EGAS MONIZ SCHOOL of HEALTH & SCIENCE | INSTITUTO UNIVERSITÁRIO EGAS MONIZ

INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO EM TECNOLOGIAS LABORATORIAIS EM CIÊNCIAS FORENSES

COMPREHENSIVE ANALYSIS OF HEPATOPROTECTIVE SUPPLEMENTS THROUGH CHEMICAL, MICROBIOLOGICAL, AND PALYNOLOGICAL APPROACH

Trabalho submetido por
Aline Samara Martins Cardoso Varela
para a obtenção do grau de Mestre em Tecnologias Laboratoriais em Ciências Forenses

novembro de 2025

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Trabalho orientado por
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Agradecimentos

A realização deste trabalho não teria sido possível sem o apoio, incentivo e contributo de várias pessoas, às quais expresso a minha mais profunda gratidão.

À minha orientadora, Professora Patrícia Serra, pela sua orientação atenciosa, pela disponibilidade constante e pela forma meiga e cuidadosa com que sempre me acompanhou. Ao Professor Paulo Mascarenhas, o verdadeiro orientador deste trabalho e também o homem das ideias e do "tudo é possível", pela criatividade e motivação. Ao meu coorientador Professor Nuno Neng, pela assertividade e por garantir que as ideias se mantivessem dentro dos limites científicos necessários. À Professora Zoé Vaz da Silva, pela paciência, compreensão e apoio em momentos cruciais. Também gostaria de agradecer o Instituto Universitário Egas Moniz, pelo espaço, recursos e suporte institucional que possibilitaram a concretização deste trabalho.

Um agradecimento especial à Camila Rossi, que esteve ao meu lado durante todo o processo, basicamente do surto à comemoração, partilhando comigo cada etapa. Aos meus grandes amigos, que, mesmo à distância, foram uma fonte de força e conforto. À minha amiga de infância Aimone Duarte, que, a partir do Porto, viveu este percurso académico comigo, partilhando longas horas de desabafos e ideias. À minha querida amiga Eliane Fernandes, pelas palavras de incentivo e afirmação que tantas vezes me ajudaram a continuar.

À minha família, o meu maior alicerce. À minha mãe, Killy Samaá, e ao meu pai, João José, pelo amor, paciência e apoio incondicional. Aos meus irmãos, Alicia e Marcelo, pelo carinho e presença constante.

A todas as mulheres da minha família, exemplos de força, resiliência e determinação, que moldaram a mulher que sou hoje. Uma menção especial à minha tia Maria Júlia (Yula), que me incentivou num momento em que as palavras de encorajamento eram mais necessárias. À minha avó Maria Conceição, matriarca da família materna, a quem devo a minha resiliência e espírito de luta.

Por fim, à *nha terra, nha kretxeu,* Cabo Verde pelas raízes que me definem e pela identidade que carrego com honra. Levo comigo o orgulho das minhas origens porque como tão bem disse Mayra Andrade *"ami é fidju de rotxa que bentu leste fulha na mar, dento mi tem 10 ilha"*

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Eu, Aline Samara Martins Cardoso Varela, referente a Tese *"Comprehensive Analysis of Hepatoprotective Supplements through Chemical, Microbiological, and Palynological Approach"* declaro que o meu trabalho se encontra financiado.

O financiamento foi concedido pelo Instituto Universitário Egas Moniz, no montante de 500 euros (€500,00).

O valor atribuído foi destinado à aquisição de reagentes e meios de cultura utilizados nas componentes química e microbiológica do projeto.

Data: 21/11/25

Resumo

Os suplementos alimentares hepatoprotetores têm vindo a ganhar destaque devido à sua alegada capacidade de auxiliar na desintoxicação hepática e na regeneração celular. Contudo, a ausência de regulamentação rigorosa e o crescente número de produtos no mercado levantam questões sobre a sua autenticidade, qualidade e segurança. O presente estudo teve como objetivo realizar uma análise abrangente de suplementos hepatoprotetores comercializados em Portugal, recorrendo a abordagens químicas, microbiológicas e palinológicas, de modo a verificar a conformidade entre a rotulagem e a composição real dos produtos.

Na vertente química, a espectroscopia de infravermelho por transformada de Fourier (FTIR) permitiu identificar bandas características de compostos fenólicos e flavonolignanos, nomeadamente silibinina e silicristina, principais constituintes do *Silybum marianum* (cardo-mariano). As amostras sólidas revelaram maior correspondência com os padrões de referência, enquanto o suplemento líquido apresentou interferência da matriz (solvente). A análise microbiológica demonstrou ausência de microrganismos patogénicos perigosos, indicando condições adequadas de fabrico e armazenamento. A abordagem palinológica revelou a presença recorrente de grãos de pólen da família *Cistaceae* em todas as amostras, com deteção adicional de *Brassicaceae* numa amostra e de esporos fúngicos noutra, o que sugere uma possível contaminação ambiental ou a presença de resíduos processuais. Embora não tenham sido identificados grãos de pólen pertencentes às famílias botânicas correspondentes aos ingredientes principais dos suplementos, a ocorrência de *Cistaceae* e *Brassicaceae* é compatível com espécies comuns nas zonas de cultivo e recolha dos extratos vegetais utilizados, podendo refletir contaminação ambiental natural durante o processamento.

A integração dos resultados evidenciou uma conformidade parcial entre os rótulos e o conteúdo real, com confirmação dos compostos bioativos declarados, mas variações na presença de resíduos biológicos. Este trabalho demonstra a relevância da aplicação combinada de métodos químicos, microbiológicos e palinológicos no controlo de qualidade e autenticidade desses suplementos, propondo a palinologia como uma ferramenta inovadora de rastreabilidade e verificação da origem botânica.

Palavras-chave: suplementos hepatoprotetores, FTIR, palinologia, *Silybum marianum*, silibinina, autenticidade.

Abstract

Hepatoprotective supplements have gained popularity due to their claimed ability to support liver detoxification and cellular regeneration. However, the lack of strict regulation and the increasing diversity of marketed products raise concerns regarding their authenticity, quality, and safety. This study aimed to perform a comprehensive analysis of hepatoprotective supplements commercialised in Portugal, combining chemical, microbiological, and palynological approaches to evaluate the correspondence between label information and actual composition.

Chemically, Fourier-Transform Infrared Spectroscopy (FTIR) identified characteristic bands of phenolic and flavonolignan compounds, particularly silybinin and silychristin, the main constituents of *Silybum marianum* (milk thistle). Solid samples displayed stronger correlation with reference standards, whereas the liquid supplement showed matrix interference caused by solvent composition. Microbiological analysis revealed the absence of dangerous pathogenic microorganisms, indicating suitable manufacturing and storage conditions. The palynological analysis revealed the recurrent presence of *Cistaceae* pollen grains in all samples, with additional detection of *Brassicaceae* pollen in one sample and fungal spores in another, suggesting possible environmental contamination or processing residues. Although pollen grains from the botanical families corresponding to the main labelled ingredients were not identified, the occurrence of *Cistaceae* and *Brassicaceae* is consistent with plant species commonly found in the cultivation and harvesting areas of the analysed extracts, likely reflecting natural environmental contamination during processing.

The integration of findings indicated partial label compliance, confirming the presence of declared bioactive compounds but revealing variability in biological residues. The combined application of chemical, microbiological, and palynological methods proved valuable for quality control and authenticity verification of those supplements. Furthermore, palynology emerges as an innovative and complementary technique for botanical traceability and authenticity assessment.

Keywords: hepatoprotective supplements, FTIR, palynology, *Silybum marianum*, silybin, authenticity.

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ADB – Agarose Dissolving Buffer

ASAE – Autoridade de Segurança Alimentar e Económica

ATR – Attenuated Total Reflectance

CFU – Colony-Forming Unit

DNA – Deoxyribonucleic Acid

dNTP – Phosphated Deoxyribonucleotides

DS – Dietary Supplements

EFSA – European Food Safety Authority

EMVS – European Medicines Verification System

FDA – Food and Drug Administration

FTIR – Fourier-Transform Infrared Spectroscopy

GC – Gas Chromatography

HPLC – High-Performance Liquid Chromatography

INFARMED – Instituto Nacional da Farmácia e do Medicamento

IRE – Internal Reflection Element

KOH – Potassium Hydroxide

LC-MS – Liquid Chromatography–Mass Spectrometry

PCR – Polymerase Chain Reaction

rRNA – Ribosomal Ribonucleic Acid

SDA – Sabouraud Dextrose Agar

TSA – Tryptic Soy Agar

1. Introduction

1.1. Dietary Supplements

The consumption of dietary supplements (DS) to complement the diet, providing essential nutrients such as vitamins, minerals, amino acids, and bioactive compounds to assist overall well-being and specific bodily functions, is increasing. This widespread acceptance is largely explained by their easy accessibility, relatively low cost, the perception of associated health benefits, and consumers' belief in the lack of harmful side effects (Hedegaard et al., 2013; Ouarda Djaoudene et al., 2023).

In most countries, the definition of dietary supplements is consistent. In the United States, the Food and Drug Administration (FDA) considers them to be a category of food products intended for ingestion that contain a 'dietary ingredient', such as vitamins, minerals, herbs, amino acids, enzymes, or metabolites, designed to provide additional nutritional value to the diet (FDA, 2023). Similarly, the European Food Safety Authority (EFSA) defines these products as concentrated sources of certain nutrients or other substances with nutritional or physiological effects, whose purpose is to supplement the normal diet (EFSA, 2025). According to National Authority for Medicines and Health Products (INFARMED), dietary supplements are "food products made up of concentrated sources of nutrients or other substances with nutritional or physiological effects, presented in measured forms (tablets, capsules, powder sachets, liquid ampoules, and similar forms)" (INFARMED, 2016). These products are intended to provide a beneficial effect on the body but should not be regarded as medicines.

Nowadays, there is a wide variety of dietary supplements easily found on the market, whether in pharmacies, supermarkets, health stores, hypermarkets, or even online. These products can be sold as single substances or as mixtures of different ingredients. The types of ingredients are quite diverse and may include vitamins, minerals, oils, amino acids, essential fatty acids, fibbers, plants, and herbal extracts (A. Petroczi et al., 2010; Ouarda Djaoudene et al., 2023).

They are widely used by individuals seeking to improve physical performance, strengthen the immune system, balance metabolism, and address nutritional deficiencies (Bailey et al., 2013; Ronis et al., 2017; Kozhuharov et al., 2023). For some populations, such as vegetarians, elderly individuals with poor absorption, pregnant women, or those who are nutritionally deprived, DS may represent an important resource to address specific nutritional deficiencies. Within the broader context of dietary supplement use,

research consistently highlights vitamins and minerals as the most consumed categories, a trend observed both in the United States and in Portugal (Falcato, 2014)

In Portugal, a nationwide study conducted in 2006 with a sample of 1,200 participants aimed to analyse the dietary supplement market. They showed that 81% of respondents had used or were currently using dietary supplements. Among these, vitamin-based supplements were the most reported, with around 65% of responses, followed by minerals with 52% (Felício, 2006). The study also investigated the main reasons for supplement use: 29% of respondents cited strengthening or preventive purposes, 26% reported fatigue or concentration as motivating factors, 22% linked use to general health, 17% mentioned other reasons, and 10% pointed to aesthetic concerns (Felício, 2006).

Later, in 2008, a study conducted in Lisbon and Vale do Tejo revealed that 48.8% of respondents consumed plant-based supplements, most often with the intention of achieving a calming effect (Santos et al., 2008).

Data from Marktest, confirm changing consumption patterns and indicates that in 2013, 12.7% of the Portuguese population over 15 years old and living on the mainland had taken dietary supplements in the previous 12 months (Marktest, 2013). By 2015, this proportion had risen to 19.5%, meaning that nearly one in five Portuguese adults were supplement users (Marktest, 2015). Overall, as shown in Figure 1, the data suggest that supplement consumption in Portugal has fluctuated over time, with peaks of higher use observed in 2008, 2014, and 2015, and lower rates recorded between 2009 and 2013. These declines coincided with the country's economic crisis, which likely influenced purchasing habits (Marktest, 2015).

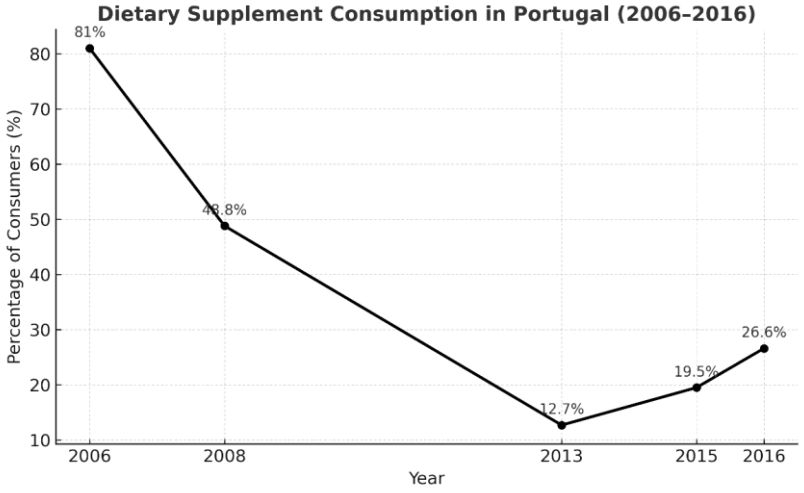

Figure 1 - Trends in dietary supplement consumption in Portugal between 2006 and 2016. Data adapted from Felício (2006), Santos et al. (2008), Marktest (2013, 2015), and FMUP et al. (2017).

More recently, data from the National Food Survey conducted between October 2015 and September 2016 revealed that 26.6% of the Portuguese population reported using dietary supplements in the last year. The survey also highlighted gender differences, showing that women were more frequent consumers (31.5%) compared to men (21.5%). Age-related patterns were evident as well, showing that 29.2% of adults were frequent consumers against 28.4% of the elderly. These groups reported higher supplement use than younger groups, with only 16.0% among adolescents and 6.2% among children as shown in Figure 2 (FMUP et al., 2017).

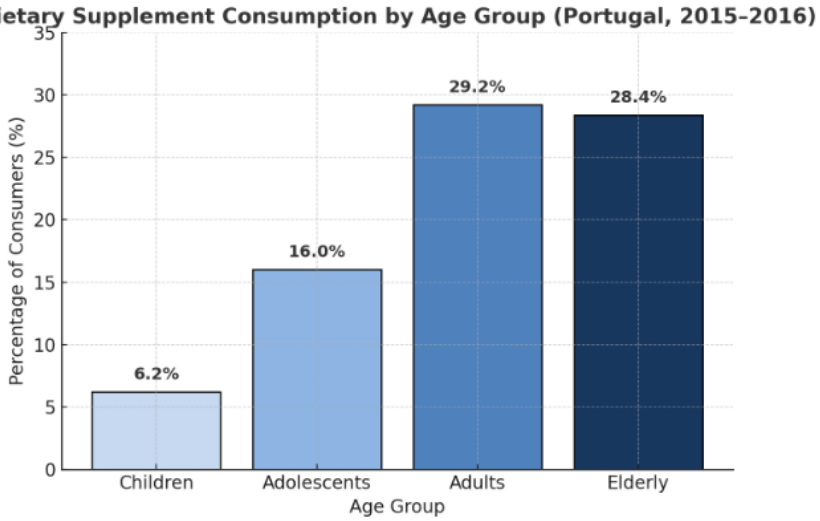

Figure 2 - Dietary supplement consumption by age group in Portugal (2015–2016). Data adapted from FMUP et al. (2017).

The nutritional supplements market can be categorised into several key segments, including vitamins, plant-based supplements, sports nutrition, minerals, meal replacement supplements, and specialised supplements (Pires et al., 2018). Among these, the vitamins segment recorded the highest sales in 2010 compared to other categories (Ouarda Djaoudene et al., 2023). This reflects the prominent role vitamins play in consumer choices, supported by factors such as ease of use and broad health benefits.

Plant-based supplements, while purchased less frequently than vitamins or minerals, are also widely used by consumers seeking to maintain or improve health. These supplements are generally considered more susceptible to microbiological hazards than those produced through bioengineering or organic synthesis. This higher vulnerability stems from both their natural origin and the processing methods applied. Such products can harbour microorganisms commonly found in soil, water, and air, including bacteria,

yeasts, and moulds, which may compromise safety and quality if not adequately controlled (Ratajczak et al., 2020)

Although widely perceived as natural and safe, plant-based supplements can present significant health risks due to side effects or interactions with medications and other supplements. These risks are particularly concerning in vulnerable groups or specific circumstances, such as the perioperative period. The use of these products is not recommended for pregnant or breastfeeding women, nor for children under 12 years of age, due to insufficient safety data. Although there is no specific guidance regarding the maximum duration of use, anyone who continues to experience symptoms for more than two weeks while taking the product should seek medical advice from a doctor or qualified healthcare professional. People who are hypersensitive to the active ingredient should avoid these supplements. In general, pant-based supplements are considered safe and non-toxic when used in small amounts and over short periods. The safety profile of many plant-derived supplements remains uncertain, especially regarding potential embryotoxic, fetotoxic, or carcinogenic effects, which are still insufficiently studied (De Smet, 2002; Kuhn, 2015).

The balance between benefits and risks depends not only on the product itself but also on possible interactions. When herbal supplements are taken alongside drugs or other dietary supplements, they may reduce or enhance therapeutic effects, posing additional dangers (Kuhn, 2015).

Health authorities have repeatedly drawn attention to these challenges. Some examples of adverse reactions to dietary supplements include gastrointestinal issues such as nausea or diarrhea; allergic reactions; kidney or liver toxicity; cardiovascular complications such as arrhythmias or hypotension; neurological problems like seizures; and, in severe cases, they may even lead to death (Barata, 2008; Cohen, 2014). The severity of an adverse reaction is always influenced by factors such as supplement-drug interactions, dosage, and health status, among others (Cohen, 2014).

1.2. Hepatoprotective Supplements

Hepatoprotective supplements are designed to protect and improve liver function, an organ essential for processes such as detoxification, nutrient metabolism, and protein synthesis. These supplements typically contain plant extracts with antioxidant, anti-inflammatory, and regenerative properties, being used by individuals seeking to prevent

or mitigate liver damage caused by factors such as excessive alcohol consumption, exposure to toxins, and hepatotoxic medications (Lucena & Navarro, 2014).

Through both traditional and modern herbal medicine, a variety of highly versatile and valuable plants with recognised hepatoprotective properties have been under ongoing investigation and use. The most common active components in these supplements include silymarin, derived from milk thistle (*Silybum marianum*), known for its ability to protect liver cells and promote regeneration (Ludovico Abenavoli et al., 2010). Curcumin, the active compound in turmeric, and alpha-lipoic acid, a natural antioxidant, are widely used to reduce oxidative stress and liver inflammation (Aggarwal & Harikumar, 2009; Shay et al., 2009). Additionally, artichoke (Cynara scolymus) has choleretic activity, meaning it stimulates the production of bile by the liver. These species have attracted attention due to the global rise in liver health concerns, with different parts of the plants demonstrating promises for therapeutic applications. While milk thistle has been widely researched for its active constituent (silymarin), artichoke is frequently consumed as a food or supplement and only to a lesser extent incorporated into herbal medicines (Foghis et al., 2023).

1.2.1. *Silybum marianum* (Silymarin)

Silybum marianum is an annual or biennial plant belonging to the *Asteraceae* family (Alemardan et al. 2013), commonly known as milk thistle and as botanically synonymous *Carduus marianus*. Native to the Mediterranean region, it is a plant that can reach up to two meters in height (Sherif et al., 2012), featuring large, dark green leaves with spiny edges as seen in Figure 3.

The plant is primarily sourced from cultivated fields, partly in Germany but mostly from China, Argentina, Romania, and some Mediterranean countries. Freshly ground seeds have a cocoa-like odour and an oily, bitter taste (Ludovico Abenavoli et al., 2010).

Figure 3 - *Silybum marianum* (milk thistle). From Mary Evan Picture Library, n.d.

The medicinal compounds of milk thistle derive from its seeds, which have been used pharmaceutically for over 2000 years (Alemardan et al., 2013). The most studied chemical from *Silybum marianum* is silymarin, widely considered the main active ingredient in the seeds (Alemardan et al., 2013; Corchete and Fernandez-Tarrago, 2013). Silymarin is an isomeric mixture of flavonolignans including silybin A, silybin B, isosilybin A, isosilybin B, silicristin, silydianin, and the flavonoid taxifolin (Alemardan et al., 2013; Sherif et al., 2013). Among these, the most abundant are the diastereoisomers silybin A and silybin B (Brantley et al., 2010) (Figures 4). Figure 5 shows their chemical structure.

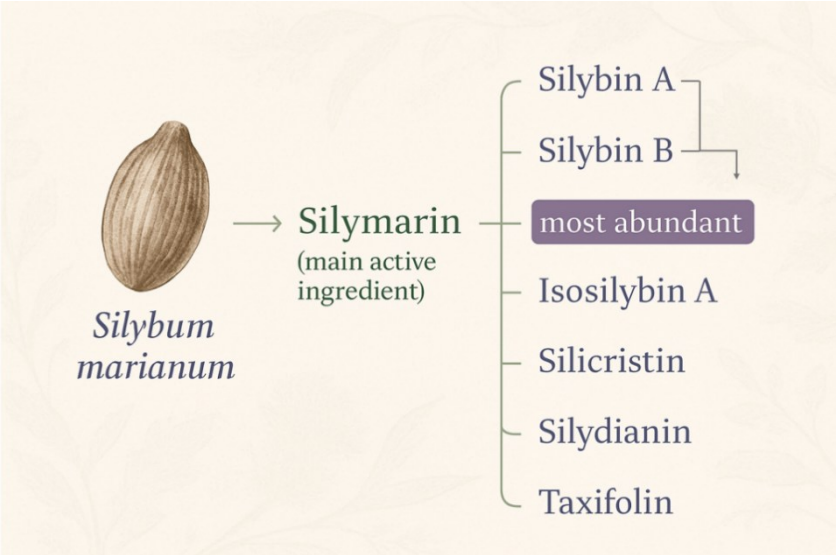

Figure 4 - Flavonolignan complex of silymarin extracted from milk thistle (*Silybum marianum*)

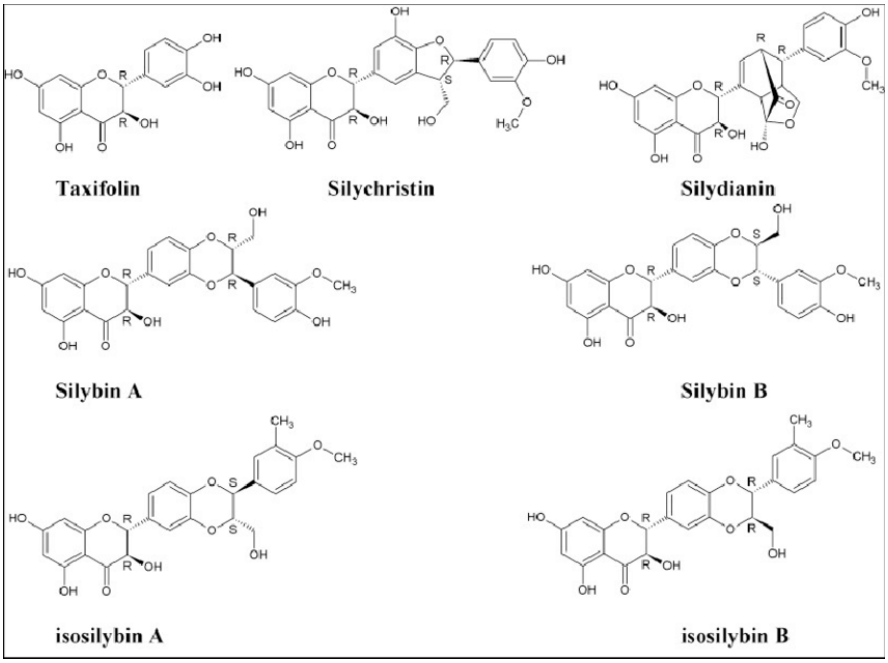

Figure 5 - Structures of known biologically active silymarin components. Adapted from Sigma-Aldrich, n.d.

Silymarin exhibits extensive biological activity. It is characterised by low toxicity, favourable pharmacokinetics, and stimulation of detoxification pathways (Theodosiou et al., 2013). Numerous studies indicate that this compound inhibits the hepatitis C virus by suppressing the inflammatory cascade triggered by viral infection. Additionally, silymarin imparts antioxidant, anti-inflammatory, and immunomodulatory properties responsible for its hepatoprotective effects. It also promotes protein biosynthesis and liver regeneration, increases lactation, and exhibits immunomodulatory activity (Alemardan et al., 2013; Ludovico Abenavoli et al., 2010). Silymarin has been found to block free radical formation and acts as an iron chelator. Research demonstrated its

ability to reduce COX-2 (Cyclooxygenase-2) expression and NF-κB (nuclear factor kappa B) pathway activity, both involved in inflammatory processes. Regarding its antifibrotic properties, silymarin decreases DNA synthesis induced by platelet-derived growth factor and TGF-β (Transforming growth factor beta), thereby reducing liver fibrosis and disease progression (Ludovico Abenavoli et al., 2010). Furthermore, it possesses cytotoxic activity against various cancer cell lines and enhances the efficacy of certain chemotherapeutic agents (Theodosiou et al., 2013).

Because of these biological activities, milk thistle is used medicinally to regulate liver function. Its primary active compound, silymarin, is employed to treat chronic liver inflammation and cirrhosis (Sherif et al., 2013). Today, it is a common ingredient in many dietary supplements aimed at weight loss, mainly due to its detoxifying properties.

Approximately 20 to 50% of silymarin is absorbed orally in humans and around 80% of the dose is excreted via bile, and about 10% undergoes enterohepatic circulation. Silybin's bioavailability is low and appears to be influenced by several factors including the presence of solubilising substances such as other flavonoids, phenolic derivatives, amino acids, proteins, tocopherol, fats, cholesterol, and other excipients in the formulation, and the concentration of the preparation itself (Ludovico Abenavoli et al., 2010).

Available in capsules, tablets, and liquid forms at various doses, milk thistle is generally non-toxic to the liver. However, some adverse effects reported include transient gastrointestinal complaints such as bloating, nausea, dyspepsia, and diarrhoea, as well as headaches, urticaria, and skin rashes. Preparations containing milk thistle are contraindicated in individuals with hypersensitivity to *Asteraceae* family plants (Patel-Rodrigues et al., 2024).

1.2.2. *Cynara scolymus* (Artichoke)

Cynara scolymus is a perennial plant commonly known as the artichoke (globe artichoke or simply artichoke in English). It belongs to the *Asteraceae* family and is also referred to botanically as *Cynara cardunculus* var. scolymus or *Cynara cardunculus subsp. scolymus*. Morphologically, it is an herbaceous plant that can grow up to 1.5 meters tall, with long leaves arranged in a rosette pattern. From the centre of the plant rises a tall flowering stalk, with a blooming period between August and September (Figure 6) (Ruiz-Cano et al., 2014).

Figure 6 - Globe artichoke (*Cynara scolymus*). From Popescu et al., 2014.

The artichoke's head and leaves are rich in flavonoids such as apigenin and luteolin, as well as glycosides. Native to the Mediterranean region, artichokes are now cultivated worldwide from seeds, being valued both for nutritional and medicinal purposes.

The nutritional and medicinal benefits of the artichoke are closely linked to the chemical composition of its head and leaves. Studies have shown that *Cynara scolymus* is a natural source of phenolic acids, mainly cynarin and chlorogenic acid (Figure 7) (Lattanzio et al., 2009; Moglia et al., 2008).

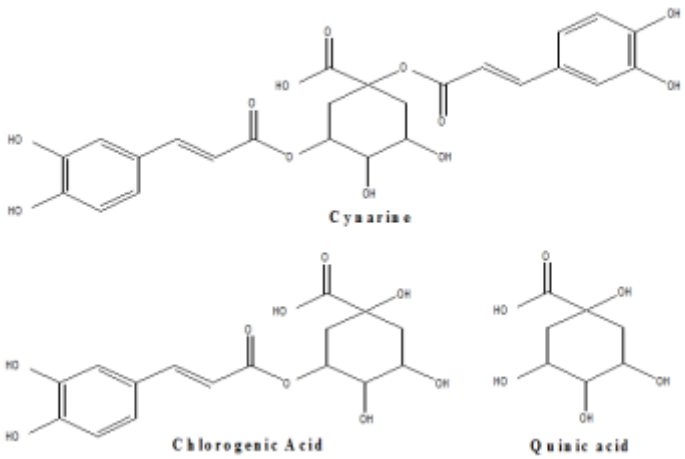

Figure 7 -Chemical structures of Cynarin and Chlorogenic from Alassadi et al., 2017

Research has also demonstrated that artichoke extract inhibits enzymes like HMG-CoA reductase and phosphatidate phosphohydrolase, which are involved in cholesterol and triglyceride biosynthesis, respectively. Some studies also report that artichoke extract

reduces oxidative stress by lowering reactive oxygen species production and lipid peroxidation. Additionally, several researchers have found that artichoke promotes insulin secretion, improves insulin sensitivity, and reduces blood glucose levels in animal models of diabetes (Doostkam et al., 2022).

Given its significant biological activities, artichoke is widely used medicinally to treat various conditions such as liver disorders, as a diuretic, and for diarrhoea management (Souza et al., 2013). It is also popular for its combined effects on weight loss, such as promoting satiety, enhancing faecal elimination, and improving fat metabolism.

1.2.3. Turmeric (curcumin)

Curcumin is a bright yellow compound found in turmeric, which comes from the plant Curcuma longa (Figure 8), a member of the ginger family (*Zingiberaceae)* native to tropical South Asia (Prasad & Aggarwal, 2025). Before turmeric can be used, its rhizomes must be processed by boiling or steaming to remove the raw odour, gelatinise the starch, and produce a more uniform colour.

Figure 8 - Turmeric (*Curcuma longa*) plant. From İpek Ece & Yasin Emre Kitis, 2023

More than 100 components have been isolated from turmeric. The main constituent of the root is a volatile oil containing turmerone, and turmeric also contains colouring agents called curcuminoids. These curcuminoids, shown in Figure 9, consist of

curcumin, demethoxycurcumin, 5'-methoxycurcumin, and bis-hydrocurcumin, all considered natural antioxidants (Ruby et al., 1995; Selvam et al., 1995). In its standard form, turmeric contains about 5–6.6% curcumin (Prasad & Aggarwal, 2025).

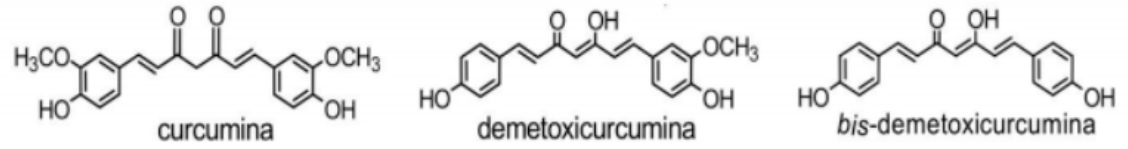

Figure 9 - Chemical structure of curcuminoid pigments isolated from the rhizome of *Curcuma longa* L. Adapted from Nascimento et al., 2020

As a dietary supplement, curcumin has gained attention for its anti-inflammatory and antioxidant properties due to its strong inhibition of NF-κB activation and TNF-mediated effects (Patel-Rodrigues et al., 2024). Diets supplemented with turmeric extract have been shown to reduce increases in lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels caused by liver injury induced by D-galactosamine (Miyakoshi et al., 2004).

Turmeric is believed to have many medicinal benefits, including boosting overall body energy, relieving gas, eliminating worms, improving digestion, regulating menstruation, dissolving gallstones, and easing arthritis. In many South Asian countries, it is used as an antiseptic for cuts, burns, and bruises, as well as an antibacterial agent. They are also used for their anti-inflammatory effects and to treat gastrointestinal discomfort associated with irritable bowel syndrome and other digestive disorders (Prasad & Aggarwal, 2025).

1.3. Counterfeiting of Supplements

While legitimate, the quality of dietary supplements can be variable and may not align with the claims made on their labels. Such products may contain inadequately dosed ingredients, contaminants, or lack the declared active compounds. Such issues pose a significant public health risk, increasing the likelihood of adverse effects and undermining the expected benefits of these supplements (Kamil, 2019; Hervé Rebiere et al., 2017).

Many dietary supplements do not comply with the associated labels or are of poor quality. Plant-based supplements for diabetes control, energy boosting, and hepatoprotective purposes are common targets for noncompliance. These products may contain synthetic chemicals to mask the absence of active ingredients or mimic the effects of genuine compounds, posing serious toxicity risks and undesirable interactions

with other medications (Lucena & Navarro, 2014). Other examples include supplements promoting weight loss, sexual performance enhancement, and muscle growth (Ronis et al., 2017). Table 1 summarises the main categories of dietary supplements most frequently adulterated, and the potential undeclared substances found in them. Such undeclared ingredients can pose severe health risks, especially when combined with other substances or consumed by individuals with preexisting medical conditions. (Rocha et al., 2015; Vanya Rangelov Kozhuharov et al., 2023)

Table 1 - Commonly adulterated dietary supplements and potential undeclared substances

Supplement type	Claimed purpose	Frequent undeclared/adulterant substances
Plant-based supplements	Diabetes control, energy boosting, liver protection	Synthetic chemicals mimicking active ingredients; drugs to mask the absence of natural compounds
Weight-loss supplements	Appetite suppression, fat burning	Banned appetite suppressants (e.g., sibutramine), laxatives, stimulants
Sexual performance enhancers	Erectile dysfunction support	Phosphodiesterase type 5 (PDE-5) inhibitors (e.g., sildenafil analogues)
Muscle growth/bodybuilding supplements	Muscle mass increase, anabolic effects	Synthetic anabolic steroids, prohormones, stimulants

The addition of sibutramine to plant-based dietary supplements is a well-documented form of adulteration that has garnered attention in recent scientific literature. Sibutramine acts as a neurotransmitter reuptake inhibitor, sharing structural similarities with amphetamines. By reducing the reabsorption of serotonin, norepinephrine, and noradrenaline, this molecule increases their concentrations within synaptic clefts, which can suppress appetite and contribute to weight loss (Mathon et al., 2014).

Another common adulteration practice involves replacing the original plant material used to produce extracts with another species, either wholly or partially. This substitution can be unintentional, such as misidentification during wild harvesting, where gatherers may confuse target plants with close relatives or lookalike species, or

deliberate, often motivated by economic incentives, especially when species are hard to distinguish (Applequist & Miller, 2013).

For all herbal products, it is essential to uphold strict microbiological quality standards, like those applied for other non-sterile pharmaceuticals. Acceptable microbial contamination limits for plant-derived medicines typically include total aerobic bacteria $\leq 10^5$ Colony-Forming Units per gram (CFU/g), yeasts and moulds $\leq 10^3$ CFU/g, and Enterobacteriaceae or other Gram-negative organisms also $\leq 10^3$ CFU/g, while *Escherichia coli* and *Salmonella* spp. must be absent. Failure to maintain microbiological quality can compromise, or even nullify, the therapeutic effectiveness of the product (Okunlola et al., 2007).

Given the tangible risks when using low-quality or adulterated natural products, rigorous quality assurance, regulatory oversight, and public education are crucial (Bugno et al., 2005). Manufacturers must minimise microbial loads in raw materials and ensure their products remain safe, effective, and consistent (Okunlola et al., 2007). Product information should clearly present details about the manufacturer, composition, storage, and proper usage. Despite these guidelines, standardising plant-based supplements remains challenging due to their complex nature and the frequently unknown role of individual constituents. The quality and quantity of bioactive compounds in the finished product depend on factors such as source material, geographic origin, cultivation practices (with or without pesticides), and post-harvest handling—including harvesting, washing, drying, and storage (Rocha et al., 2015).

1.4. Relevant legislation

The exponential growth in the number of food supplements marketed in Europe, coupled with the differing national regulations of each Member State, alerted the European Union to the need for harmonising legislative measures governing these products. The aim was to allow free circulation within the internal market and prevent unequal competitive conditions. Consequently, Directive 2002/46/EC of the European Parliament and of the Council, concerning the approximation of the laws of the Member States relating to food supplements, was introduced on June 10, 2002, and revised on March 30, 2006. This directive provides only partial harmonisation of these products, covering their presentation, labelling, advertising, and the conditions for the use of vitamins and minerals (Silano et al., 2011). In fact, among the range of nutrients and

other ingredients that can be present in dietary supplements, this directive sets specific standards only for vitamins and minerals.

Mandatory labelling information must be written in Portuguese and appear on the exterior packaging and cannot be replaced by information inside the package. These include the food category designation "Food Supplement", list of ingredients, indication of ingredients or technological aids that cause allergies or intolerances, quantity of certain ingredients or ingredient categories, net quantity, minimum durability date or use-by date, special storage and/or usage conditions, usage instructions, designation of nutrient categories or substances characterizing the product or specific reference to the nature of those nutrients or substances, recommended daily intake, quantity of nutrients or substances with nutritional or physiological effects present, country of origin or provenance, name or company name and address of the responsible food business operator, and last a batch number. Additionally, precautionary statements must be included in the label, such as: “Food supplements do not replace a balanced and varied diet”; “The daily intake should not be exceeded”; “Keep out of reach of children”. It is permissible to include a consumer information leaflet inside the packaging, provided its content complies with legal requirements, especially regarding health claims. For safety reasons and traceability, the blister pack or individual dosage must at least contain the product name, minimum durability date, and batch identification (*REGULAMENTO UE N. O 1169/2011,* 2011; Decreto-Lei N.º 118/2015, 2015).

It is important to highlight that food supplements do not require approval by the FDA for market placement. The manufacturer is responsible for the authenticity of the claims on the packaging and must have evidence to substantiate them and ensure the quality and safety of their supplements. If a safety issue occurs, the FDA acts to verify that the product is hazardous (De Smet, 2002). Moreover, supplements are subject to all provisions of food legislation. This means that, beyond complying with the law on food supplements, they must also adhere to the General Food Law related to food hygiene, novel foods and new food ingredients, the addition of vitamins, minerals, and other substances to food, and community regulations on food additives, contaminants, and pesticides (Camilo, 2009; Silano et al., 2011).

If an economic operator intends to introduce a new supplement to the Portuguese market (or modify an existing one), or to import food supplements, they must contact the Directorate of Standardization and Food Safety Services of the Planning and Policy Office of the Ministry of Agriculture. Decree–Law No. 296/2007 of August 22

designated this Office as the competent body for submitting supplement labels before market entry. The Food and Economic Safety Authority (ASAE) have the mission of inspecting, assessing, and communicating risks within the food chain (Santos et al., 2008).

1.5. Assessment Methods

The identification of counterfeit supplements is based on advanced analytical methods and rigorous inspections to ensure product compliance with safety and quality standards. In the quality assessment of herbal dietary supplements, a wide range of analytical methods has been reported in the literature. These include chromatographic approaches such as High-Performance Liquid Chromatography (HPLC), spectroscopic tools such as Fourier Transform Infrared Spectroscopy (FTIR), mass spectrometry-based techniques, microbiological testing, targeted analyses for specific bioactive compounds, and validation strategies to ensure analytical reliability. More recently, palynology has also emerged as an innovative complementary tool, offering insight into botanical origin and authenticity. (Martino et al., 2010; Parthik et al., 2011).

Analytical methods are essential for ensuring the safety and authenticity of dietary supplements, particularly because many products may contain undeclared or adulterated compounds. Chromatographic techniques coupled with mass spectrometry, are among the most powerful tools for detecting pharmaceutical adulterants in botanical supplements. These methods allow precise separation, identification, and quantification of compounds, making them highly effective in monitoring known adulterants and ensuring compliance with regulations (Vaclavik et al., 2014; Rocha et al., 2015). In addition to targeted methods, which focus on detecting specific known adulterants, non-targeted or screening approaches are increasingly important, as they enable the identification of unexpected or novel synthetic analogues added to supplements (Vaclavik et al., 2014; Rocha et al., 2015). Validation of these analytical protocols, including parameters such as sensitivity, precision, accuracy, and matrix effects, is fundamental to guarantee trustworthy results and to support regulatory enforcement. These methodologies are crucial for consumer protection, as they not only verify label compliance but also safeguard against the presence of undeclared pharmaceuticals that could pose serious health risks (Rocha et al., 2015).

In Portugal, the INFARMED is the entity responsible for oversight. INFARMED collaborates with the European and international network to combat medicine

counterfeiting, using databases and tools such as the European Medicines Verification System (EMVS), which authenticates products through two-dimensional barcodes (Santos et al., 2008).

1.5.1. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR is a technique used to identify functional groups in compounds based on the absorption of infrared light at different wavelengths. It can verify the presence of active components by comparing the spectral signature of the sample with a legitimate standard, making it useful for the identification of organic compounds (Figure 10) (Stuart, 2004; Smith, 2011; Russo 2017).

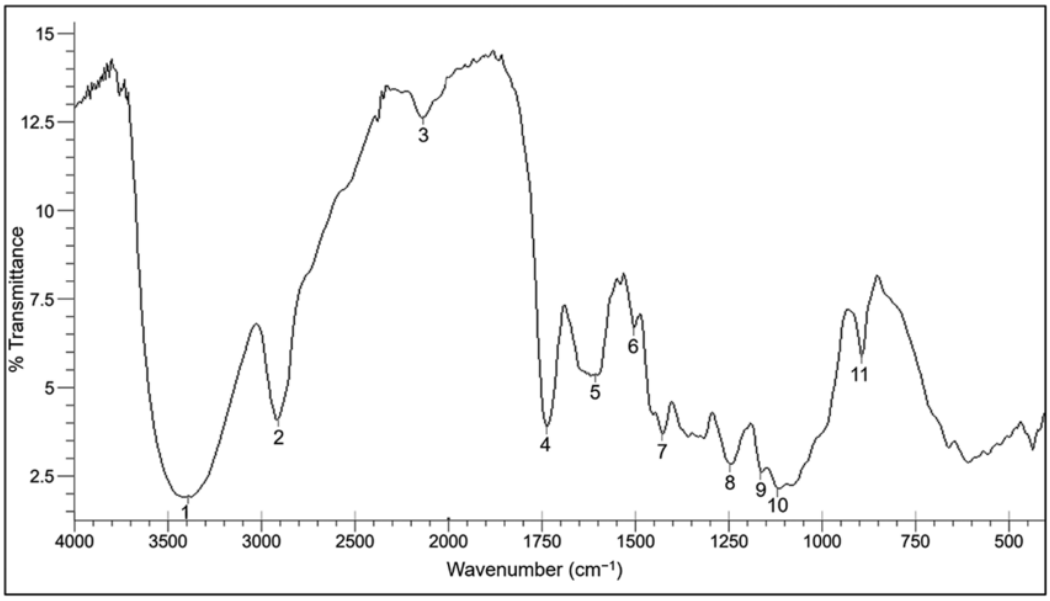

Figure 10 - FTIR spectrum of the fibbers obtained from the flower heads of milk thistle showing the % transmittance vs. wavenumber in cm^{-1}. Identified peaks are numbered 1 through 11. From Alzarieni et al., 2023

FTIR spectroscopy enables the identification of functional groups and molecular structures through their characteristic vibrational frequencies. In plant-based supplements, key absorbance regions include the broad O–H stretching band (3200–3600 cm^{-1}) associated with phenolic and hydroxyl groups, C–H aliphatic stretching (2950–2850 cm^{-1}), the conjugated C=O/C=C region around 1650–1600 cm^{-1} typical of flavonoids and flavonolignans, and the strong C–O and C–O–H stretching bands between 1260 and 1000 cm^{-1}. Additionally, out-of-plane C–H bending below 900 cm^{-1} provides information on aromatic ring substitution patterns (Table 2). These spectral regions are particularly relevant for the identification of compounds such as silibinin, silychristin, and other phenolic constituents present in hepatoprotective formulations (Li et al., 2021).

Table 2 - FTIR characteristic absorption regions relevant to the identification of phenolic compounds and flavonoid-based structures such as silybinin, silychristin, and apigenin.

Wavenumber range (cm^{-1})	**Vibrational assignment**	**Associated functional group**
3200–3600	O–H stretching (broad band)	Phenolic hydroxyl groups
2950–2850	C–H stretching (aliphatic)	CH_2 / CH_3 groups in organic matrices
1650–1600	Conjugated C=O stretching and/or aromatic C=C stretching	Flavonoids and flavonolignans
1560–1500	Aromatic C=C stretching	Phenolic aromatic rings
1260–1000	C–O and C–O–H stretching	Ethers, alcohols, and phenolic groups
900–650	Out-of-plane C–H bending	Substituted benzene rings
~2300	Atmospheric CO_2	—

One of the most significant advantages of FTIR is its broad applicability. A wide variety of molecules exhibit strong absorbances in the mid-infrared region, which is why spectra are typically collected in this range. Infrared spectroscopy can be applied to an extensive range of sample types, including solids, liquids, gases, semi-solids, powders, polymers, organic and inorganic compounds, biological materials, pure substances, and mixtures (Smith, 2011; Pavia et al., 2015). Another key advantage is the wealth of information contained within infrared spectra. The peak positions provide insights into the molecular structures present in a sample, peak intensities reflect the relative concentrations of molecules, and peak widths are sensitive to the sample's chemical environment, including pH and hydrogen bonding (Stuart, 2004; Smith, 2011). In addition to being information-rich, infrared spectroscopy is also rapid and straightforward. Although the sample nature and the chosen sampling technique influence ease and speed, many spectra can be collected within five minutes or less. In some cases, high-quality spectra are obtainable within seconds. Cost-effectiveness is another important advantage. FTIR is less expensive to operate than many chromatographic methods, making it accessible for routine analysis. Besides that, FTIR offers high sensitivity, which refers to the minimal amount of material needed to generate a usable spectrum.

Despite its significant analytical capabilities, FTIR spectroscopy, does have some important limitations. Certain substances, such as noble gases and monatomic ions, do not produce detectable infrared spectra because they lack molecular vibrations. These species are chemically unbound and therefore invisible in the mid-infrared region (Pavia et al., 2015).

Homonuclear diatomic molecules like oxygen and nitrogen exhibit only symmetric stretching, resulting in zero intensity peaks in the infrared, but this feature is advantageous since these gases make up the bulk of Earth's atmosphere and would otherwise interfere strongly with analytical spectra (Smith, 2011).

A major practical disadvantage of FTIR lies in the analysis of complex mixtures. As sample composition becomes more intricate, the resulting spectra also increases in complexity, making it difficult to assign peaks to specific molecules. Besides, the inability of FTIR to directly identify atoms or monoatomic ions means complementary methods, such as atomic spectroscopy, are often required when analysing such species (Smith, 2011).

The disadvantages and advantages are listed in Table 3.

Table 3 - Advantages and limitations of FTIR spectroscopy

Advantages	Limitation
Broad applicability	Can not detect substances without molecular vibrations (e.g., noble gases, monatomic ions).
Provides rich molecular information (structure, concentration, chemical environment).	Homonuclear diatomic molecules (e.g., O_2, N_2) show no infrared activity.
Rapid and simple, with spectra obtained in seconds to minutes	Difficult to interpret spectra from complex mixtures due to overlapping peaks.
Cost-effective compared to many chromatographic methods.	Often requires complementary methods for full characterisation.
High sensitivity	-

1.6. Forensic Palynology

Palynology is the scientific discipline that studies plant pollen, spores, and certain microscopic planktonic organisms, such as diatoms, in both living and fossil forms (Boi,

2018). This field is linked to plant sciences as well as geological sciences, notably those aspects related to stratigraphy, historical geology, the study of organic microfossils (palynomorphs) extracted from ancient coals, palaeontology, and archaeology. Palynology is exceedingly broad, with applications across botany and related sciences, and it has also generated significant interest in forensic science and crime scene investigation (Boi, 2018).

In forensic contexts, palynology refers to the application of pollen and spore studies to legal matters, which continues to be used to establish connections among objects, people, and places by analysing and identifying pollen (Vaishnavi, 2022).

Pollen grains are particularly useful in forensic applications due to their exceptional resistance to chemical attacks. They can remain at crime scenes long after the events under investigation. Additionally, pollen provides a regularly transferred source of material often involved in exchanges of mud, soil, and debris particles (D.C. Mildenhall et al., 2006). Furthermore, pollen can be directly transferred through contact with plant parts containing spores or pollen. Pollen grains are ideal forensic traces because of their small size, high variability, and presence on objects exposed to or interacting with air (Alotaibi et al., 2020).

Like any emerging scientific application, forensic palynology has advantages and limitations. The technique has gained recognition as an important crime investigation tool, with benefits outweighing drawbacks, largely due to the dispersion of pollen grains by their small size and strong adherence to various surfaces, skin, and clothing fibres. Both pollen and spores possess highly resistant structures that withstand environmental factors such as heat, cold, washing, and degradation, preserving them for many years.

Conversely, difficulties include incomplete information regarding sampling, location, and standardised collection methods, alongside a shortage of specialists and academic centres for scientific training, which limits its use (Walsh and Horrocks, 2008).

Plant species identification assists in determining the geographic origin of specimens, linking crime scenes and suspects, establishing alibis, controlling the trade of protected species, and resolving civil and criminal cases (Coyle et al., 2001). Even when the efficacy of botanical evidence is proven and legally accepted, many investigators still underestimate its potential. This is exacerbated by pollen grains lodging in fabrics and small footwear crevices, held by static charges and surface morphology, rendering removal difficult even after washing. This persistence secures their value as location or surface indicators (Alotaibi et al., 2020).

Moreover, botanical recognition can correlate samples with geographic origins, help prove presence at locations, differentiate between accidental, suicidal, or homicidal deaths, estimate burial dates, and identify bodies (Ferri et al., 2009; Alotaibi et al., 2020). While matching pollen between suspects and crime scenes reveals prior presence, it does not imply guilt, so forensic palynology is often considered a supplementary tool rather than a replacement for traditional investigative techniques. The shortage of trained palynologists remains a core challenge. Additionally, most plant evidence, except pollen, may deteriorate or change following collection (Alotaibi et al., 2020; Vaishnavi, 2022).

As noted, palynology is used widely in forensic scenarios. Palynological evidence enables the establishment of relationships among crime scenes and suspects/victims, objects, or locations by comparing forensic and control samples (Vaishnavi, 2022). Qualitative assessment of pollen content can confirm alleged crime scene locations cited by victims and evaluate suspect testimonies and alibis. Palynological evidence may also support other evidence types in sexual assault cases, helping to establish or verify the crime location indicated by victims (Alotaibi et al., 2020).

Bodies effectively trap pollen and spores, whether in living individuals or cadavers in early decomposition stages. The human body efficiently captures pollen content across various tissues, permitting sampling for palynological analysis from different biological tissues even in advanced decomposition or skeletonisation (Boi, 2018).

Besides biological tissues, non-biological materials are relevant. Soil serves as an effective palynomorph storage source, naturally or through rain deposition. After plant death and degradation, pollen grains accumulate in the soil, making forensic palynological analyses commonly performed alongside geological forensic investigations (D.C. Mildenhall et al., 2006).

Applications of forensic palynology have extended to illicit substance use and poisoning cases. By collecting multiple samples from the victim's gastric contents and objects from their residence, various psychotropic substances from illicit plants and fungi manipulated by the victim were identified. The presence of illegal drug plantations, such as marijuana, can also be monitored via palynological analysis. Aerobiological sampling to identify marijuana pollen can indicate nearby cultivation sites (Vaughnm-Bryant & Mildenhall, 1998).

Besides, vehicles can also be sampled for palynology, collecting residues from pedals, wheels, mats, seats, and filters (Adams-Groom, 2012).

In Table 4 are listed some advantages and limitations of this technique.

Table 4 - Advantages and Limitations of Forensic Palynology

Advantages	Limitations
High persistence of pollen grains due to resistant exine (can survive for decades or centuries).	Requires specialised expertise; few trained forensic palynologists worldwide.
Pollen is highly diverse, species-specific, and ubiquitous in the environment, acting as a natural geographic marker.	Lack of standardised protocols for sampling, storage, and analysis.
Small size and strong adherence allow transfer to clothing, hair, vehicles, and other objects, making it difficult to remove even after washing.	Complex spectra in mixed samples may complicate interpretation.
Can link suspects, victims, objects, and crime scenes through comparative analysis.	Results are often considered supplementary evidence and rarely sufficient on their own in court.
Applicable in a wide range of cases: homicides, sexual assaults, drug trafficking, terrorism, and environmental crimes.	Some investigators underestimate or overlook its evidential potential.
Cost-effective technique compared to many other forensic methods.	Plant materials other than pollen (e.g., leaves, fibres) deteriorate quickly, reducing their evidential value.

1.7. Aim

This study evaluated hepatoprotective supplements commercialised in Portugal through a multidisciplinary approach. It aimed to identify active phenolic compounds using FTIR-ATR spectroscopy, assess microbiological quality through culture and molecular characterisation, and determine botanical authenticity while detecting potential environmental contaminants using forensic palynology.

While label compliance verification is pursued, quantification of active components and comprehensive screening for undeclared adulterants requires complementary chromatographic techniques beyond the scope of this exploratory study.

2. Materials and Methods

2.1. Criteria for Supplement Selection

Four dietary supplements with alleged hepatoprotective action were acquired from local parapharmacies and independent shops in Lisbon:

- **ArkoPharma:** Hepatic detox supplement containing milk thistle.
- ***GOTAS* Wells:** Drops for gallbladder and liver with hydrophilic and dry extracts of *Silybum marianum* (milk thistle), artichoke, and others.
- **Bioherba:** Capsules with milk thistle extract and vitamin B6.
- **BambooLabs:** Capsules with extracts of artichoke, dandelion, *desmodium,* and milk thistle.

Only hepatoprotective supplements were included in this study, given the liver's central role in the metabolism of xenobiotics, including medications, drugs, and environmental contaminants. All selected supplements contained plant-based ingredients with either traditional use or scientific evidence supporting hepatoprotective effects. Specifically, *silybum marianum* (milk thistle), *cynara scolymus* (artichoke) and *taraxacum officinale* (dandelion) that has been traditionally used to support digestive and liver function. Also, *desmodium adscendens* that is a plant with ethnopharmacological reports suggesting liver protection.

The selected products were easily obtainable in the Portuguese market and represented a range of brands, including well-established brands distributed in parapharmacies (Arko, Wells) as well as alternative or independent brands available in specialised shops (Bioherba, BambooLabs), reflecting the diversity of commercially available hepatoprotective supplements. Table 5 summarises their main characteristics, active ingredients, and the effects stated on their labels.

Table 5 - Selected dietary supplements for analysis, main ingredients, and alleged hepatoprotective effect.

Product/ Source	Main Ingredients	Hepatoprotective Effect	Dosage Form
Arko (Parapharmacy)	*Silybum marianum* (milk thistle)	Liver detoxification; protection against oxidative stress	Capsules
***Gotas* Wells (Parapharmacy)**	*Silybum marianum, Cynara scolymus* (artichoke), other plant extracts	Support for liver and gallbladder function; stimulation of bile secretion	Oral drops
Bioherba (Independent shop)	*Silybum marianum* + Vitamin B6	Metabolic support; antioxidant; liver regeneration	Capsules
BambooLabs (Independent shop)	*Cynara scolymus* (artichoke), *Taraxacum officinale* (dandelion), *Desmodium adscendens, Silybum marianum*	Liver protection; digestive support; anti-inflammatory effect	Capsules

2.2. Chemical Approach

2.2.1. Analytical standards

Three reference standards were used as analytical markers for comparative evaluation of the samples: silychristin, 10 mg (Sigma-Aldrich, St. Louis, MO, USA), silybin A ≥98% (Sigma-Aldrich, St. Louis, MO, USA), and apigenin 100 µg/mL in Acetonitrile:Methanol (Dr. Ehrenstorfer, UK) (Figures 11,12,13). These compounds served as model references for identifying and interpreting the main functional groups present in the analysed formulations. The comparison of their vibrational profiles by Fourier Transform Infrared Spectroscopy (FTIR) enabled a qualitative assessment of structural similarities between the compounds under study.

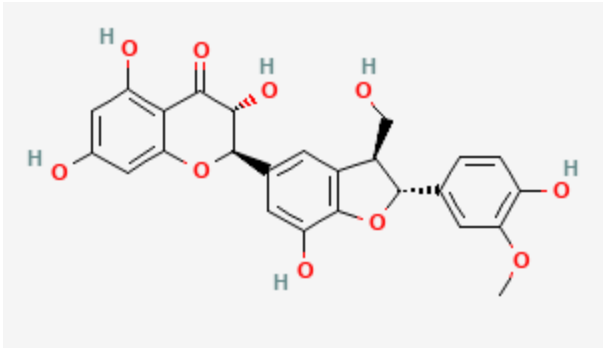

Figure 11 - Chemical structure of silychristin. Image retrieved from the PubChem database (CID: 54685836)

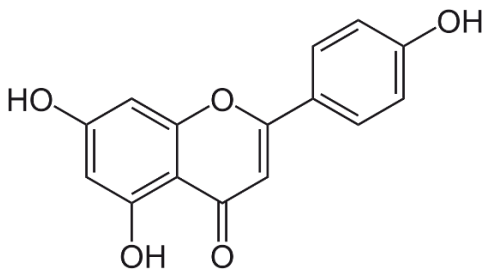

Figure 12 - Chemical structure of Apigenin. Retrieved from Pubchen, n.d.

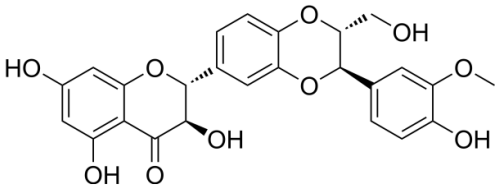

Figure 13 - Chemical structure of silybin A (silibinin). Retrieved from Silybin A (MCE Product Page), by MedChemExpress, n.d.

2.2.2. Experimental Procedure (FTIR-ATR)

Spectroscopic analyses were conducted using a FTIR spectrometer, Spectrum 65 (PerkinElmer, Waltham, MA, USA) equipped with an Attenuated Total Reflectance (ATR) accessory, eliminating the need for pellet preparation. Although infrared spectra can be represented in transmittance or absorbance, the present work adopts the transmittance format, as it provides clearer visualisation of peak maxima and facilitates the qualitative interpretation of characteristic vibrational bands. In ATR-FTIR, transmittance spectra are commonly preferred because they minimise distortions associated with strong bands in transmittance mode and allow more straightforward comparison of functional group assignments reported in the literature for flavonoids and phenolic compounds.

Samples and standards were directly applied to the ATR crystal without dilution or pretreatment, ensuring intimate contact between the sample and the crystal surface.

Spectra of the analytical standards were first collected for reference. Each measurement consisted of 32 scans accumulated under a resolution of 4 cm^{-1} within a spectral range of 4000–400 cm^{-1}.

Following the acquisition, spectra corresponding to both reference standards (silychristin, silybin, and apigenin) and samples were processed using the instrument's analytical software Spectra Manager™ Software, version 2.15 (PerkinElmer, Waltham, MA, USA). Baseline correction and intensity normalisation were applied to allow direct comparison across spectra. Absorption peaks were analysed within the 4000–400 cm^{-1}

region, focusing on characteristic vibrational modes associated with O–H, C=O, C–O, and aromatic/phenolic C–H bonds (Li et al., 2021).

The relative positions and intensities of these characteristic bands were compared between reference and sample spectra to evaluate structural similarities and confirm the presence of functional groups typical of hepatoprotective compounds.

2.3. Microbiological Approach

2.3.1. Inoculation in Culture Media

Each supplement was diluted in buffered peptone water (Scharlau, Sentmenat, Barcelona, Spain), and 100 µL of each dilution was inoculated in duplicate onto plates of Tryptic Soy Agar (TSA) (Biolife Italiana S.r.l., Milan, Italy) and Sabouraud Agar with Chloramphenicol 50 mg/L (SDA w/CAF) (Biolife Italiana S.r.l., Milan, Italy), using the surface spread plate technique. All inoculation procedures were performed under aseptic conditions in a class II laminar flow cabinet. The plates were incubated at 37°C and 25°C, respectively, for 48 hours. Bacterial growth was observed only on the TSA plates.

2.3.2. Microscopic Analysis

2.3.2.1. Methylene Blue Staining

From the cultures obtained on TSA, slides were prepared for microscopic observation. The samples were stained with 1% methylene blue for 2 minutes, washed with distilled water, and observed under an optical microscope, model CX23LED (Olympus Corporation, Tokyo, Japan), at 1000x with immersion oil, to assess bacterial morphology.

2.4.2.1. Gram Staining

For the determination of cell wall type, Gram staining was performed according to the classical protocol: crystal violet (1 min), iodine (1 min), washing with alcohol-acetone (20 sec), and counterstaining with fuchsin (30 sec). The slides were again observed under the microscope (Cappuccino et al 2014).

2.3.3. Genomic DNA Extraction

Bacterial DNA extraction was performed using the phenol-chloroform method (ThermoFisher, 2025). Colonies were collected with a sterile loop and resuspended in 180 µL of TE buffer to promote cell wall lysis. This was followed by incubation with 20 µL of 10% SDS and 5 µL of Proteinase K (20 mg/mL) at 55°C for 60 minutes. Protein denaturation was achieved by extraction with 200 µL of phenol:chloroform:isoamyl

alcohol (25:24:1) (Sigma-Aldrich, St. Louis, MO, USA), followed by precipitation with one volume of isopropanol and incubation at -80°C for 10 minutes. The DNA was then washed with 70% ethanol and centrifuged in centrifuge for Microtubes (Eppendorf, Hamburg, Germany) for 20 minutes at 13,000 rpm. The pellet was air-dried overnight and subsequently resuspended in 25 µL of ultrapure water. DNA quantification was performed by spectrophotometry using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Verona Road, MA, USA), ensuring an A260/A280 ratio between 1.8 and 2.0.

2.3.4. Polymerase Chain Reaction (PCR)

2.3.4.1. Master Mix Preparation

PCR amplification was performed using universal bacterial 16S rRNA primers 27F and 1492R, targeting approximately 1,400 bp of the 16S rRNA gene. Two PCR protocols were carried out, both using GoTaq® DNA Polymerase protocol (Promega, Madison, WI, USA) (Promega, 2025):

- **First PCR:** Final volume of 50 µL (4 µL DNA + 46 µL Master Mix).
- **Second PCR:** Final volume of 30 µL (3 µL DNA + 27 µL Master Mix).

The composition of the Master Mix for each protocol is detailed in Table 6, with the components listed from highest to lowest concentration.

Table 6 - Composition of the PCR Master Mix

Component	1st PCR (50 µL)	2nd PCR (30 µL)
Ultrapure Water	15.75 µL	9.45 µL
5 x Green GoTaq Reaction Buffer	10 µL	6 µL
dNTPs (10 mM)	10 µL	6 µL
$MgCl_2$ (25 mM)	4 µL	2,4 µL
Primer Forward	2.5 µL	1.5 µL
Primer Reverse	2.5 µL	1.5 µL
GoTaq Polymerase	0.25 µL	0.15 µL

The Master Mix was prepared with a multiplication factor of ×6 for the first run and ×8 for the second, according to the number of samples, including negative and positive controls and error margins.

2.3.4.2. PCR Program

After preparation, the tubes were gently vortexed and centrifuged (spin-down) before being placed in the thermal cycler, T-1 *Thermoblock* (Biometra Analytik GmbH, Göttingen, Germany). The amplification program used is demonstrated in table 7:

Table 7 - PCR Thermal Cycler Program

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	95	2 min	1
Denaturation	95	30 s	35
Annealing	55	30 s	-
Extension	72	1 min 30 s	-
Final Extension	72	5 min	1

2.3.5. Agarose Gel Electrophoresis

The analysis of amplified products was performed by agarose gel electrophoresis at 2%, prepared with 1× TAE buffer (Thermo Fisher Scientific, Waltham, MA, USA). The gel was stained with GreenSafe Premium Nucleic Acid Stain (NZYTech, Lisbon, Portugal) and the products visualised using a UV transillumination system. The Biotin Hyperladder 100 bp (Bioline, London, UK) molecular marker was used to determine fragment sizes, with the expected amplicon being approximately 1.5 kb. The Power Supply used, was PS 3003 (Biometra Analytik GmbH, Göttingen, Germany)

2.3.6. DNA Band Purification

The bands corresponding to the amplicons of interest were excised from the gel with a sterile blade and stored in Eppendorf tubes. The bands were incubated at 50°C for 2 minutes with 300 µL of ADB Buffer. Subsequently, 100 µL of isopropanol was added and the mixture was centrifuged at 13,000 rpm for 1 minute at room temperature in a purification column. Washing was performed with 750 µL of PE buffer, followed by incubation at room temperature for 5 minutes and a second centrifugation. The column was then dried by an additional 3-minute centrifugation at 13,000 rpm. Finally, DNA was eluted with 22 µL of ultrapure water, incubated for 2 minutes, and centrifuged for final recovery (Green, M. R., & Sambrook, J., 2025).

The purified samples were sent for Sanger sequencing at STABvida – *Investigação e Serviços em Ciências Biológicas, Lda*. (Caparica, Portugal). For each sample, 10 µL of purified DNA and 3 µL of the 1492R (reverse) primer were sent. Sequence analysis was

subsequently performed using bioinformatics tools, specifically SnapGene® Software (version 7.0.2; GSL Biotech LLC, Chicago, IL, USA) and BLASTn (NCBI, Bethesda, MD, USA)

2.4. Palynological Approach

The palynological analysis was conducted to isolate, purify, and identify pollen grains and spores present in the supplement samples under study. This type of analysis is particularly relevant for verifying the authenticity of products of plant origin and for identifying possible environmental contaminants. The applied protocol was based on classical methods of palynomorph preparation, with specific adaptations introduced according to the characteristics of the analysed samples.

2.4.1. Sample Preparation

Approximately 1 g of each powdered supplement was homogenised in 10 mL of ultrapure water. The suspension was centrifuged in the Centrifuge 5810 R (Eppendorf, Hamburg, German) at 3000 rpm for 10 minutes to concentrate the solid fraction. The sediment obtained was reserved for subsequent steps. This initial step allowed the reduction of soluble particles and the concentration of material potentially containing palynomorphs.

For the liquid samples, a volume of 10 mL was filtered through a 10 µm pore-size membrane filter, and the residue retained on the filter was transferred to a test tube and centrifuged for 10 minutes at 2500 rpm.

2.4.2. Potassium Hydroxide (KOH) Treatment

To digest non-pollen organic matter, the sediment was treated with 2 mL of 10% KOH solution, sufficient to cover the sample. The treatment was carried out in a water bath at 60 °C for 60 minutes, to accelerate the degradation of organic matter. The use of KOH is justified by its ability to dissolve cellular components and plant debris without compromising the structural integrity of the pollen exine.

After treatment, the sample was centrifuged at 3000 rpm for 5 minutes, and the supernatant was carefully discarded. The pellet was then washed with ultrapure water and centrifuged under the same conditions for a total of three cycles, ensuring complete removal of KOH residues that could interfere with the subsequent steps.

2.4.3. Chemical Processing (Acetolysis)

In accordance with classical palynological protocols, acetolysis was applied to further remove cellulose and other organic material surrounding the pollen grains. The pellet

was treated with a mixture of acetic anhydride and concentrated sulfuric acid (9:1 v/v) and incubated at 90 °C for 5 minutes.

This step promotes the selective digestion of cellulose, leaving the exine intact and enhancing the morphological visualisation of palynomorphs.

Following acetolysis, the suspension was washed three times with ultrapure water by centrifugation (3000 rpm, 5 minutes each) to remove residual reagents and neutralise the sample (Erdtman, 1960).

2.4.4. Palynomorph Purification

The purification of palynomorphs was performed by phase separation using chloroform as the organic solvent. This technique exploits the density differences between palynomorphs and other debris, promoting the concentration of the elements of interest. The sediment was resuspended in chloroform and centrifuged at 2500 rpm for 10 minutes.

Following centrifugation, the resulting fractions were separated, and the retained material was washed with ultrapure water to remove any residual chloroform. This step was crucial to reduce the number of unwanted particles improving the quality of the microscopic preparation.

2.4.5. Microscopic Preparation.

The purified material was resuspended in glycerol, used as the observation medium due to its stability and favourable refractive properties for microscopic observation. To enhance the visualisation of palynomorphs, the samples were stained with liquid fuchsin in glycerol, prepared in a 1:4 ratio (fuchsin: glycerol). This staining method highlights the cell wall of pollen grains and spores, allowing better observation of exine morphological details.

A drop of the stained suspension was placed on a microscope slide and covered with a coverslip. Slides were examined under an optical microscope, model CX23LED (Olympus Corporation, Tokyo, Japan), initially at 100× for general orientation and subsequently at 400× and 1000× for detailed characterisation of pollen morphology.

To ensure the reliability of the results, quality controls were included. As a negative control, solutions without pollen material were processed to exclude the possibility of external contamination.

3. Results

3.1. Chemical Analysis

3.1.1. Spectral analysis of the reference standards (FTIR)

The FTIR spectrum of the silybinin (figure 14) standard displayed a broad and intense absorption band at approximately 3448 cm^{-1}, corresponding to O–H stretching vibrations from phenolic hydroxyl groups engaged in strong hydrogen bonding. A smaller absorption at 2970 cm^{-1} was associated with aliphatic C–H stretching from CH_2 and CH_3 groups. The distinct band at 1631 cm^{-1} can be assigned to conjugated C=O stretching and/or aromatic C=C vibrations, characteristic of the flavonolignan backbone of silybinin. Intense absorptions observed in the 1245–1019 cm^{-1} region correspond to C–O stretching vibrations typical of ether and phenolic groups, confirming the oxygenated aromatic nature of the molecule. The bands between 828–710 cm^{-1} are attributed to out-of-plane C–H bending, consistent with substituted aromatic rings. A weak signal near 2300 cm^{-1} corresponds to atmospheric CO_2 and was therefore excluded from structural interpretation.

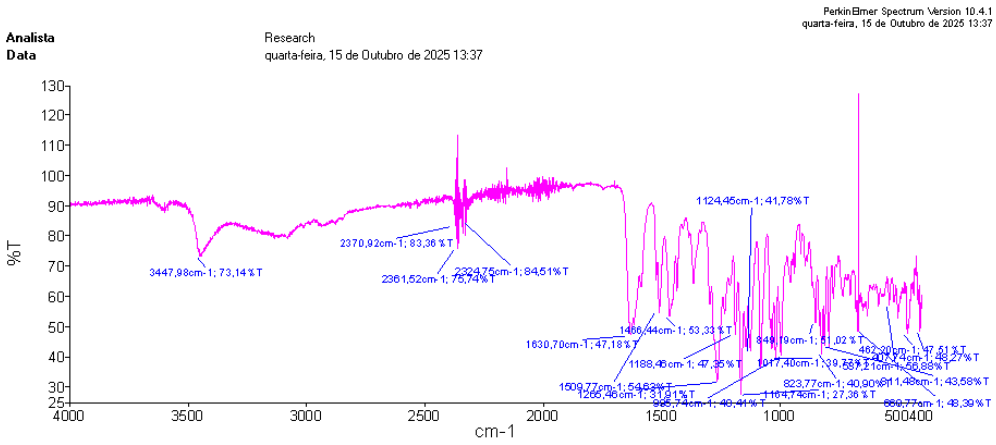

Figure 14 - FTIR spectrum of silybinin (reference compound). Wavenumber range: 4000–400 cm^{-1}.

The FTIR spectrum of the silychristin standard (Figure 15) exhibited a broad band around ≈3328 cm^{-1}, assigned to the phenolic O–H stretching vibration with strong hydrogen bonding, consistent with the polyphenolic nature of the molecule. A well-defined band in the ≈1630–1600 cm^{-1} region confirmed conjugated C=O/C=C vibrations within the aromatic system, a characteristic functional marker of flavonolignans.

Intense bands observed between 1500 and 1450 cm^{-1} corresponded to aromatic C=C vibrations and CH bending within the benzene ring. High-intensity absorptions in the ≈1260–1000 cm^{-1} region were attributed to C–O and C–O–H stretches, aligning with the highly hydroxylated lignan-flavonoid structure. Bands below ≈900 cm^{-1} represented out-of-plane C–H deformations, compatible with substituted aromatic rings. Peaks detected at ≈2350 cm^{-1} were identified as atmospheric CO_2 artefacts and excluded from structural interpretation.

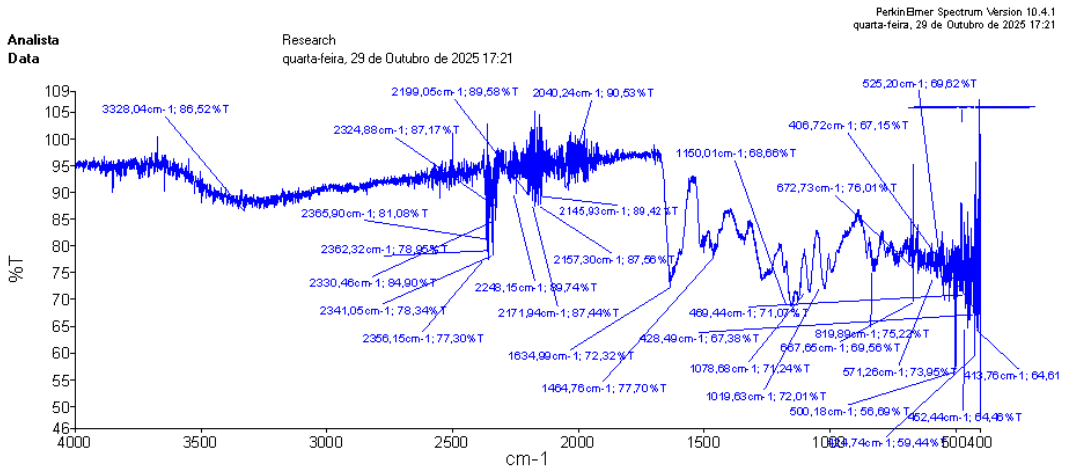

Figure 15 -FTIR spectrum of silychristin (reference compound). Wavenumber range: 4000–400 cm^{-1}.

The FTIR spectrum of the apigenin standard (Figure 16) showed intense bands within the fingerprint region (1028 and 968 cm^{-1}), assigned to characteristic C–O vibrations and aromatic deformations of the hydroxylated flavone structure. Bands observed between 917 and 668 cm^{-1} correspond to out-of-plane C–H bending typical of substituted aromatic rings.

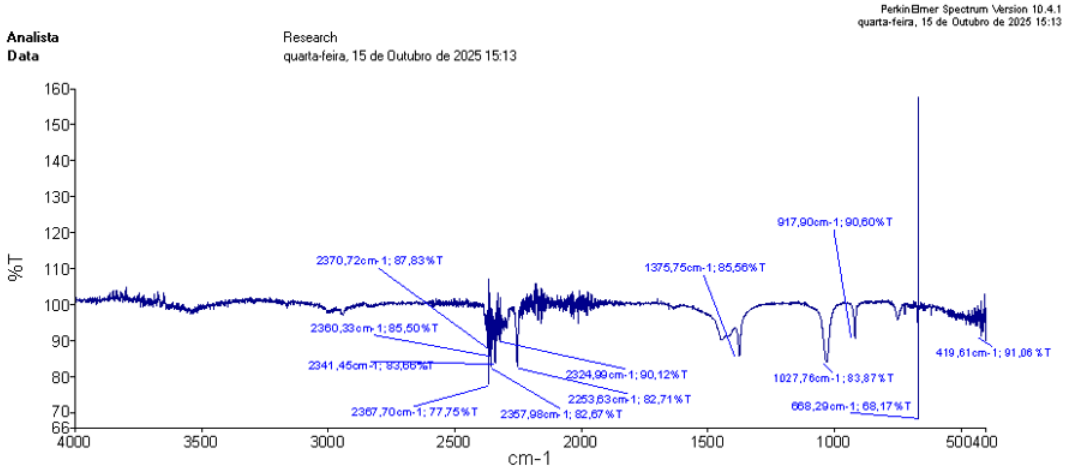

Figure 16 - FTIR spectrum of apigenin (reference compound). Wavenumber range: 4000–400 cm^{-1}.

3.1.2. Comparative evaluation of the reference standards and the samples

The FTIR spectra of the four hepatoprotective supplement samples were analysed by direct comparison with the reference standards (silibinin, silychristin, and apigenin). Characteristic bands attributed to aromatic phenolic compounds were observed in all samples, indicating the presence of flavonoids or flavonolignans consistent with most of the ingredients studied declared on the labels (Figure 17,18,19 and 20).

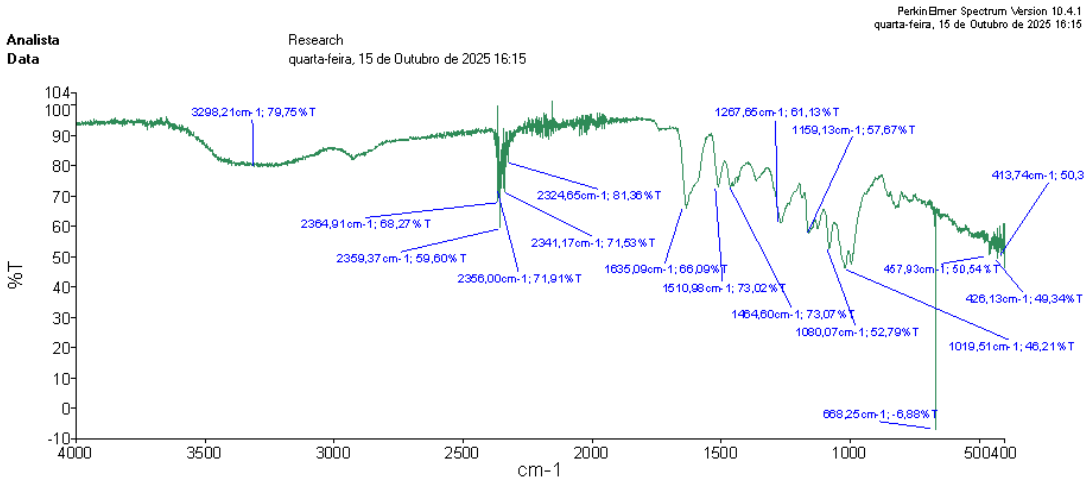

Figure 17 - FTIR spectrum of Sample 1 – solid hepatoprotective supplement from BambooLabs. Wavenumber range: 4000–400 cm^{-1}.

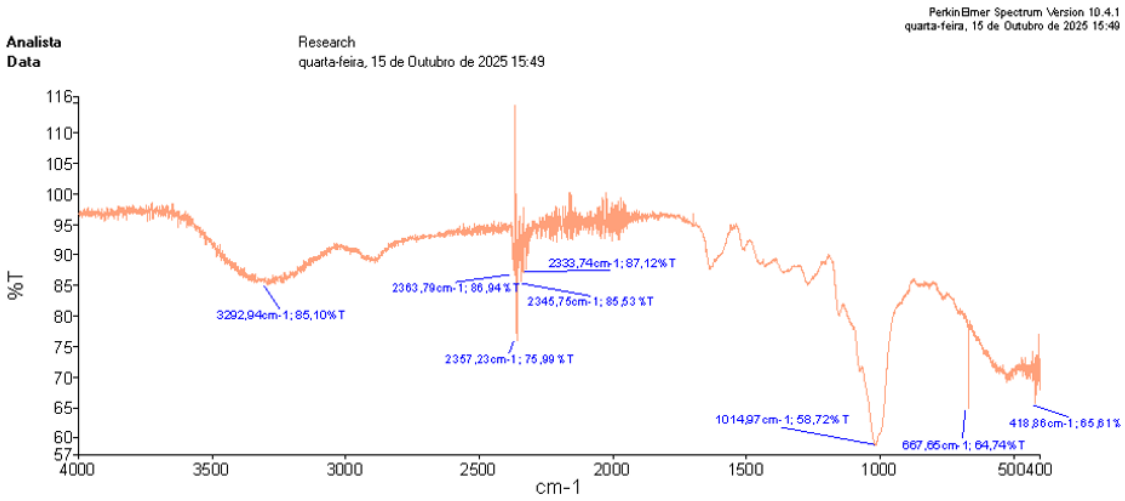

Figure 18 - FTIR spectrum of Sample 2 – solid hepatoprotective supplement from BioHerba. Wavenumber range: 4000–400 cm^{-1}.

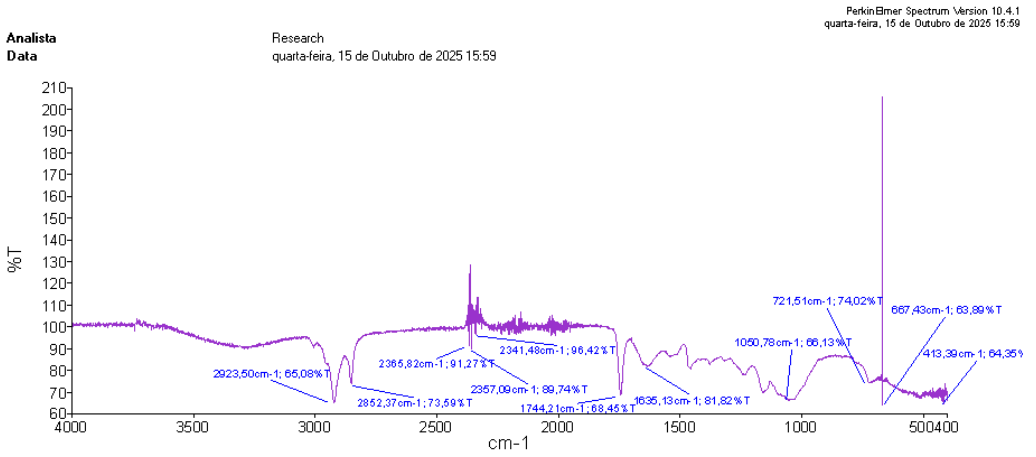

Figure 19 - FTIR spectrum of Sample 3 – solid hepatoprotective supplement from ArkoPharma. Wavenumber range: 4000–400 cm^{-1}.

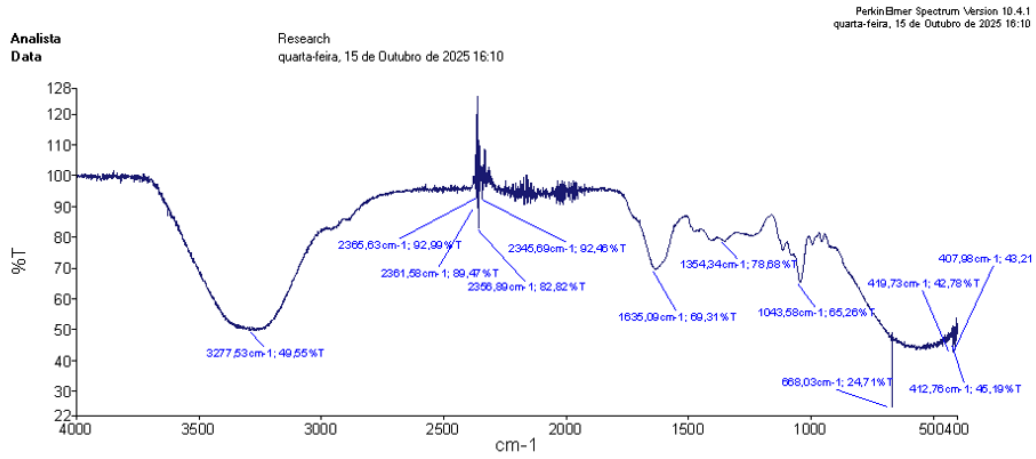

Figure 20 – FTIR spectrum of Sample 4 – liquid hepatoprotective supplement from Wells. Wavenumber range: 4000–400 cm^{-1}.

Table 8 summarises the correspondence between reference standards and supplement samples based on key vibrational markers.

Table 8 - FTIR correspondence between reference standards and supplement samples based on key vibrational markers.

Sample (Brand)	Silybinin (C=O/C=C at ~1630 cm^{-1}; C–O 1260–1000 cm^{-1}; aromatic <900 cm^{-1})	Silychristin (similar flavonolignan markers)	Apigenin (C=O ~1650–1660 cm^{-1}; C–O 1200–1000 cm^{-1})
Sample 1 (BambooLabs)	High correspondence – clear flavonolignan bands present	High correspondence	Low correspondence – apigenin-specific C=O band not observed
Sample 2 (BioHerba)	Moderate correspondence - weaker flavonolignan intensity	Moderate correspondence	Low correspondence
Sample 3 (ArkoPharmacies)	High correspondence – strong flavonolignan profile	High correspondence	Low correspondence
Sample 4 (Wells)	Moderate correspondence – bands affected by solvent matrix	Moderate correspondence	Moderate correspondence – matrix interference prevents clear attribution

3.2. Microbiological Analysis

The four selected hepatoprotective supplements were subjected to initial microbiological assays to assess the presence of viable microorganisms. For this purpose, each sample was inoculated in duplicate on Tryptic Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) media.

After the incubation period, microbial growth was observed only on the TSA plates, with no growth detected on the SDA plates. This result indicates the presence of bacteria but the absence of fungi in the samples analysed. A negative control was also prepared, which showed no growth, confirming the sterility of the media and the absence of contamination during the experimental procedure.

Colony counts on the TSA plates revealed very low bacterial growth in all samples, with values below 30 colonies per plate (Table 9). According to the European Pharmacopoeia, these values are below the reliable count limit and only indicate the presence of a very low number of viable microorganisms.

Samples inoculated on SDA did not show visible growth, confirming the absence of fungal contamination.

Table 9 - Colony counts obtained on TSA and SDA agar for the analysed supplement samples

Brand	Medium	Colony count	Interpretation
Arko	TSA	1	Low bacterial growth
Bamboolabs	TSA	2	Low bacterial growth
Bioherba	TSA	>10 but less than 30	Low bacterial growth
Wells	TSA	3	Low bacterial growth
All	SDA	0	No fungal growth

3.2.1. Bacterial Morphology

Microscopic observation of colonies stained with 1% Methylene Blue revealed the presence of two predominant bacterial morphologies:

- Cocci (spherical cells), often found in clusters, characteristic of bacteria of the species Staphylococcus;
- Short rods (bacilli) observed singly or in small groups, compatible with bacteria of the species Bacillus.

The images obtained during this analysis are presented in Figures 21 and 22, representing the different morphological types observed.

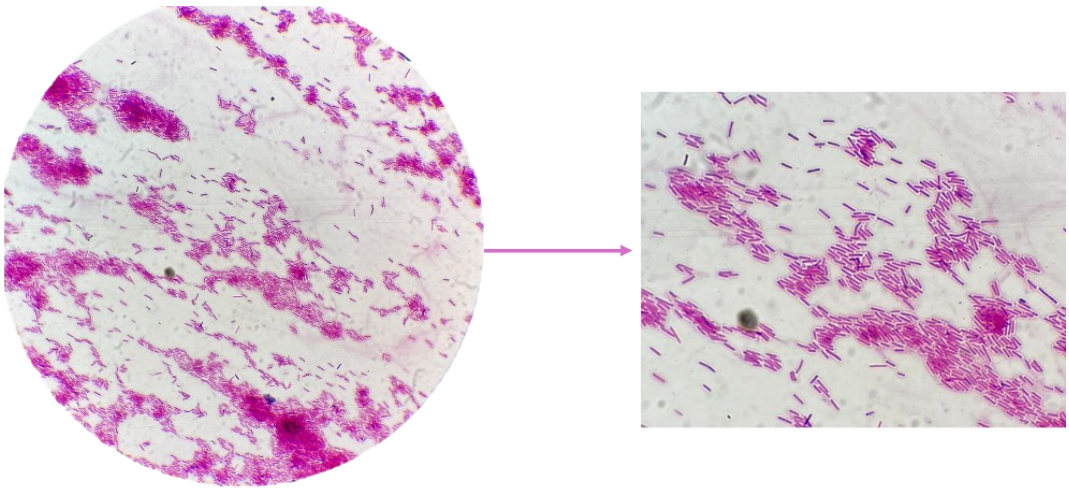

Figure 21 - Bacterial cells with bacillary morphology observed after Gram staining (1000×)

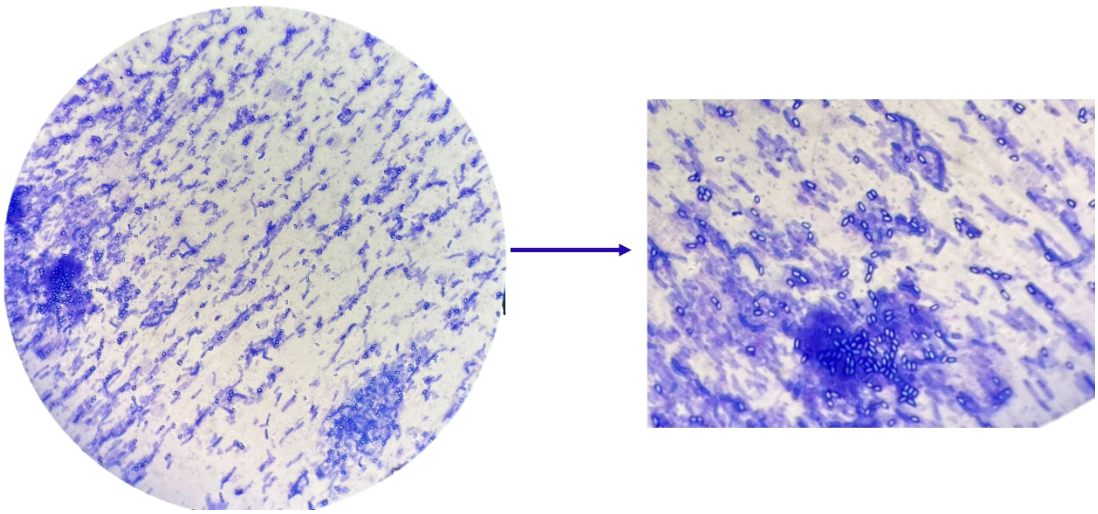

Figure 22 - Bacterial cells with coccoid morphology observed after methylene blue staining (1000×).

3.2.2. Cell Wall Determination (Gram Staining)

The results of cell wall determination using Gram staining showed all samples-stained violet, indicating the presence of Gram-positive bacteria (Figure 23).

Retention of crystal violet suggests the bacteria have a thick peptidoglycan-rich cell wall, typical of species in the genera Staphylococcus and Bacillus, confirmed later by molecular methods.

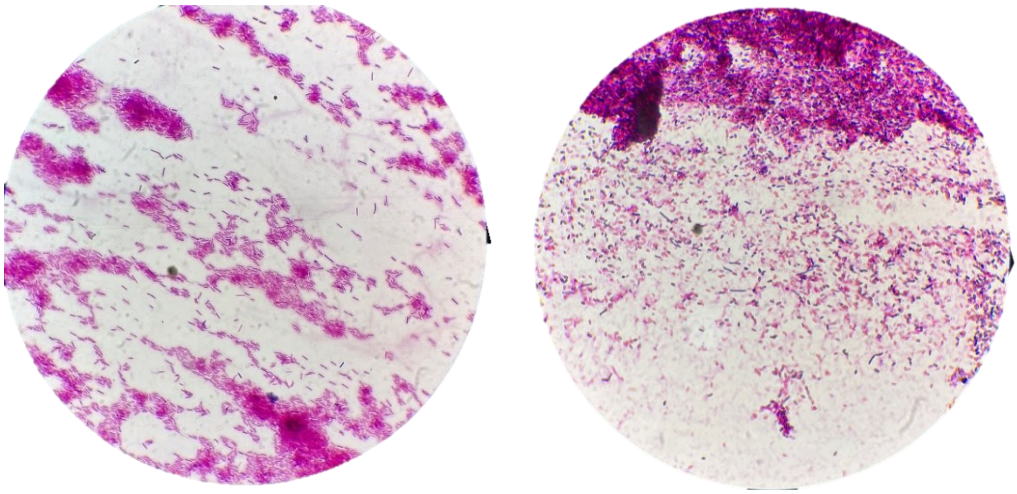

Figure 23 - Gram-positive bacteria observed after Gram staining (1000×).

3.2.3. Molecular Identification

Following the observation of bacterial growth in TSA plates and the microscopic characterisation of the isolates, molecular identification was carried out through PCR amplification of the 16S rRNA gene, a highly conserved genetic marker widely used for bacterial taxonomic classification. The amplification products were confirmed by

agarose gel electrophoresis, and the resulting bands were subsequently purified and sequenced.

The nucleotide sequences obtained were analysed using SnapGene software (GSL Biotech, USA) and the NCBI GenBank database using the BLAST algorithm (Basic Local Alignment Search Tool for nucleotides) which showed the following results in Table 10.

The analysis revealed high sequence similarity values, allowing precise identification of the bacterial species present in each supplement sample.

Table 10 - Molecular identification of bacterial strains isolated from different supplement brands and sample types by 16S rRNA gene sequencing.

Supplement Brand	Sample Type	Molecular Identification (BLAST)	Similarity Percentage	Observed Species/Morphology
Arko	Capsules	*Staphylococcus pasteuri*	98%	Gram-positive cocci
BambooLabs	Capsules	*Staphylococcus* sp. *mutant XU1*	100%	Gram-positive cocci
Bioherba	Capsules	*Bacillus amyloliquefaciens*	100%	Gram-positive bacilli
Wells	Liquid	*Bacillus subtilis*	99%	Gram-positive bacilli

In summary, all analysed samples showed growth of Gram-positive bacteria, with molecular identification belonging to the genera Staphylococcus and Bacillus. Samples from the brands Arko and BambooLabs were dominated by species of the group Staphylococcus, while the brands Bioherba and Wells revealed species of the group Bacillus.

No evidence of fungal growth was found in any of the tested samples. Although the number of colonies was very low (<30), the presence of bacterial DNA detected by PCR and sequencing confirms the existence of residual microbial contamination, possibly resulting from environmental contact or from the plant raw material before processing.

3.3. Palynological Analysis

Microscopic analysis of the samples subjected to extraction and staining protocols revealed the presence of various biological elements, predominantly pollen grains and,

to a lesser extent, fungal spores. The morphology, size, and surface ornamentation of the retained elements were evaluated after purification and staining with glycerol-fuchsin.

Morphological comparison was based on micrographs acquired during analysis and corresponding plates from the pollen atlas "*Pollen et spores d'Europe et d'Afrique du nord*" by Maurice Reille. Identification was assigned at the botanical family level, considering exine ornamentation, shape, size, and type of aperture (colpus or pore) observed.

In all samples, a spherical pollen grain exhibiting fine reticulate ornamentation and a diameter of approximately 40–50 µm was observed (Figure 24). These features agree with the diagnostic profile of pollen from the *Cistaceae* family (genus Cistus).

However, due to the plane of focus, the number of apertures (colpi/pores) could not be determined.

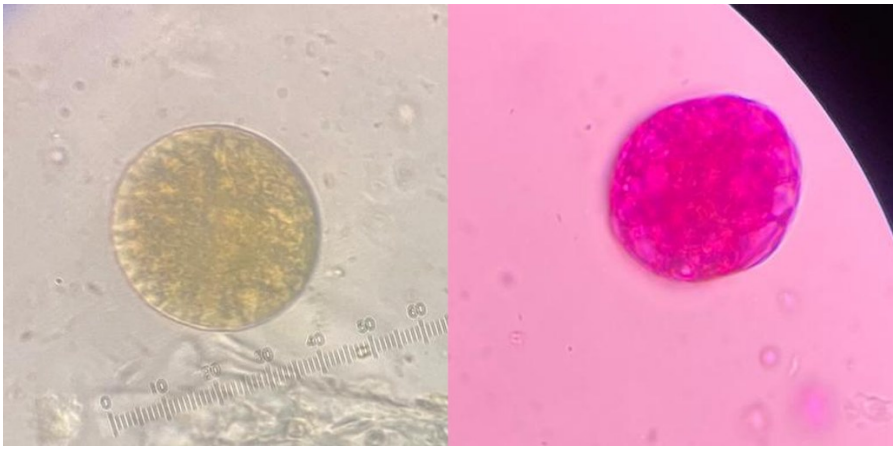

Figure 24 - Spherical to subspherical pollen grain (approximately 40–50 µm) with a smooth to finely reticulate exine (1000×).

In one of the samples, a sub-spherical pollen grain with an estimated diameter between 20–30 µm and possessing a psilate or microreticulate exine (i.e., smooth or finely patterned surface) was found (Figure 25). Such characteristics align with typical *Brassicaceae* pollen. The apertures could not be resolved due to the grain's orientation.

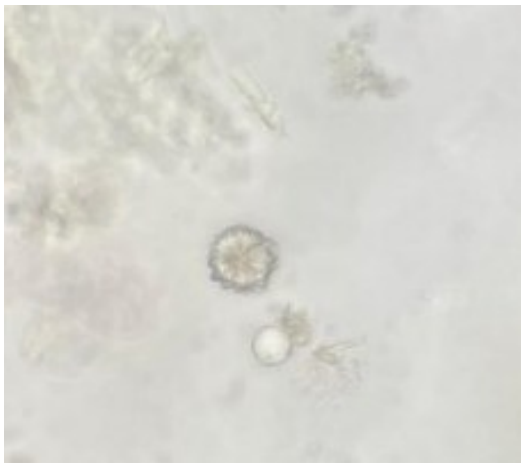

Figure 25 - Small subspherical pollen grain (20–30 µm), with psilate to micro-reticulate exine, visualised under light microscopy at 1000× magnification.

In addition, one sample contained an oval or oblong element with a thick wall, homogeneous staining after fuchsin application, and lacking both reticulate ornamentation and visible apertures, consistent with a fungal spore (Figure 26). The detection of this fungal spore may be attributed to contamination during supplement processing or storage, although its precise origin could not be established.

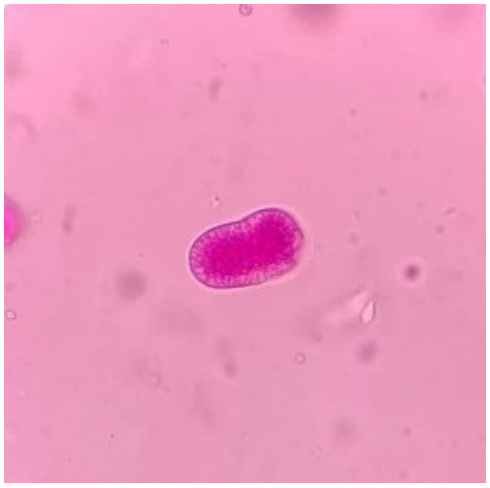

Figure 26 - Oval-shaped, intensely stained structure observed at 1000×, showing a smooth wall and lacking diagnostic pollen features.

In summary, microscopic examination of the samples allowed the identification of distinct palynomorph types, including pollen grains from two angiosperm families (*Cistaceae* and *Brassicaceae*) as well as a single fungal spore (Table 11). Collectively, these findings support the botanical authenticity of the supplements while reflecting natural environmental exposure during plant cultivation and processing.

Table 11 - Morphological and dimensional characteristics, surface ornamentation, and presumptive family assignment of pollen grains and fungal spores identified in the analysed samples following purification and glycerol-fuchsin staining.

Image	Observed Morphology	Size Range	Ornamentation	Assigned Family
24	Spherical	40–50 µm	Fine reticulation	*Cistaceae*
25	Subspherical	20–30 µm	Psilate/microreticulate	*Brassicaceae*
26	Oval/oblong	8–12 µm (est.)	Smooth, homogeneous wall	Fungus (spore)

4. Discussion

4.1. Chemical Approach

Analysis of the reference standards allowed the identification of robust spectral markers characteristic of flavonolignans such as silibinin and silychristin, as well as less defined but recognisable features associated with the flavone apigenin. These markers provided a qualitative framework for interpreting the spectra of the four supplements. Although FTIR does not allow full confirmation of all label-listed ingredients, the comparison enabled the identification of spectral features consistent with the presence of the main phenolic constituents declared, particularly those associated with *Silybum marianum* extracts.

Results indicate that all samples exhibited characteristic bands of aromatic phenolic compounds, namely an O–H stretch (3200–3600 cm^{-1}), intense C–O/C–O–H stretches (1260–1000 cm^{-1}), and aromatic ring bands below 900 cm^{-1}, confirming the presence of plant-derived ingredients. However, notable differences were observed among the samples in the presence and intensity of the ~1630 cm^{-1} band, attributed to the conjugated C=O/C=C system typical of milk thistle flavonolignans, which are the main active constituents expected in hepatoprotective supplements (Radek Gazak et al., 2007; Singh & Mendhulkar, 2015).

Samples 1 and 3 showed strong correlation with silibinin and silychristin standards, simultaneously displaying the main structural markers of these molecules. These findings align with the labelling that clearly indicates silymarin or milk thistle as the principal ingredient. Sample 2 also showed flavonolignan markers, albeit with reduced intensity around ~1630 cm^{-1}, possibly reflecting a lower relative amount of active extract or a formulation with more excipients overlapping in the critical spectral region. Sample 4 exhibited spectral features consistent with phenolic compounds but with reduced definition, particularly in the 1700–1500 cm^{-1} region. This can be explained by the liquid state of the matrix, where the high presence of water, glycerol, or other vehicles leads to broadening of the O–H band, band overlapping in the C–O region, and increased baseline noise (Sun et al., 2025).

To summarise (Table 8), when comparing all spectral features to the reference compounds, Samples 1 and 3 (Figure 17 and 18) showed the closest resemblance to the silybinin and silychristin standards. Sample 2 (Figure 19) demonstrated similarities with

a lower degree of alignment. Sample 4 (Figure 20) displayed partial correspondence within the same spectral markers, although with broader and less defined bands.

Thus, although phenolic compounds are evident, FTIR was insufficiently discriminative to unequivocally confirm flavonolignans in this formulation.

Regarding apigenin, correspondence with the supplements was virtually absent. Even in Sample 1 (BL), which contains dandelion known as a natural source of this compound, specific flavone markers such as the C=O band at ~1650–1660 cm^{-1} with appropriate separation and intensity were not observed. This outcome can be explained by three main factors:

- low relative concentration of apigenin compared to milk thistle flavonolignans;
- glycosylation or complexation of apigenin within plant extracts, causing shifts and intensity loss of bands;
- liquid/solution phase, a condition that may reduce band intensity and shift peak positions due to solvent overlap.

Therefore, FTIR is suitable for confirming flavonolignans in solid, less complex matrices but has limitations in detecting minor constituents like apigenin and liquid matrices with high aqueous or organic solvent fractions (Dharmastuti Cahya et al., 2022; Almeida & Gonçalves, 2023). Complementary analysis using HPLC-DAD or LC-MS is necessary to definitively confirm the presence or absence of apigenin in samples, particularly in sample 1 (BambooLabs) where it is claimed.

Overall, the results partially corroborate label specifications, confirming the presence of characteristic active compounds from milk thistle in all four samples analysed.

4.2. Microbiological Approach

The results demonstrate that the four hepatoprotective supplement samples showed very low bacterial growth on TSA plates, as all counts were below 30 colonies per plate, and no growth was observed on SDA medium. This indicates that while viable microorganisms were present, they existed at very low levels, consistent with a residual microbial load rather than active contamination.

Gram staining revealed that all isolated bacteria were Gram-positive, and morphological observation indicated two major forms: cocci and short bacilli. Molecular identification coupled these morphologies to the species *Staphylococcus pasteuri* (98%), *Staphylococcus* sp. *mutant XU1* (100%), *Bacillus amyloliquefaciens* (100%), and *Bacillus subtilis* (99%).

The very low viable count (<30 colonies) suggests that although microorganisms were present, their levels did not reach the threshold required for reliable CFU/g quantification according to the European Pharmacopoeia (which typically considers 30–300 colonies per plate as statistically valid). This implies that interpretation should focus on residual presence and compliance verification rather than significant contamination.

As previously noted, for non-sterile food or herbal products, acceptable limits generally include total aerobic bacteria $\leq 10^5$ CFU/g, yeasts/fungi $\leq 10^3$ CFU/g, and Enterobacteriaceae and other Gram-negative bacteria $\leq 10^3$ CFU/g, with no detection of *Escherichia coli* or *Salmonella spp.* (Okunlola et al., 2007).

The values observed in this study were very below these risk thresholds, indicating effective microbiological control and efficient manufacturing or storage practices. Nevertheless, the detection of microorganisms, even at low levels, warrants attention, as Staphylococcus and Bacillus genera are frequently reported as contaminants in dietary supplements and plant-based raw materials (Nwankwo & Olime, 2019).

The presence of *Staphylococcus pasteuri* and *Staphylococcus* sp. *mutant XU1* suggests possible contamination related to human handling or the manufacturing environment, as many Staphylococcus strains colonise the skin, hands, mucosae, or equipment surfaces. The detection of *Staphylococcus* sp. *mutant XU1* might also reflect accidental contamination during laboratory handling. Although this lineage is not taxonomically recognised, it likely represents a genetic variant within the Staphylococcus genus, possibly related to coagulase-negative species such as *S. epidermidis* or *S. pasteuri*. The detection of this organism, though at low abundance, is likely to reflect environmental or operator-related contamination during processing. In studies of plant-based supplements, Staphylococcus has often been identified as an indicator of inadequate manufacturing practices (O. Chesneau et al., 1993; Becker et al., 2014). However, the low colony counts in this study suggest that contamination levels were minimal.

The two samples identified as *Bacillus amyloliquefaciens* and *Bacillus subtilis* are typical of soil, dust, or plant raw material sources and are commonly associated with supplements or environmental formulations because of their strong sporulation capacity. Several studies identify these species as frequent contaminants in processed plant materials. Although often considered low-risk organisms or even used as probiotics, their presence in supplements that do not explicitly list them raises concerns about labelling accuracy, traceability, and contamination control (Nwankwo & Olime, 2019).

Despite the low microbial load, some implications should be considered:

- The presence of microorganisms, even at low levels, indicates that manufacturing or storage may have permitted microbial survival or introduction, which could reflect partial non-compliance with Good Manufacturing Practices.
- For vulnerable populations, like immunocompromised individuals or the elderly, even small quantities of opportunistic microorganisms could pose a risk, especially with prolonged consumption or inadequate storage.
- The absence of growth on SDA medium indicates no fungal or yeast contamination under the tested conditions, a positive finding that reduces one potential microbial risk factor.

In summary, the findings of this study show that although the microbial load in the analysed supplement samples was very low the detectable presence of Gram-positive bacteria from the genera Staphylococcus and Bacillus highlights the need for continued vigilance. Even at low counts, molecular detection of these bacteria underscores potential weaknesses in manufacturing, storage, or handling procedures and emphasises the importance of maintaining strict microbiological quality control, ingredient traceability, and ongoing compliance with international microbial safety standards.

4.3. Palynological Approach

Palynological analysis allowed the identification of structures consistent with pollen grains and fungal spores, confirming the effectiveness of the extraction and staining protocol applied. The use of liquid fuchsin in glycerol enabled clear visualisation of exine ornamentation and contours, facilitating discrimination between different pollen morphologies and non-pollen structures.

Pollen grains identified as belonging to the *Cistaceae* family exhibited spherical morphology, finely reticulate exine ornamentation, and diameters ranging from 40 to 50 µm. These features correspond with those described for the genus *Cistus*, a family prevalent in Mediterranean environments and commonly found in ecosystems near to agricultural areas where *Silybum marianum* (milk thistle) and *Cynara scolymus* (artichoke) are produced (Guzmán & Vargas, 2009; Lombardo et al., 2018; Pasquariello et al., 2025).

Despite both main ingredients being from the *Asteraceae* family, no echinate pollen typical of this family was detected. This absence can be explained by two critical factors in the industrial process:

- The main ingredients are purified extracts, meaning pollen was removed during filtration.
- Chemical and thermal treatments degrade more delicate pollen structures.

Palynology alone cannot verify authenticity of purified plant extracts; it is effective only for detecting crude plant materials or identifying adulterations through unexpected pollen profiles. For determining label compliance of purified extracts, chromatographic methods targeting specific marker compounds (silybinin, silychristin) are required. Therefore, the absence of *Asteraceae* pollen does not contradict supplement authenticity but indicates that the final raw material no longer contains reproductive tissues of the original plant (Bryant, 2014).

Moreover, the pollen identified, specifically from the *Cistaceae* and *Brassicaceae* families, are spontaneous or weedy species related to cultivation, behaving as natural environmental contaminants. Hence, the presence of these pollen grains is expected and consistent with the nature of the product analysed (Guzmán & Vargas, 2009; Al-Shehbaz, 2012).

In summary, the identification of pollen from the *Cistaceae* and *Brassicaceae* families, strongly linked to Mediterranean and European Atlantic regions, aligns with the production chain of the supplements analysed. Thus, the results suggest that the supplements have genuine plant origins compatible with European provenance, with no evidence of substitution by products from other continents or regions with less sanitary control.

The presence of an isolated fungal spore reflects the plant-based nature of the supplements and their inevitable exposure to environmental microorganisms throughout the production chain. Its residual occurrence and the absence of fungal growth structures suggest no active biological contamination, supporting the adequacy of manufacturing and storage procedures (Bryant, 2014).

4.4. Integrated Assessment of Label Compliance

To synthesise the findings across all analytical methods, a comparative label compliance assessment was carried out and is shown in Table 12.

Table 12 - Label Compliance Assessment

Brand	Declared Ingredients	Detected by FTIR	Detected by PCR/16S	Detected by Palynology	Compliance Status	Notes
BambooLabs	Artichoke (*Cynara scolymus*), Dandelion (*Taraxacum officinale*), Desmodium (*Desmodium adscendens*), Milk thistle (*Silybum marianum*)	Clear flavonolignan markers (~1630 cm^{-1}; 1260–1000 cm^{-1}; <900 cm^{-1})	Bacterial DNA detected; no pathogenic species identified	*Cistaceae* pollen present	Partially compliant	Presence of milk thistle confirmed; apigenin (dandelion) markers absent—possibly low concentration or overlapping signals.
BioHerba	Milk thistle, vitamin B6	Weak flavonolignan bands (~1630 cm^{-1}) detected	Bacterial DNA detected; no pathogenic species identified	*Cistaceae* + *Brassicaceae* pollen present	Partially compliant	Chemical support limited; palynology suggests mixed/ambient input
ArkoPharmacy	Milk thistle	Strong flavonolignan profile (~1630 cm^{-1} and 1260–1000 cm^{-1})	Bacterial DNA detected; no pathogenic species identified	*Cistaceae* pollen + single fungal spore(s)	Compliant	Consistent with declared composition and expected profile for silymarin-containing supplements. Fungus is likely airborne
Wells	Milk thistle, artichoke, other plant extracts	Broad O–H and weak C=O/C=C peaks; solvent interference evident	Non-pathogenic bacterial DNA detected	*Cistaceae* pollen present	Partially compliant	Liquid matrix limits FTIR definition

This table provides a systematic summary of compliance between the declared ingredients on product labels and the analytical findings obtained through FTIR spectroscopy, microbiological sequencing, and palynological examination. Samples 1 and 3 demonstrated the strongest correspondence with the declared *Silybum marianum* content, while Samples 2 and 4 exhibited lower spectral definition and evidence of matrix interference or excipient masking. Palynological results confirmed plant-derived origins and environmental consistency but also highlighted occasional contamination by airborne spores. Overall, most samples were partially compliant, revealing no major falsification but notable compositional variability among brands.

4.5. Study Limitations

This study has a few important limitations that must be acknowledged. Only four supplements were analysed, limiting generalisability to the broader Portuguese market. A minimum of 10-15 samples representing different brands and formulations would be more representative.

FTIR spectroscopy provides qualitative identification of functional groups but there wasn't a quantification of active compounds. Complementary methods (HPLC-DAD, LC-MS) are essential for complete characterisation, and a further quantification of the active compounds may be necessary. Liquid matrices (Sample 4) exhibited compromised spectral quality due to solvent interference. Minor compounds (apigenin) below FTIR sensitivity thresholds cannot be definitively ruled out. Also, excipients and processing additives were not characterised.

While culture-based colony counts were below reliable detection limits (<30 CFU), molecular detection (PCR/16S rRNA) identified bacteria in all samples. The discrepancy between methods complicates risk assessment, particularly for immunocompromised consumers.

Palynology cannot verify the identity of purified plant extracts because industrial processing removes diagnostic pollen. This technique is valuable only for crude materials or for detecting unexpected pollen profiles indicating adulteration. Asteraceae pollen (from declared ingredients) was not detected, so botanical authenticity claims cannot be supported by palynology alone.

Despite these limitations, the complementarity of the three applied techniques provided useful exploratory data on the botanical nature, microbiological status, and phenolic compound profiles of four hepatoprotective supplements marketed in Portugal. The

results demonstrate that while basic safety criteria appear to be met (quantitative microbial loads remain below regulatory limits), sensitive molecular and chemical methods reveal previously undetected microbial populations and spectral limitations that warrant further investigation with advanced techniques.

5. Conclusion

The present study evaluated the authenticity and compliance of hepatoprotective supplements marketed in Portugal through a multidisciplinary methodological approach based on chemical, microbiological, and palynological techniques. Together, these methods enabled a comprehensive characterisation of the products analysed, contributing to quality control, safety assurance, and traceability of dietary supplements with hepatoprotective claims.

Regarding the chemical approach, Fourier Transform Infrared (FTIR) spectroscopy proved to be an effective technique for the qualitative identification of phenolic compounds present in the supplements. The spectra obtained confirmed the presence of characteristic bands corresponding to milk thistle flavonolignans, namely silybinin and silychristin, in solid samples, providing analytical evidence that the formulations contain the main bioactive ingredients declared by manufacturers. However, limitations were observed in detecting minor constituents, especially apigenin, and in analysing the liquid supplement, where the solvent matrix significantly interfered with spectral resolution. Thus, FTIR proved suitable as a preliminary chemical identity confirmation method, with further development recommended through complementary application of selective chromatographic techniques such as HPLC-DAD or LC-MS, which would allow rigorous quantification of active compounds and more detailed discrimination among different silymarin components.

Microbiological evaluation revealed that all four supplements contained cultivable Gram-positive bacteria (*Staphylococcus pasteuri, Staphylococcus* sp. *mutant XU1, Bacillus amyloliquefaciens, Bacillus subtilis*) despite colony counts remaining below the reliable detection threshold (<30 CFU/plate). Molecular detection via 16S rRNA sequencing confirmed the presence of viable bacterial populations in all samples. While quantitative loads remain below European Pharmacopoeia action limits ($\leq 10^5$ CFU/g for aerobic bacteria), the universal presence of bacterial DNA indicates exposure to environmental contamination, possibly from raw plant materials, and/or suboptimal but not critically deficient manufacturing practices. These findings do not constitute a food safety violation but indicate that manufacturing protocols may benefit from enhanced microbial control measures, particularly for vulnerable consumer populations (immunocompromised and elderly).

Lastly, the palynological approach demonstrated innovation in the context of dietary supplement analysis, proving useful for identifying and interpreting vegetal-origin particles in samples. The observation of structural elements such as pollen contributes to tracing botanical ingredients utilised and serves as an indicator of authenticity and quality control, potentially allowing detection of undeclared adulterations or substitutions.

In conclusion, the complementarity of the three applied techniques enabled a comprehensive characterisation of the studied hepatoprotective supplements, demonstrating microbiological compliance and the presence of characteristic milk thistle bioactive phenolic compounds. Additionally, palynology emerged as a promising tool to enhance traceability and authenticity control.

It is anticipated that, with the application of advanced quantitative methods, such as HPLC-DAD or LC-MS to quantify silybinin, silychristin and apigenin concentrations and compare against label claims and regulatory threshold, and expansion of sample numbers, these results may contribute to continuous improvement in inspection and quality assessment practices for dietary supplements intended for liver protection.

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Attachment

Table A 1 - Experimental Peaks and Functional Group Assignments of Silybinin

Experimental Peak (cm^{-1})	Assignment (Reference Range)	Observation	Confidence
3448	O–H stretching (3200–3600)	Phenolic and alcoholic O–H groups; silybinin contains multiple hydroxyls. Broadband due to hydrogen bonding.	High
2970	C–H aliphatic stretching (2950–2850)	CH_2/CH_3 groups from the molecular skeleton or excipients.	Medium
1631	C=O conjugated / C=C stretching (1650–1600)	Conjugated carbonyl and aromatic C=C vibrations; characteristic of flavonolignans.	High
1546	Aromatic C=C stretching / Amide II region (1560–1500)	May reflect aromatic ring vibrations or minor amide signals from impurities.	Medium
1383	CH bending (1400–1380)	Methyl/methylene deformation; common in organic matrices.	Medium
1245 / 1198	C–O stretching (1250–1200)	Phenolic and ether/ester groups typical of flavonoid structures.	High
1061 / 1019	C–O / C–O–H bending (1100–1000)	Alcohol or ester C–O stretching; possible contribution from excipients (e.g., starch).	High
828 / 779 / 710	C–H out-of-plane bending (900–650)	Aromatic ring deformations typical of substituted phenolic rings.	High
~2300	Background artifact (CO_2)	Atmospheric CO_2 absorption — disregarded in the analysis.	—

Table A 2 - Experimental Peaks and Functional Group Assignments of Silychristin

Experimental Peak (cm^{-1})	**Reference Range (cm^{-1})**	**Observation**	**Confidence**
3328	O–H stretch (3200–3600)	Phenols with strong hydrogen bonding characteristic of silychristin's polyphenolic structure.	High
≈1630–1600	C=O conjugated / C=C (1700–1600)	Conjugated vibrations within the aromatic system confirm the key flavonolignan group.	High
≈1500–1450	Aromatic C=C / CH bending	Indicates substitute aromatic rings.	High
≈1380–1250	CH bending (lipids/proteins)	Aliphatic and phenolic bending are common in oxygenated aromatic compounds.	Medium
1260–1200	C–O stretch / phenols & ethers	Intense band associated with the molecule’s strong hydroxylation.	High
1150–1000	C–O / C–O–H bending (carbohydrates/phenols)	Fingerprint region of polyphenols may include residual excipients.	High
900–850 (multiple peaks)	Out-of-plane C–H bending (900–650)	Aromatic ring substitution pattern typical of flavonolignans.	High
2364–2340	Artifact (atmospheric CO_2)	Background absorption, excluded from structural interpretation.	—

Table A 3 - Experimental Peaks and Functional Group Assignments of Apigenin

Experimental Peak (cm^{-1})	Reference Range (cm^{-1})	Observation	Confidence
≈3400 (not clearly defined in image)	O–H stretch (3200–3600)	Apigenin contains phenolic groups; band may appear weak due to strong intramolecular bonding and/or low sample humidity.	Medium
≈1660–1630 (not clearly annotated but expected)	C=O conjugated / aromatic C=C (1700–1600)	Marker for the flavone carbonyl group.	Low
1376	CH bending (1400–1380)	Bending of aliphatic and phenolic groups is common in flavonoids.	Medium
1028 / 968	C–O / C–O–H bending (1100–1000)	Bands typical of phenolic compounds with C–O bonds and hydroxyl groups.	High
917 / 668	Out-of-plane C–H bending (900–650)	Substituted aromatic rings are characteristic of flavones.	Medium-High
2341–2370	Artifact (atmospheric CO_2)	Background absorption, disregarded for structural analysis.	—
419	Skeletal vibrations (low wavenumber)	Less informative region, used only as secondary fingerprint.	Low