow to pink if the microorganisms were viable. The minimum bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) were also determined. These were defined as the lowest concentrations required to kill the microorganisms (Pires et al., 2018).

3.3.2 Cytotoxic activity

A. hippocastanum extracts were tested against three human tumour cell lines, namely AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), and MCF-7 (breast adenocarcinoma). Non-tumour cell cultures were also tested: Vero (African green monkey kidney) and PLP2 (primary pig liver culture), and for both, the sulforhodamine B (SRB) assay was performed.

All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL), except for Vero, maintained in DMEM medium supplemented with fetal bovine serum (10%), glutamine and antibiotics. The culture flasks were incubated at 37 ºC, with 5% CO_2, under a humid atmosphere. The cells were used only when they reach 70 to 80% of confluence. A known mass of each of the extracts (8 mg) was dissolved in H_2O (1 mL), to obtain the stock solutions with a concentration of 8 mg/mL. From this stock solution, successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8 mg/mL). Each of the extract concentrations (10 µL) was incubated with the cell suspension (190 µL) of the tested cells in 96-well microplates for 72 hours. The microplates were incubated at 37 ºC, with 5% CO_2, in a humid atmosphere, after checking the adherence of the cells. All the cells were 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 collected: 100 µL of cool TCA (10% w/v) was added to the wells and the plates were incubated for 1 hour at 4 ºC. Afterwards, the plates were washed with water and, after drying, the SRB solution (0.057%, m/v; 100 µL) was added, and 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, the adhered SRB was solubilized with Tris (10 mM, 200 µL) and the absorbance was read at 540 nm in a Biotek ELX800 microplate reader. The results were expressed as the concentration of extract that inhibits the cell growth in 50% - GI_{50}. As a positive control of the use of an ellipticin. **Figure 14**, shows the microplate used in cytotoxicity evaluation.

Figure 14. Microplate used in cytotoxicity evaluation.

3.3.3 Anti-inflammatory activity

The anti-inflammatory activity was performed using the procedure described by (Taofiq et al., 2016). The extracts (8 mg) were dissolved in H_2O to obtain a final concentration of 8 mg/mL. From this stock solution, successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8 mg/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 37 ºC, with 5% CO_2, and under a humid atmosphere. According to the Trypan Blue exclusion test, cells were discharged with a cell scavenger, the experimental density of the cells was set at 5×10^5 cells/mL, and the fraction of dead cells was less than 1% in each experiment. An aliquot of the cell suspension of macrophages (300 µL) was placed in each well (96-well plate). The microplate was incubated for 24 hours with the conditions previously described, to allow an adequate adherence and multiplication of the cells. After that, cells were treated for 1 hour with *A. hippocastanum* extracts, followed by 24 hours of stimulation with lipopolysaccharide (LPS) (30 µL; 1 µg/mL). Controls were made without the addition of LPS to see if they induced changes in nitric oxide levels at baseline (NO). Dexamethasone (50 mM) was used as a positive control and samples in the absence of LPS were used as negative control. A Griess Reagent System Kit including sulfanilamide, N- (1-naphthyl) ethylenediamine hydrochloride (NED), and nitrated solutions was used to test the presence of NO. At room temperature, the cell supernatant (100 µL) was added to the plate and mixed with sulfanilamide and NED solution for 5 to 10 minutes each. The amount of nitric oxide produced was measured at 540 nm using an ELX800 Biotek microplate reader and compared

to a standard calibration curve. The results were calculated through the graphical representation of the percentage of inhibition of nitric oxide production *versus* the sample concentration and were expressed as the concentration of the extract that causes the 50% inhibition of nitric oxide production - IC_{50}.

3.4 Statistical analysis

The results obtained throughout the different evaluation studies were analyzed by applying statistical tools, selected according to the degree of complexity of the results and considering the defined research purposes. The optimization protocol was performed using Design Expert 11 (Stat-Ease, Minneapolis, MN, USA), and relied on the Response Surface Methodology using Box-Behnken models for the design of experiments followed by an optimization of the maximization of the response. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 25 (IBM Corporation, New York, USA). Each preparation employed three samples, and all experiments were performed in triplicate. The results were reported as mean values plus standard deviation (SD), with the number of decimal points determined by the SD's magnitude. The significance level for all of the tests was set as $p<0.05$. When possible, an analysis of variance (ANOVA) was utilized to assess differences between the researched mushroom species or different co-culture situations. Preliminary tests for homoscedasticity (using the Levene test) and normal distribution (using the Shapiro Wilks test) were carried out. The Welch test was used to ensure that statistically significant differences existed. When homoscedasticity was validated or not, the ANOVA results were categorized using the Tukey HSD test or Games-Howell.

IV Results and discussion

4.1 Experimental data for extraction optimization by RSM

For RSM optimization of the Soxhlet and UAE processes, the Saponins (aescin) content obtained with the 17 runs of the experimental design matrix for the horse chestnut seed samples was used as the response variable. The results for both extraction procedures, using both ethanol and methanol are discussed below in **Table 5.**

Table 5. Experimental design for the ultrasound extraction displaying the responses for methanol and ethanol extractions.

Ultrasonic Experimental Design				
Factors			Responses	
Solvent (% v/v) (χ_1)	Power (W) (χ_2)	Time (min) (χ_3)	Saponin Content (g/100g) Methanol (R_1)	Saponin Content (g/100g) Ethanol (R_1)
0	275	75	0.240	0.362
100	500	38.5	5.228	~~2.575~~
0	275	2	0.238	0.049
50	50	2	0.262	0.218
50	500	2	0.196	0.0755
50	275	38.5	0.614	0.673
50	50	75	1.067	0.458
50	500	75	0.456	0.788
50	275	38.5	0.463	0.748
0	500	38.5	0.477	0.254
50	275	38.5	0.674	0.963
50	275	38.5	0.689	0.623
0	50	38.5	0.689	0.0529
50	275	38.5	0.0733	~~0.072~~
100	275	2	0.0733	1.409
100	50	38.5	9.920	1.254
100	275	75	7.821	1.858

The design was based on a 17 run Bex-Behnken model with 5 repetitions (green lines in the tables) of the center point to improve the precision of the model. **Table 5** shows the experimental design in random order, including the three varying factors solvent (χ_1 (A) varying between 0 and 100%), ultrasonic power (χ_2 (B) varying between 50 and 500 W) and time (χ_3 (C) varying between 2 and 75 minutes), as well as the response, namely saponin content (Y1). The design was the same for

both solvents thus, the presentation of the response for the extraction with ethanol and methanol. The green highlighted factors correspond to the center point repetitions.

Considering the first assay, the optimization of saponin yield using methanol, the data needed a natural log transformation to reach a quadratic model, allowing for a R^2 of 0.83 (meaning that 83% of the variability is explained by three factors) and an adjusted R^2 of 0.62 (results from an adjustment of R^2 to the model and its terms), a non-significant lack of fit and an adequate precision of 6.2. The model determined the following **Equation 1**:

Equation 1. Equation of coded factors of Saponin content for ultrasound extraction with Methanol

$$Ln(Saponin\ Content) = -0.0270 + 0.92 * \chi_1 - 0.2689 * \chi_2 + 0.8655 * \chi_3 - 0.0683 * \chi_1\chi_2 + 1.17 * \chi_1\chi_3 - 0.1391 + \chi_2\chi_3 + 0.8525 * \chi_1^2 + 0.7842 * \chi_2^2 - 0.7786 * \chi_3^2$$

The equation of coded factors allows an understanding of the contribution of each factor and can be used to predict outcomes. In this design, the factors with most influence on the outcome are the (χ_1), the amount of solvent, followed by the extraction time (χ_3), being the intensity of the ultrasound (χ_3) the least important factor.

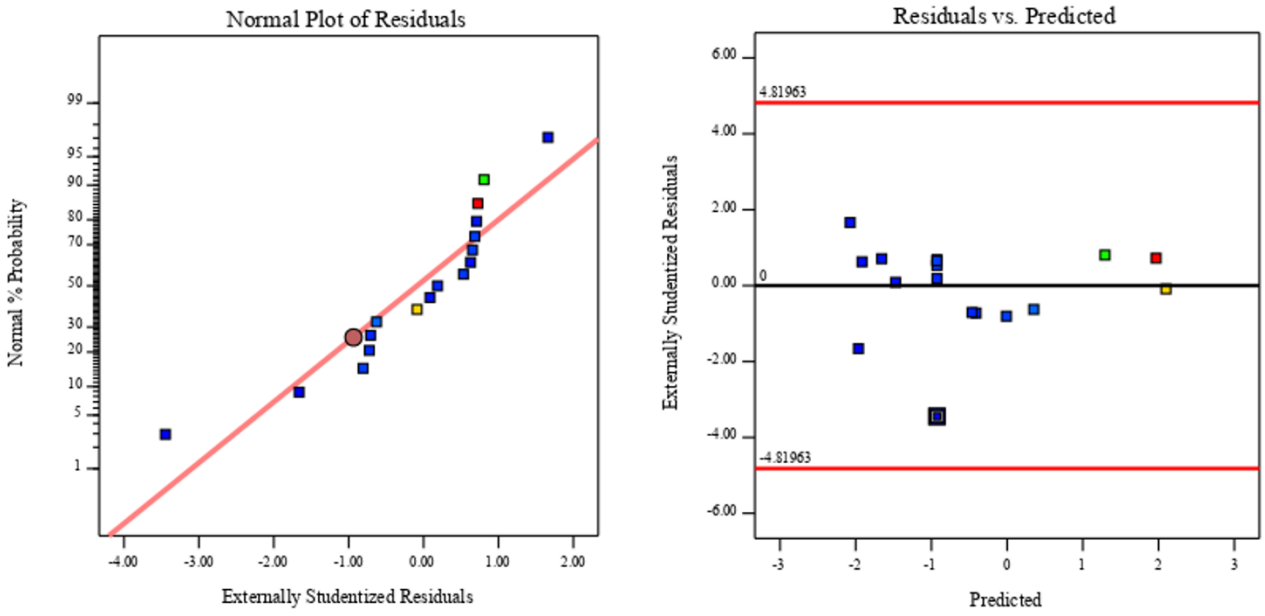


Figure 15. Plots of externally Studentized residuals and predicted vs. actual residuals.

Figure 15 shows the plots of the residuals, in which it is possible to ascertain, on the left chart that the residuals are in near proximity of the trendline, with only two residuals which are somewhat

far from the line. The right chart shows the predicted residuals on the x axis and the accuracy of the prediction on the y axis. The prediction of the residuals shows and accurate prediction, except for two residuals which are lower down near the inferior limit of -4.81963.

To predict the optimal points, the maximize function was executed for the response (saponin content-R1), resulting in 95% of methanol, 129 Watts of ultrasound intensity and 73 minutes. The model predicted that this optimal point would allow an extraction yield of 9.92 g/100g of saponin content (**Figure 16**), which is beyond the maximum yield, obtained for run 16. **Figure 17** shows the 3D plots of paired factors, with color schemes that show higher yield zones (red), average yield zones (green) and low yield zones (blue). **Figure 17a** shows that higher amounts of solvent increase the yield, especially at over 80%, while the power does not seem to have influence in the middle ranges, showing higher output at low intensity and at intensities of 500W, as seen in the coded equation. A similar effect was sough in **Figure 17b**, where once again amounts over 80% of methanol increase the yield, being the same verified at 75 minutes. Finally, when plotting power vs. time, **Figure 17c**, low intensities and long extraction times of 75 minutes promote the yield.

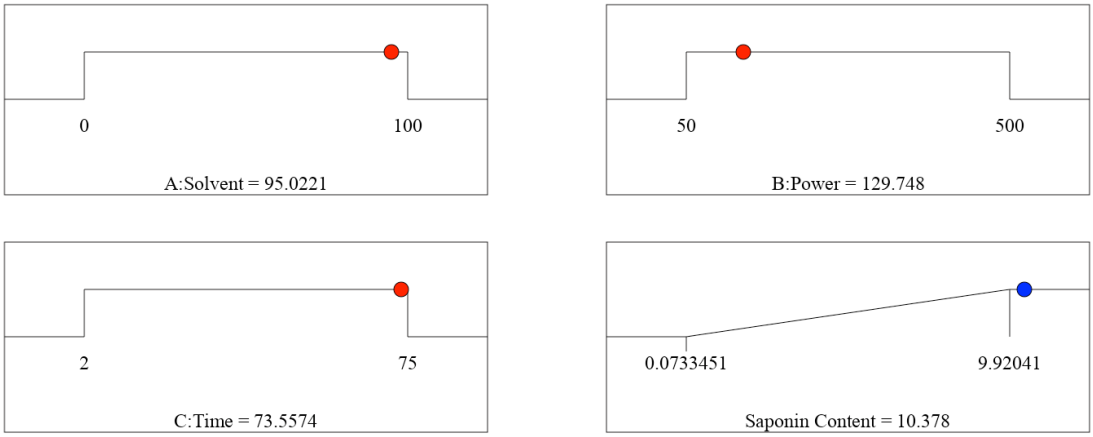


Figure 16. Optimal points and predicted yield of Saponin content for ultrasound extraction with Methanol.

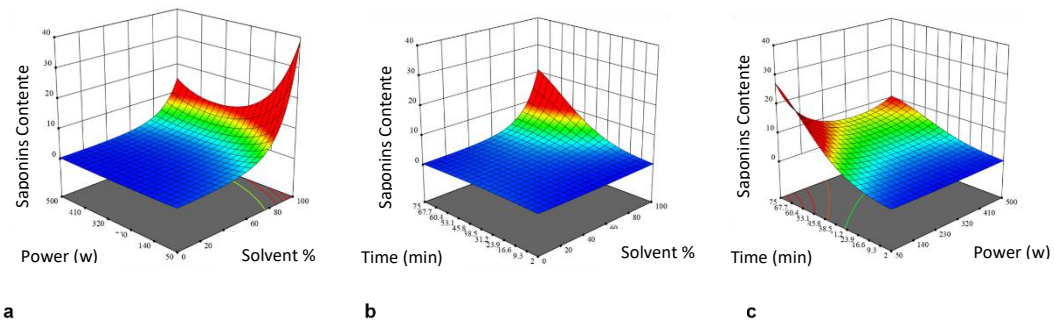


Figure 17. 3D plots of the paired factors for the ultrasound extraction using methanol.

Figure 18 show the three contour graphs, using the same colors to describe the outcome. These charts are another way of interpreting the data in a 2D scale, understanding the effects of each factor through the black lines. **Figure 18a** shows that the contour is higher at high quantities of solvent and low power, while **Figure 18b** shows the highest yielding range between 80 to 100% of methanol and extraction times between 31 to 75 minutes. Finally, **Figure 18c** reveals an almost uniform (red zone) high desirability zone along the different ultrasonic intensities (reason why this factor did not contribute much to the model), but only in extraction times over 31 minutes.

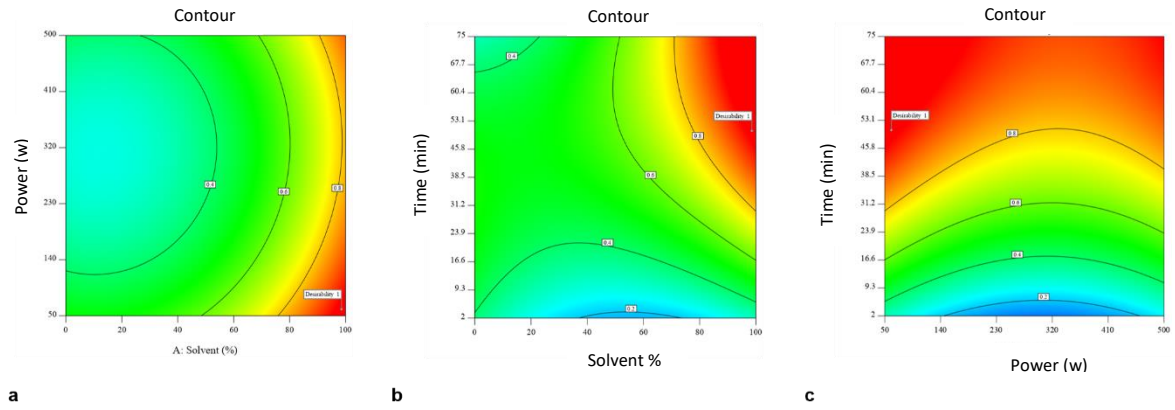


Figure 18. Contour plots of the paired factors for the extraction using methanol and ultrasound.

Overall, the model predicted that solvent percentage and extraction time were the most influential factors, which seem to promote a higher Saponin amount at longer extraction times and high amount of methanol. This could constitute high savings in terms of the use of lower intensities of ultrasonic power.

Considering the ethanol ultrasound assay, the data required a square root, but only obtained a linear model. Thus, two outliers (run 2 and 14) were removed from the model to increase predictability, hence the strikethrough on the responses in **Table 5**. After removal of the outliers, a quadratic model was obtained that did not require any transformation, showing an R2 of 0.97, an adjusted R2 of 0.93, a non-significant lack of fit and an adequate precision of 15.5. The model determined the following **equation 2**:

Equation 2. Equation of coded factors of Saponin content for ultrasound extraction with Ethanol.

$$Saponin\ Content = +0.7524 + 0.6723 * \chi_1 + 0.0885 * \chi_2 + 0.2144 * \chi_3 + 0.0296 * \chi_1\chi_2 + 0.0337 * \chi_1\chi_3 + 0.1183 + \chi_2\chi_3 + 0.2834 * \chi_1^2 - 0.2515 * \chi_2^2 - 0.1156 * \chi_3^2$$

The coded equation showed that, once again, the higher influence on the yield of saponins derives from variation in factor 1 (0.67), while factor 3 (extraction time) only showed one third of the influence (0.2144), and factor 2, (ultrasound power) showed very low influence. The optimization function showed that the predicted points with higher yield were situated at 100% of the solvent, 332 watts of power and 71 minutes (**Figure 19**).

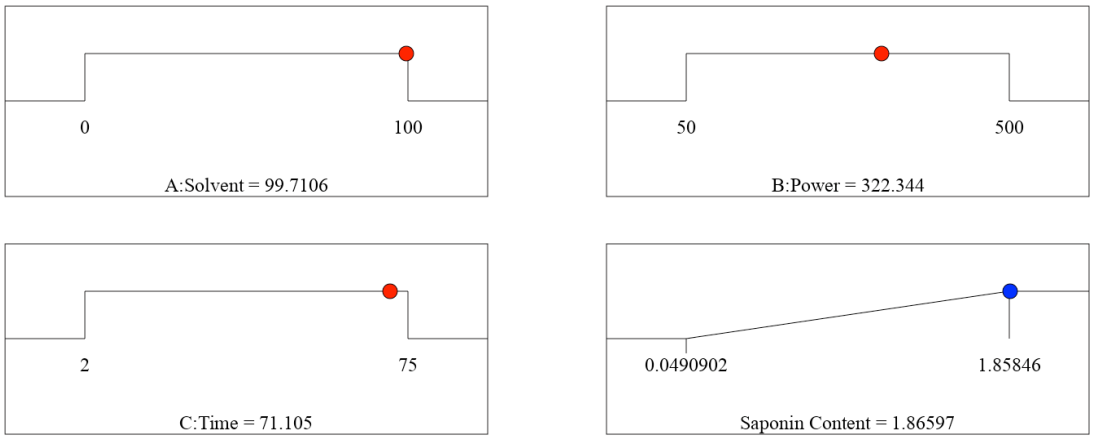
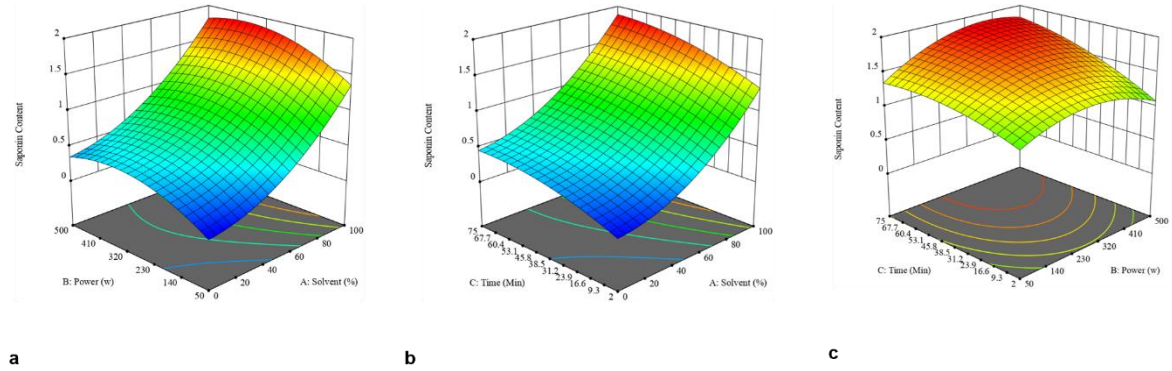


Figure 19. Optimal points and predicted yield of saponin content for ultrasound extraction with ethanol.

The model seems to predict that, to obtain a maximum yield, the ultrasound power should be more than double the one for methanol, although the amount of solvent required was the same. Still, this optimal point only predicts a maximum yield of 1.85 g/100 g while the extraction with methanol would achieve 10.38 g/100 g, almost a 10-fold increase. Thus, methanol is a better solvent to extract saponins when combined with ultrasound extraction.



a b c

Figure 20. 3D plots of the paired factors for the ultrasound extraction using ethanol.

Figure 20 shows the response surface (or 3D) graphs of the ultrasound extraction with ethanol. Analyzing **Figure 20a**, higher amounts of solvent promote the yield, while intermediate intensity of ultrasound are enough, thus showing higher yields in these intermediate intensities than for the highest possible yields, meaning that at lower intensities the extractability with ethanol is compromised, but also that higher intensities may destroy Saponins. Comparing with the methanol extraction, in which the yield was promoted by lower intensities, the lower extraction yield of ethanol allows for higher ultrasound intensity to maximize Saponin yield. The same behavior was registered when plotting time and solvent, meaning that higher yields of solvent promote higher yields and long extracting times are also beneficial to obtaining higher yields (**Figure 20b**). Finally, in **Figure 20c**, power intermediate power intensities did favor higher yields, but according to the coded equation, time showed a higher influence, and thus the red region, corresponding to higher yields, is promoted by extraction time over 30 minutes.

The contour plots corroborate the claims of the surface plots and the coded equation, that the solvent showed a higher influence on the extraction yield, while the ultrasound did not influence much, represented with a red zone almost encompassing the entirety of the range of values (**Figure 21a**).

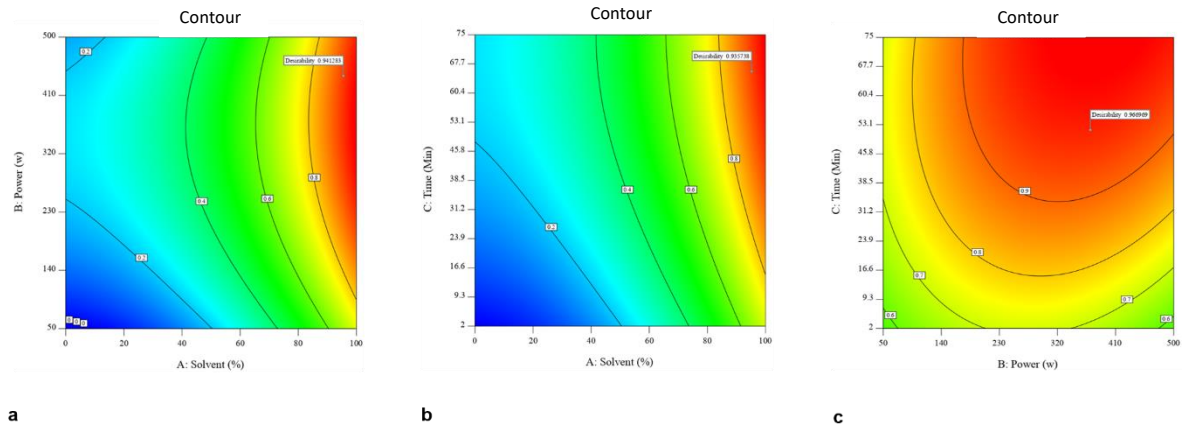


Figure 21. Contour plots of the paired factors for the extraction using ethanol and ultrasound.

A similar profile was sought for **Figure 21b**, plotting time and solvent, where high yields were found upwards of 16 minutes while high amounts of solvent, over 90% promote high yields. Finally, when plotting time with ultrasound intensity, the desirability is located near 350 WATS and 53 minutes, but very low yield zones (green color) are only fond near the extremes of the intensity range, showing that intermediate intensities favor the yield.

Overall considering the two solvents, methanol is 10-fold better in extracting saponins with an ultrasound equipment. Still, ethanol is a greener solvent, and while methanol can be used to obtain

food grade ingredients, ethanol is safer. Still, low to intermediate intensities of ultrasound are enough to achieve considerable amounts of saponins. The most important factor is high percentages of either of the solvents, which highly promote extractability, followed by extraction time which also promotes it. While methanol allows for higher yields and lower intensities, ethanol is a greener solution that may require higher ultrasound power. Further studies should be carried out using a mixture of both solvents, which could promote the extraction of different saponins, and eventually have a higher extraction yield, due to specific affinity of some saponins to one of these two solvents.

Regarding the extraction through Soxhlet apparatus with methanol and ethanol, the experimental design is shown in **Table 6**. The three factors were (χ_1) solvent percentage (A) which ranged from 0 to 100%, (χ_2) solid to liquid ratio (B) which varied from 5 to 50 g/L, and finally (χ_3) extraction time (C) which was comprehended between 30 and 720 minutes. Once again, the green highlighted lines correspond to the repetitions center points of the experimental design. The 17 individual runs were performed randomly to reduce operator error.

Table 6. Experimental design for the Soxhlet extraction displaying the responses for methanol and ethanol extractions.

SoxHlet Experimental Design				
Factors			Responses	
Solvent (% v/v) (χ_1)	S/L Ratio (g/L) (χ_2)	Time (min) (χ_3)	Saponin Content (g/100g) Methanol (R_1)	Saponin Content (g/100g) Ethanol (R_1)
50	27.5	375	1.000	1.201
50	50	720	1.273	1.156
100	50	375	1.563	1.236
100	5	375	1.428	1.236
100	27.5	30	~~9.067~~	1.255
50	5	30	1.087	1.344
50	27.5	375	0.709	1.112
0	27.5	720	0.117	0.145
50	27.5	375	0.961	1.347
50	5	720	0.954	1.05
50	50	30	0.806	1.311
0	5	375	0.058	0
50	27.5	375	0.986	1.298
100	27.5	720	0.986	1.286
0	27.5	30	0	0
0	50	375	0.986	0.357
50	27.5	375	1.046	1.059

Considering the extraction with methanol, the quadratic model allowed an R^2 of 0.93 and an adjusted R^2 of 0.83, a non-significant lack of fit and an adequate precision of 10.7, following the deletion of one run which corresponded to an outlier. The model determined the following **Equation 3:**

Equation 3. Equation of coded factors of Saponin content for Soxhlet extraction with Mehtanol

$$Saponin\ Content = 0.9407 + 0.4675 * \chi_1 + 0.1378 * \chi_2 + 0.0642 * \chi_3 - 0.1983 * \chi_1\chi_2 - 0.0139 * \chi_1\chi_3 + 0.1500 + \chi_2\chi_3 - 0.2277 * \chi_1^2 + 0.2959 * \chi_2^2 - 0.2061 * \chi_3^2$$

By analyzing the equation, once again, and as like the extraction with ultrasound, the solvent showed the highest influence on the yield of Saponins, followed by the solid-liquid ratio.

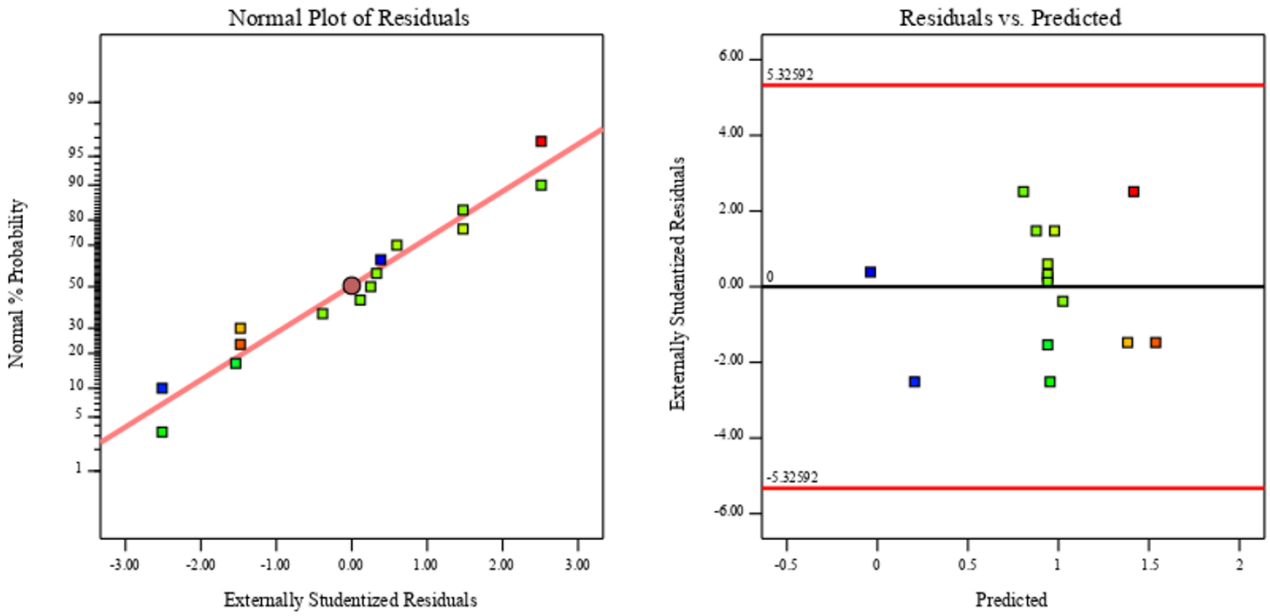


Figure 22. Plots of externally studentized residuals and predicted vs. actual residuals.

Analyzing the normal plot of the residuals in **Figure 22**, all of them are in near the trendline and the dispersion occurs along the whole spectrum, meaning the model fitted them correctly. Furthermore, the predicted residuals are also well within the upper and lower limits, meaning an acceptable prediction by the model.

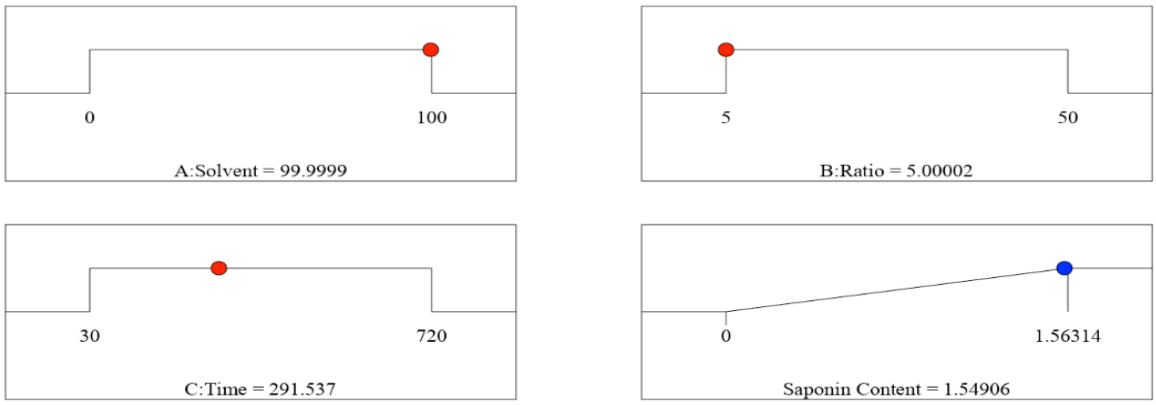


Figure 23. Optimal points and predicted yield of saponin content for Soxhlet extraction with methanol.

Figure 23 shows the optimal points calculated by the model, being 99% of methanol, 5 g/L and about 291 minutes of extraction time through the Soxhlet apparatus. Overall, the highest amount was achieved with low amounts of solid extracts, meaning that saturation is achieved at a very low amount, thus allowing for a considerable saving in maintaining the solid to ratio low. The model predicted that at these optimal points the extraction yield would be 1.56 g/100g of Saponins.

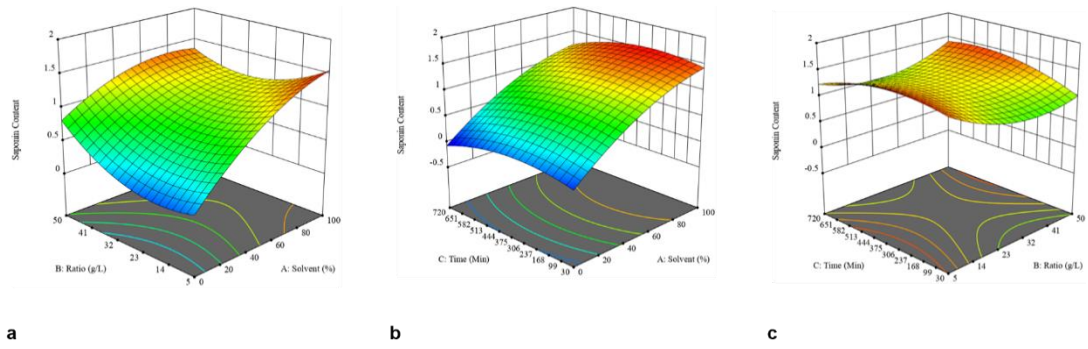


Figure 24. 3D plots of the paired factors for the Soxhlet extraction using methanol.

Figure 24 shows the response surface of the methanol extracted saponins through Soxhlet apparatus. **Figure 24a** shows that the yield in Saponins is promoted by high percentages of methanol, while low rations of solid to liquid seems to increase the extractability. **Figure 24b** plots the solvent percentage with extraction time, and once again shows that high solvent percentages increase extractability and therefore, the yield, while median extraction times are better at improving the yield than long or short extraction times. Finally, **Figure 24c** shows the small variation between the S/L ration and extraction time. Still, it is clear that low S/L rations and median extraction times promote higher quantities of Saponins.

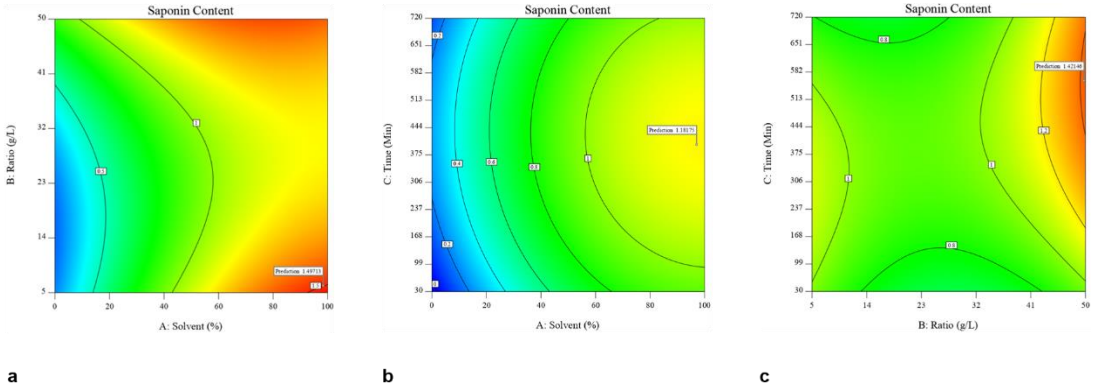


Figure 25. Contour plots of the paired factors for the extraction using methanol and Soxhlet.

The contour plots of the methanol extracted saponins confirm the highest yields for almost 100% of methanol at very low S/L rations, although high ratios did also show a higher extractability zone (**Figure 25a**). In **Figure 25b**, the highest yields were achieved in mid-ranges of extraction time and high solvent percentage, although the optimal zone is quite wide. Finally, when plotting S/L ratio and extraction time (**Figure 25c**), the optimal point is located at high S/L ratios and upper range of extraction time. Overall, although high ratios of solid to liquid did show improved extractability, when combining the three factors, this capacity is diluted by the high solvent percentage, which shifts the optimal points to lower ratios, meaning that there is lower gain in higher ratios when compared to higher solvent percentages, which, industrially is a much better option due to the lower waste of saponin extract and the cheaper cost of methanol that can be recycled and lower extraction time.

Considering the Soxhlet extraction with ethanol, this was the only assay that showed two runs with no saponin extraction, while the methanol only showed one. The ultrasound assisted extraction was able to extract saponins in all runs, while the Soxhlet did not. This absence of extractability could reduce the accuracy of the model, but still, a quadratic model was achieved, showing a non-significant lack of fit, and R^2 of 0.97 and an adjusted R^2 of 0.92 and an adequate precision o 12.6. The model determined the following **Equation 4**:

Equation 4. Equation of coded factors of Saponin content for Soxhlet extraction with Etahnol.

$$Saponin\ Content = 1.20 + 0.5639 * \chi_1 + 0.0540 * \chi_2 - 0.0340 * \chi_3 - 0.0895 * \chi_1\chi_2 - 0.285 * \chi_1\chi_3 + 0.0349 + \chi_2\chi_3 - 0.5199 * \chi_1^2 + 0.0238 * \chi_2^2 - 0.0120 * \chi_3^2$$

The coded equation shows that once again, of the three factors, the one that stands out the most is solvent percentage, with 56% of the influence, while the other two do not even reach 10%.

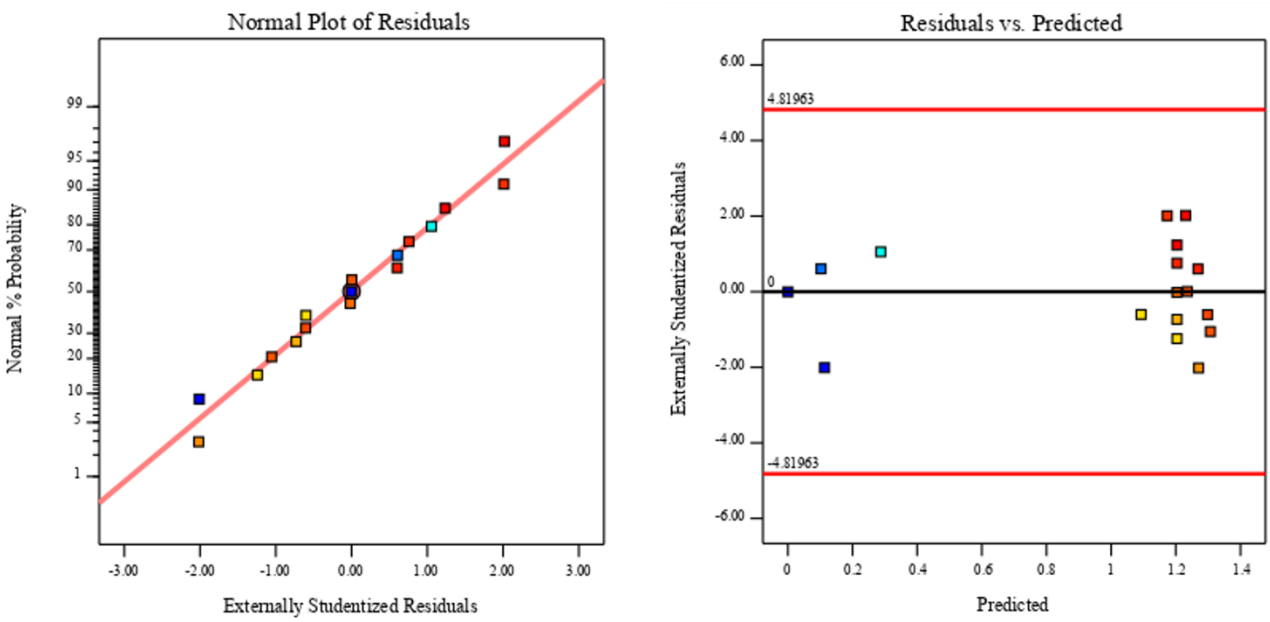


Figure 26. Plots of externally studentized residuals and predicted vs. actual residuals.

Regarding the plot of residuals, although the model did have two ignored runs, it did show a continuous scattering of residuals along the trendline meaning it adequately fit the data. Still, the predicted residuals albeit being within the upper and lower limits did show a clustering on both ends of the predicted values. This could reduce accuracy of the model, but the lack of fit was below the limits, meaning there was an adequate fit.

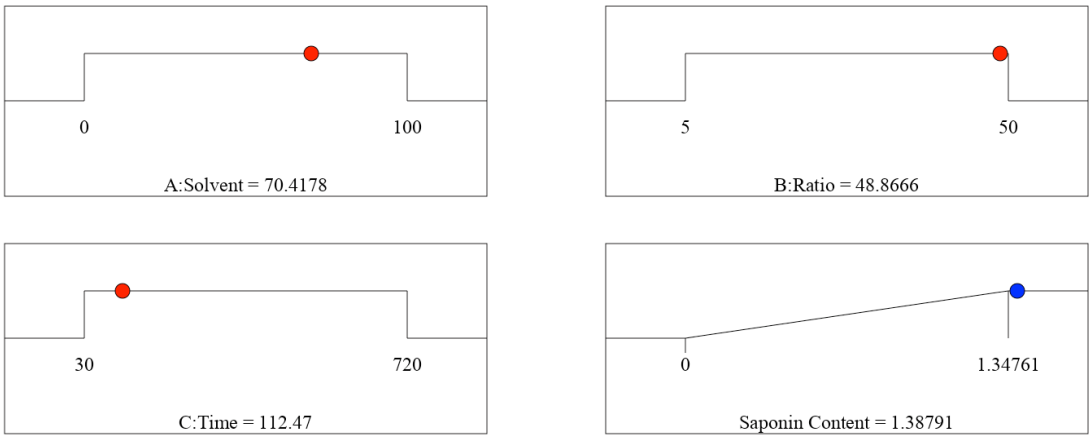
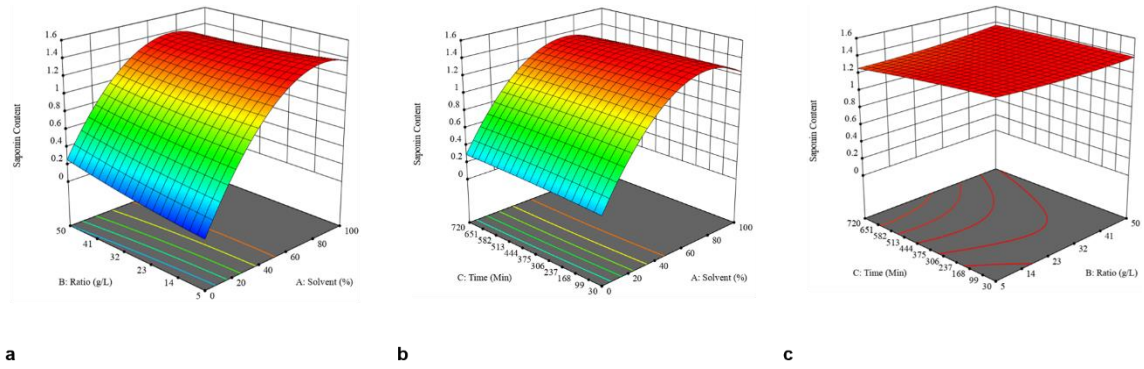


Figure 27. Optimal points and predicted yield of saponin content for Soxhlet extraction with ethanol.

The optimal points for the Soxhlet extraction with ethanol show that the highest yield in saponins requires 70% of ethanol, 49 g/100 g of extract and 112 minutes of extraction time. According to the model, these points predict a yield above the one achieved in the experimental design, reaching 1.35 g/100 g of saponins. Interestingly, this is the model that predicted the lowest amount of solvent for optimization of the yield. Furthermore, the high extraction yield found in high ratios for methanol

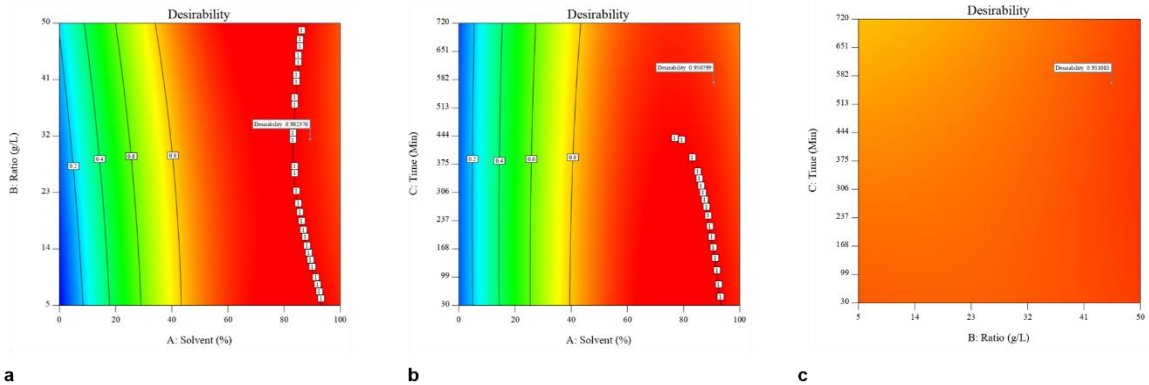
that was dissolved by the high percentages of solvent, shifting the optimal points away from long extractions, was, in the case of ethanol not dissolved, even though this factor did not have such a high influence.



a b c

Figure 28. 3D plots of the paired factors for the Soxhlet extraction using ethanol.

The response surface plots show that when plotting solvent and ratio, higher percentages of ethanol promote the extractability, although, contrarily to the other assays, there is a reduction, meaning that over about 70% there is a reduction in this phenomenon (**Figure 28a**). Still, Ratio did not show much influence. In **Figure 28b**, a similar surface is sought when plotting extraction time with solvent, once again the solvent shows a peak near 70% and the decreases, while no change is verified for the range in S/L ration. Thus, when plotting the S/L ratio with extraction time, a linear surface is plotted (**Figure 28c**), which shows the very low influence that these two factors have on the extractability of saponins.



a b c

Figure 29. Contour plots of the paired factors for the extraction using ethanol and Soxhlet.

The contour plots for the Soxhlet extraction with ethanol corroborates the findings from the surface plots, in which an increasing yield is found over the range of solvent, with close to no influence of the S/L ratio (**Figure 29a**), the same occurring for **Figure 29b**, where the yield also

increases over the solvent with low influence of the extraction time. Finally, (**Figure 29c**) the plot of time and S/L ratio shows that due to their very low influence, the yield is almost constant in the whole area of the plot.

Overall, the best extraction method seems to be ultrasound assisted extraction, although this was only perceptible when using methanol, where the model predicted an extraction at the optimal points of 9.9 g/100 g. Still, UAE with ethanol showed the same magnitude of extraction as did the Soxhlet apparatus. Thus, the most important factor to consider when trying to extract saponins is the amount of solvent, which was constants throughout all the assays, meaning that high percentages of ethanol or methanol extract saponins better than any other factor. Between ethanol and methanol, the latter seems to have a slight better affinity, allowing for a higher yield. This is especially evident when ultrasounds are applied, albeit at low intensities.

4.2 Saponins analysis

Saponins identification was carried out by chromatographic comparison with the aescin standard (injected at 100 μg/mL; **Figure 30**). As previously referred, aescin is composed of two major groups of isomers, β-aescin containing aescin Ia and aescin Ib, and α-aescin formed by isoaescin Ia and isoaescin Ib.

For quantitative analysis, seven-level calibration curves were constructed based on the UV-Vis signal for the available standard: aescin 1a ($y = 92371x - 27844$; $r^2 = 0.9995$; limit of detection (LOD) = 1.42 μg/mL; limit of quantification (LOQ) = 4.30 μg/mL), aescin 1b ($y = 87703x - 46743$, $r^2 = 0.9999$, LOD = 0.78 μg/mL, LOQ = 2.35 μg/mL), isoaescin 1a ($y = 120190x + 11457$; $r^2 = 0.9999$; LOD = 0.66 μg/mL; LOQ = 1.99 μg/mL), isoaescin 1b ($y = 26242x - 61474$; $r^2 = 0.9998$; LOD = 0.89 μg/mL; LOQ = 2.70 μg/mL). The results were expressed in mg/g of extract.

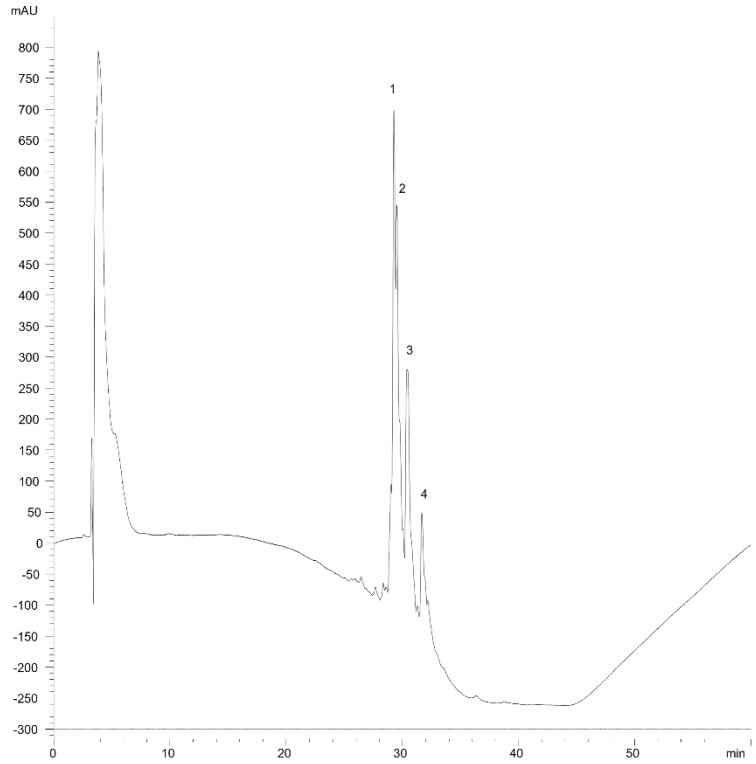


Figure 30. Aescin standard profile obtained by UFLC recorded at 220 nm.
Peak identification: 1- aescin Ia; 2- aescin Ib; 3- isoaescin Ia; and 4- isoaescin Ib.

Figure 31 represents the results obtained to determine the saponin content in the extracts under study, both for Soxhlet and UAE extraction, using methanol as the extraction solvent. The chromatograms obtained for all the tested conditions, including both extraction techniques and extraction solvents, were very similar. In all cases, it was possible to identify the four peaks characteristics of aescin isoforms (β-aescin - peak 1: aescin Ia and peak 2: aescin Ib; and α-aescin - peak 3: isoaescin Ia and peak 4: isoaescin Ib).

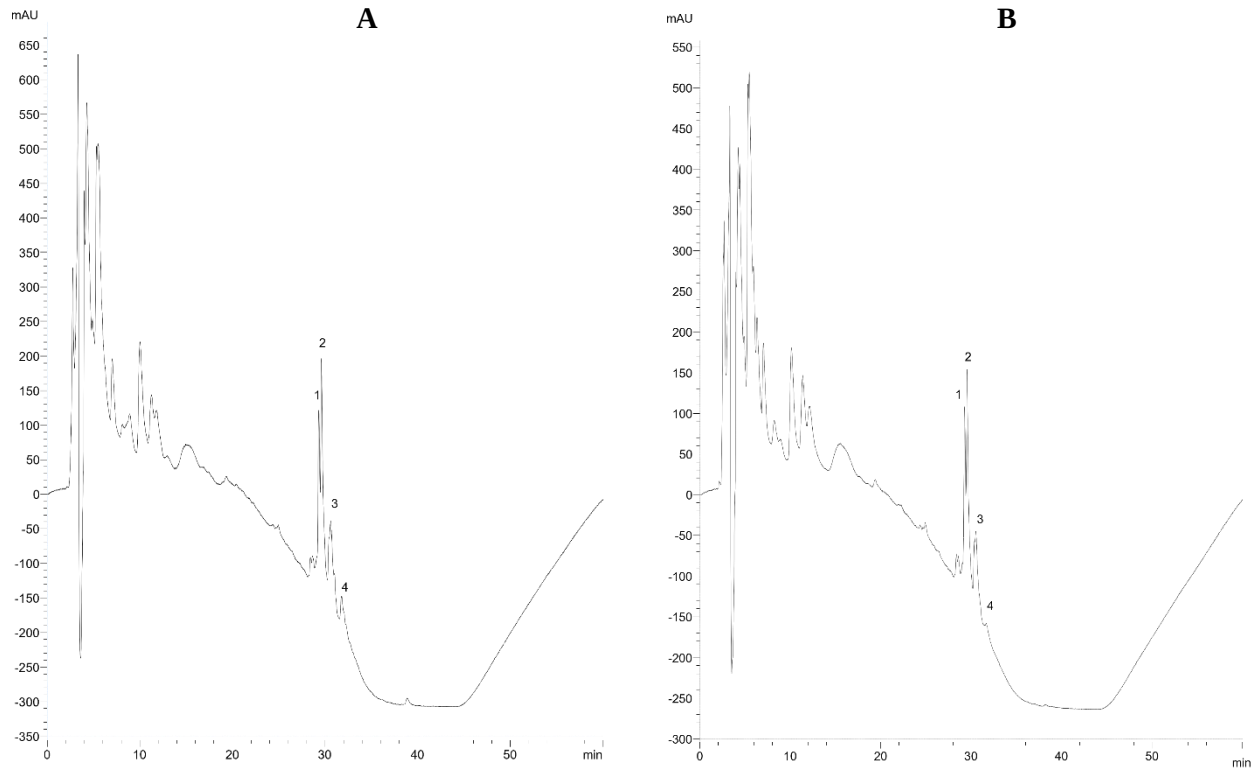


Figure 31. UFLC saponin profile of horse chestnut extract, obtained by (A) soxhlet extraction and (B) by UAE extraction, using methanol as solvent and recorded at 220 n*m.*
Peak identification: 1- aescin Ia; 2- aescin Ib; 3- isoaescin Ia; and 4- isoaescin Ib.

The main differences verified were regarding the contents of aescin obtained. Regarding UAE and analyzing the total values obtained for aescin (results not shown due to its extension), a total of approximately 29 mg/g of *A. hippocastanum* extract was obtained in the methanol extraction, while in the ethanol extraction, a total of approximately 12 mg/g extract were obtained. For the Soxhlet extraction, approximately 24 mg/g extract was obtained with methanol and 16 mg/g extract with ethanol.

Thus, it can be concluded that the best results were obtained using the UAE; however, methanol remains the most suitable solvent for the extraction of saponins (Abudayeh et al., 2015); Colson et al., 2019)

Although the intention was to reduce the use of methanol and replace it with ethanol (greener), the fact is that in much of the literature found methanol is still the chosen solvent.

4.3 Bioactivity of the most promising extracts

4.3.1 Antimicrobial activity

The antibacterial activity of *A. hippocastanum* extracts (pulp, skin, and bark) has been performed against clinical isolates of Gram-positive and Gram-negative bacteria. As referred in the material and methods section, the bioactivity was evaluated for both methanolic and ethanolic extracts obtained by Soxhlet and UAE, according to the optimal points given by the RSM design. The obtained results are presented in **Table 7** and **Table 8**.

Analyzing both tables, it was possible to conclude that the MIC (minimal inhibitory concentration) or MBC (minimum bactericidal concentration) for most extracts was 10 or up to 10 mg/mL. Nonetheless, some of the tested bacteria were more sensitive to the extracts, namely *Escherichia coli* (**Table 7**). Moreover, the skin extract obtained by Soxhlet using ethanol as solvent (SXETS) inhibited the growth of *E. coli* (MIC = 5 mg/mL), *Klebsiella pneumoniae* (MIC = 10 mg/mL), *Morganella morganii* (MIC = 5 mg/mL), and *Proteus mirabilis* (MIC = 2.5 mg/mL), as well as *Enterococcus faecalis* (MIC = 5 mg/mL), *Listeria monocytogenes* (MIC = 10 mg/mL), and MRSA (MIC = 1.25 mg/mL). Among the tested bacteria, the gram-positive were the most sensitive to the tested *A. hippocastanum* extracts.

Considering the analyzed parts and the types of extraction carried out, we cannot reach a satisfactory conclusion regarding the best extraction solvent and methodology. Thus, it is believed that the antimicrobial properties of *A. hippocastanum* extract may not only be conferred by the saponins present but also by other compounds (*e.g.* phenolic compounds (Gagić et al., 2021).

Table 7. Antibacterial activity (MIC and MBC, mg/mL) of A. hippocastanum extracts (pulp, skin and bark), obtained by Soxhlet and UAE and extracted with ethanol (maximum tested concentration, 10 mg/mL).

	Soxhlet extraction						UAE				Ampicillin (20mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	Skin		Pulp		Bark		Pulp		Bark							
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																
Escherichia coli	5	>10	1.25	>10	1.25	>10	0.3	>10	2.5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	10	>10	>10	>10	10	>10	>10	>10	10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	5	>10	10	>10	5	>10	>10	>10	10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	2.5	>10	10	>10	10	>10	10	>10	>10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria																
Enterococcus faecalis	5	>10	10	>10	5	>10	10	>10	5	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	10	>10	5	>10	10	>10	10	>10	10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	1.25	>10	5	>10	5	>10	5	>10	5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MRSA – Methicillin-resistant *Staphylococcus aureus*; MSSA – methicillin-susceptible *Staphylococcus aureus*; MIC – minimal inhibitory concentration; MBC – minimum bactericidal concentration; nt – not tested.

Table 8. Antibacterial activity (MIC and MBC, mg/mL) of A. hippocastanum extracts (pulp, skin and bark), obtained by Soxhlet and UAE and extracted with methanol (maximum tested concentration, 10 mg/mL).

	SXMETS		**SXMETP**		**SXMETB**		**UAMETS**		**UAMETP**		**Ampicillin (20mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria																
Escherichia coli	10	>10	5	>10	2.5	>10	10	>10	2.5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>10	>10	>10	>10	10	>10	>10	>10	>10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	5	>10	>10	>10	10	>10	10	>10	>10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>10	10	>10	>10	>10	10	>10	>10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria																
Enterococcus faecalis	10	>10	10	>10	10	>10	10	>10	10	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	2.5	>10	10	>10	>10	>10	10	>10	5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	5	>10	5	>10	5	>10	2.5	>10	2.5	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MRSA – Methicillin-resistant *Staphylococcus aureus*; MIC – minimal inhibitory concentration; MBC – minimum bactericidal concentration; nt – not tested.

In terms of the antifungal potential, the results obtained for *A. hippocastanum* extracts (pulp, skin, and bark), obtained by Soxhlet and UAE, using ethanol and methanol are presented in **Table 9**.

Once again, extracts obtained from different parts of the species and extracts obtained by Soxhlet and UAE with different solvents revealed some antifungal potential. The MIC values ranged from 5 and >10 mg/mL, while the minimum fungicidal concentrations (MFC) were slightly higher (> 10 mg/mL). Even so, it can be concluded that *Aspergillus brasiliensis* is slightly more sensitive to the tested extracts.

Table 9. Antifungal activity (MIC and MFC, mg/mL) of *A. hippocastanum* extracts (pulp, skin, and bark), obtained by Soxhlet and UAE and extracted with ethanol and methanol (maximum tested concentration, 10 mg/mL, and 1 mg/mL for ketoconazole).

			Aspergillus brasiliensis		***Aspergillus fumigatus***	
			MIC	***MFC***	***MIC***	***MFC***
Soxhlet	**Ethanol**	**Pulp**	10	>10	>10	>10
		Skin	10	>10	>10	>10
		Bark	>10	>10	>10	>10
	Methanol	**Pulp**	5	>10	>10	>10
		Skin	>10	>10	>10	>10
		Bark	>10	>10	>10	>10
UAE	**Ethanol**	**Pulp**	10	>10	>10	>10
		Skin	nt	nt	nt	nt
		Bark	10	>10	>10	>10
	Methanol	**Pulp**	5	>10	>10	>10
		Skin	10	>10	>10	>10
		Bark	nt	nt	nt	nt
		Ketoconazole	0.06	0.125	0.5	1

MIC – minimal inhibitory concentration; MFC – minimal fungicidal concentration: nt – not tested.

4.3.2 Cytotoxic activity

The potential cytotoxicity of the ethanolic and methanolic extracts of *A. hippocastanum* (pulp, skin, and bark), obtained by Soxhlet and UAE, are presented in **Table 10**. These properties were tested against three human tumor cell lines and two non-tumor cell cultures. AGS (gastric adenocarcinoma), CaCo-2 (colorectal adenocarcinoma), and MCF-7 (breast adenocarcinoma) were the tested human tumor cell lines; Vero (African green monkey kidney) and PLP2 (primary pig liver culture) were the non-tumor cells tested. After performing the screening through the SRB assay, it was verified all the bark extracts revealed the lowest GI_{50} values for the tumor cells. However, some of these extracts also revealed the lowest GI_{50} values for the non-tumor cells. The pulp extracts showed higher GI_{50} values in the non-tumor cells, meaning those were less toxic.

Table 10. Cytotoxic activity (mean ± SD, µg/mL; GI_{50} values, n=9) of *A. hippocastanum extracts* (pulp, skin, and bark), obtained by Soxhlet and UAE and extracted with ethanol and methanol, against different cell cultures.

			AGS	CaCo-2	MCF-7	Vero	PLP2
Soxhlet	**Ethanol**	**Pulp**	202 ± 4^{b}	>400	>400	233 ± 21^{c}	174 ± 11^{e}
		Skin	70 ± 5^{e}	276 ± 9^{a}	230 ± 20^{c}	257 ± 7^{b}	>400
		Bark	31 ± 1^{h}	62 ± 6^{f}	48 ± 2^{g}	47 ± 3^{g}	52 ± 5^{g}
	Methanol	**Pulp**	221 ± 12^{a}	>400	>400	223 ± 15^{c}	222 ± 12^{c}
		Skin	35 ± 3^{h}	239 ± 24^{b}	214 ± 4^{d}	222 ± 16^{c}	292 ± 22^{a}
		Bark	41 ± 2^{g}	67 ± 6^{f}	66 ± 6^{f}	56 ± 1^{fg}	46 ± 1^{g}
UAE	**Ethanol**	**Pulp**	177 ± 7^{c}	>400	>400	287 ± 4^{a}	>400
		Skin	50 ± 5^{f}	240 ± 25^{b}	261 ± 26^{b}	231 ± 23^{c}	>400
		Bark	35 ± 1^{h}	192 ± 2^{d}	82 ± 6^{e}	172 ± 1^{e}	157 ± 13^{f}
	Methanol	**Pulp**	83 ± 8^{d}	>400	336 ± 7^{a}	192 ± 13^{d}	188 ± 14^{d}
		Skin	70 ± 6^{e}	223 ± 7^{c}	225 ± 13^{c}	176 ± 11^{e}	239 ± 11^{b}
		Bark	45 ± 5^{g}	183 ± 8^{e}	89 ± 2^{e}	60 ± 1^{f}	175 ± 12^{e}
		Ellipticine	1.23 ± 0.03	1.21 ± 0.02	1.02 ± 0.02	1.41 ± 0.06	1.41 ± 0.06

GI_{50} values correspond to the extract concentration causing 50% of growth inhibition in human tumor cell lines. In each column, different letters mean statistically significant differences among the samples. Significance level was set as $p<0.05$.

4.3.3 Anti-inflammatory activity

The results for the anti-inflammatory assays of the inducible nitric oxide pathway activation with murine macrophages RAW 264.7 cells (mean ± SD, µg/mL; GI_{50} values; n = 9) are presented in **Table 11**. Generally, and once, the bark extracts from *A. hippocastanum* revealed the lowest GI_{50} values. These results agree with those of the antioxidant activity and, once again, given that it is in the bark that we can have a higher concentration of bioactive compounds, these may be contributing (simultaneously with saponins) to the cytotoxic and anti-inflammatory activity of the species under study.

Table 11. Anti-inflammatory activity (mean ± SD, µg/mL; IC_{50} values; n = 9) of *A. hippocastanum* extracts (pulp, skin, and bark), obtained by Soxhlet and UAE and extracted with ethanol and methanol, tested in RAW 264.7 cells.

			RAW 264.7
Soxhlet	**Ethanol**	**Pulp**	151 ± 10
		Skin	>400
		Bark	56 ± 3
	Methanol	**Pulp**	>400
		Skin	>400
		Bark	57 ± 5
UAE	**Ethanol**	**Pulp**	>400
		Skin	>400
		Bark	164 ± 13
	Methanol	**Pulp**	287 ± 21
		Skin	>400
		Bark	237 ± 3
Ellipticine			6.3 ± 0.4

IC_{50} corresponds to the concentration of the extract that causes the 50% inhibition of nitric oxide production.

V Conclusions and future perspectives

The main goal of this study was to optimize the saponins extraction from *Aesculus hippocastanum*, trying to understand why conventional techniques are still being used worldwide instead of environmentally-friend methods. Soxhlet extraction and UAE were performed, and methanol was the used as extraction solvent to test its replacement by ethanol.

Regarding the saponin amounts obtained for all the extractions, UAE showed the best yields. When analyzing the UFLC saponin profiles, there were no significant differences between Soxhlet and UAE, since it was possible to quantify the four isoforms of aescin in all samples, recorded at 220 nm. Therefore, we can conclude that saponins can be successfully extracted by UAE. The RSM design confirmed that UAE was a good choice to extract saponins, being the solvent one of the most influencing the extraction process. However, when both extraction solvents were tested, methanol still gave the best results, which could be a limitation for its replacement by ethanol which is a greener one.

After obtaining the optimal points for both extraction techniques and solvents, the bioactivity of the extracts was tested.

Regarding the antimicrobial properties, it was impossible to infer which part of the *A. hippocastanum* has the best potential or the best extraction conditions to improve these properties. However, gram-positive bacteria and *A. brasiliensis* seemed to be more sensitive pathogens to this type of extracts. These results may be due to possible synergistic effects between saponins and other bioactive compounds.

Concerning the cytotoxic activity, some extracts inhibited the growth of the tested tumor cell lines. Generally, the bark extracts revealed the lowest GI_{50} values for the tumor cells. However, these extracts also revealed some toxicity in the no-tumor cells. Therefore, the use of these extracts without any further processing may be limiting. The pulp extracts showed to be less toxic in Vero and PLP2 cells. The anti-inflammatory activity of the tested extracts was according to the verified cytotoxicity since the bark extracts revealed the lowest IC_{50} values. Once again, synergistic effects with other bioactive compounds may have influenced the results obtained.

Thus, the present study showed that UAE is an efficient extraction methodology for obtaining saponins from *A. hippocastanum* and, therefore, should be an option compared to conventional methods. However, other studies need to be carried out to increase the extractability of this type of compounds with solvents other than methanol.

In future work, and as mentioned above, the replacement of methanol by greener alternatives for saponins extraction should be a study target. Furthermore, other green techniques can be studied

to reduce the time and amount of solvent used. The survey for saponins other than aescin could also be a goal. Analyzing the results obtained by UFLC-PDA, it was possible to verify the existence of other peaks that could correspond to different mixtures of saponins.

The study of bioactive properties could be extended, predicting for example the antioxidant and hypocholesterolemic potential.

One of the objectives of this work was the valorization of bio-waste as a source of bioactive compounds of interest. It was expected to study the unsaponifiable fraction of horse chestnut; however, it was impossible due to time limitations and problems with equipment. In this way, this work could be carried out in the future.

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