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Phytochemical Analysis of Medicinal Plant Extracts *Plantago major* and *Physalis peruviana* and their Assessment of Antigenicity and Antimicrobial Activity.

Trabajo de integración curricular presentado como requisito para la obtención del título de Biólogo

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DEDICATORY

"I dedicate this work to my loved mom, Guadalupe del Consuelo Haro Ruiz, who supported me all my life. Her love and guidance forged the person I am now."

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I extend my deepest gratitude to those who were part of my life during this long journey. To my parents Gustavo and Consuelo, who supported me all this time. I love you so much. To my loved brothers, Iván, Geovanni, and Alex, who always encouraged, helped and never let me give up. Thanks for everything you have done for me.

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ABSTRACT

The study of medicinal plants in Ecuador has the potential to generate valuable knowledge on the development of new therapeutic agents. The extracts from various plants have been widely studied due to the secondary metabolites they possess. This work has focused on the study of two plants used in popular medicine, but poorly studied in traditional Ecuadorian medicine, *Physalis peruviana*, commonly called uvilla, and *Plantago major*, better known as llantén. The phytochemical analysis of the leaves and roots of both plants was carried out using two extraction methods, Soxhlet and maceration, using 70% ethanol as a solvent. For the immunological tests, only the extracts of the leaves of each plant were used, prepared through the maceration process, since a greater presence of secondary metabolites was observed according to the phytochemical study. The humoral response of the plant extracts was carried out by immunizing them in Balb/c mice with complete and incomplete Freud's adjuvant. Once the hyperimmune sera were obtained, the presence of specific antibodies against that antigen was verified using the ELISA method. A cross-reaction between both plants was also determined by the indirect ELISA test with the hyperimmune sera. In the same way, the cytotoxicity analysis was carried out at concentrations of 5 ug/ml and 10 ug/ml. Additionally, antimicrobial activity tests of the extracts were carried out against two bacteria, *E. coli* and *S. aureus*. Tests demonstrated some antimicrobial activity for *S. aureus*, which is more susceptible to the extracts, at a concentration of 1 and 2 mg/ml.

Keywords: Ethanolic extract, indirect ELISA, Physalis peruviana, Plantago major, antimicrobial activity.

RESUMEN

El estudio de las plantas medicinales en Ecuador tiene el potencial de generar conocimientos valiosos sobre el desarrollo de nuevos agentes terapéuticos. Los extractos vegetales de varias plantas han sido ampliamente estudiados debido a los metabolitos secundarios que poseen. Este trabajo se ha centrado en el estudio de dos plantas de uso en la medicina popular, pero poco estudiadas en la medicina tradicional ecuatoriana, *Physalis peruviana*, comúnmente llamada uvilla, y *Plantago major*, mejor conocida como Llantén. El análisis fitoquímico de las hojas y raíces de ambas plantas se realizó mediante dos métodos de extracción, Soxhlet y maceración, utilizando etanol al 70% como solvente. Para las pruebas inmunológicas solo se usaría el extracto de las hojas de cada planta, elaborado mediante el proceso de maceración, ya que se observó una mayor presencia de metabolitos secundarios de acuerdo al estudio fitoquímico. La respuesta humoral de los extractos vegetales se realizó por inmunizaciones de estos en los ratones Balb/c con adyuvante completo e incompleto de Freud. Una vez obtenidos los sueros hiperinmunes, se verificó mediante el método ELISA la presencia de anticuerpos específicos contra ese antígeno. También se determinó una reacción cruzada entre ambas plantas por la prueba de ELISA indirecta con los sueros hiperinmunes. De igual modo se realizó el análisis de citotoxicidad en concentraciones de 5 ug/ml y 10 ug/ml. Adicionalmente, se realizaron pruebas de actividad antimicrobiana de los extractos contra dos bacterias, *E. coli* y *S. aureus*. Las pruebas demostraron cierta actividad antimicrobiana para *S. aureus* , la cual es más susceptible a los extractos, a una concentración de 1 y 2 mg/ml.

Palabras clave: Extracto etanólico, ELISA indirecta, *Physalis peruviana*, *Plantago major*, actividad antimicrobiana.

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1. INTRODUCTION

Since the beginning of civilizations plants have been widely used as a source of food but also as a cure of several illnesses.(Jamshidi-Kia et al., 2017). Parts of the plants such as leaves, stems, flowers, roots, and fruits have been studied in the field of pharmacology for the secondary metabolites that they possess, such as alkaloids, glycosides, polyphenols, and terpenes (Tiwari & Rana, 2015). (Jimenez-Garcia et al., 2013). Plant secondary metabolites play a significant role in medicine, offering a rich source of bioactive compounds that have the potential to be developed into effective treatments for various health conditions. Their diverse pharmacological properties make them valuable resources for drug discovery and development. For example, alkaloids are able to generate an immune response in the body through various mechanisms. According to (Zhou et al., 2020), demonstrated that the alkaloids from *Alstonia scholaris* exhibit anti-inflammatory properties and can regulate the innate immune response, so helping to inhibit influenza A virus replication and lung immunopathology.

For the extraction of different metabolites and active principles of plants several methods have been reported, such as, maceration, digestion, infusion, percolation, decoction, percolation, Soxhlet extraction, Ultrasound-assisted extraction, Microwave-assisted, Supercritical fluid extraction (SFC). Pressurized liquid extraction (PLE) extraction (Bitwell, Indra, et al., 2023; Q. W. Zhang et al., 2018).

Different types of solvents can be used depending on the metabolite to be extracted. One of the most used is ethanol, due to the security over another organic solvents. This solvent is a protic organic solvent with a polarity index value of 5.2, which has the ability to extract a wide range of compounds, including glycosides, polyacetylenes, sterols, polyphenols, tannins, flavonols, terpenoids, and alkaloids (Hikmawanti et al., 2021).

Ecuador is a country with a diverse range of medicinal plants, these plants contain secondary metabolites that have been found to have various therapeutic properties, such as anti-inflammatory or anticancer activities(Bailon-Moscoso et al., 2015; Velu et al., 2018). Some examples of medicinal plants found in Ecuador include *Equisetum giganteum L.*, *Crotalaria sp. L.*, *Marsdenia condurango*, *Annona montana*, *Cinchona officinalis* (Bailon-Moscoso et al., 2015). However, several plants have not received the same attention from researchers despite their therapeutic potential, such as *Physalis peruviana* and *Plantago major* (Marengo et al., 2022).

1.1 BACKGROUND

1.1.1 Extraction methods

Plant material can be extracted using a variety of techniques, each with their own benefits. Depending on the metabolites to be extracted, different solvents can be used, like ethanol to extract a wide range of compounds, such as flavonoids and phenolics, is a commonly used technique known as solvent extraction (Chaves et al., 2020). Another popular technique is distillation, which uses heat to separate volatile substances like essential oils. This makes it especially helpful for extracting low-molecular-weight phytochemicals and aromatic compounds (Q. W. Zhang et al., 2018).

Maceration, a simpler method, entails soaking plant material in a solvent, suitable for extracting thermolabile components for 3 days (minimum), at room conditions. Following the extraction process, the mixture is strained using a net of sieves. (Bitwell, Sen Indra, et al., 2023).
Decoction, involving heating plant material in water, is valuable for polar compounds, offering simplicity and scalability. Infusion, achieved by steeping plant material in a solvent like water or alcohol, is also suitable for polar compounds and shares the advantage of simplicity and scalability. Percolation and Soxhlet extraction (Constant refluxing method) involve passing a solvent through plant material. Soxhlet method is considered to have a better performance, however, with percolation it is possible to extract effectively thermolabile components (Ponphaiboon et al., 2023).

Some other non-conventional methods of extraction include: Microwave assisted extraction, Ultrasound assisted extraction, Pulsed electric field extraction, Enzyme assisted extraction, which can be used with aqueous and non-aqueous solvents (Goti & Dasgupta, 2023).
The chosen extraction method depends on several factors, including the desired specific compounds, the properties of the plant material, and the intended use of the extract.
The solvent used during this research is methanol. Ethanolic extracts are prepared using ethanol as a solvent which is able to extract hydrophilic compounds and bioactive compounds from plant materials (Sasidharan et al., 2011).

1.1.2 Physalis peruviana

The genus *Physalis*, which belongs to the family Solanaceae, consist of herbaceous plants, from which approximately 120 species are reported, mainly distributed in America tropical and temperate regions (W.-N. Zhang & Ong, 2016).

Physalis peruviana, also known as uvilla in Ecuador, uchuva in Colombia or goldenberry in some English-speaking countries, is a plant of big interest for its fruit. In Equator, *P. peruviana* is grown on more than 200 hectares in the Ecuadorian provinces of Azuay, Carchi, Cotopaxi, Imbabura, and Pichincha. This is a backyard plant in most countries, despite its potential; however, the fruits have recently become extremely expensive in some international markets, such as Europe. The fruits of Physalis peruviana are typically eaten as fresh fruit, they can also be found in canned fruit, salads, cooked foods, desserts, jams, and natural snacks (Carrillo-Perdomo et al., 2015). Physalins, alkaloids, flavonoids, carotenoids, vitamins, and polysaccharides are among the several types of compounds reported in their flowers. Phytochemical composition is linked to the plant's health benefits (Kasali et al., 2021).
P. peruviana L. is said to possess a wide range of therapeutic qualities, including antispasmodic, diuretic, antiseptic, sedative, analgesic and also to treat intestinal parasites and amoeba. Additionally, antidiabetic properties have been reported, suggesting five fruits a day. As of right now, no research has shown any potential negative effect. Some of its therapeutic uses in various parts of Colombia include kidney blood purification, albumin reduction, cataract cleaning, calcification, and amebiasis control (Puente et al., 2011). In Equator, uvilla Flower is used as a disinfectant and for healing of wounds (Kasali et al., 2021).

1.1.3 Plantago major

Plantago major L. is an herb perennial plant which belongs to genus Plantago and the family of Plantaginaceae (Adom et al., 2017). This is a species native to Europe and Asia, but has been introduced to other parts of the world, including Ecuador. Some of their characteristics include a short stout erect stem surrounded by a rosette of spirally arranged, glabrous or pubescent leaves (Rojas-Sandoval & Major, 2023)(Sagar & Harper, 1964).
Some of the therapeutic used of Plantago major L. include: Anti-inflammatory, diuretic, hepatic, stomachache (Rios et al., 2017). This is a significant medicinal plant due to the wide range of bioactive substances that have, such as vitamins, fatty acids, polysaccharides, alkaloids, phenolic compounds (derived from caffeine), flavonoids, and iridoid glycosides. Parts of the plat such us

leaves, flowers, seeds and roots contains these substances. These chemical components are responsible for Plantago major's bioactivities. (Adom et al., 2017).

1.1.4 Impact of secondary metabolites in human health

Secondary plant metabolites are crucial for both plant survival and human health. These compounds, spanning categories like alkaloids, flavonoids, essential oils, glycosides, tannins, and resins, offer various health benefits. Alkaloids, with diverse biological effects, find applications as drugs, flavors, and fragrances. Flavonoids, acting as plant pigments, possess antioxidant properties, mitigating the impact of chronic diseases and aging. Essential oils, exemplified by lavender and eucalyptus, boast therapeutic uses spanning centuries(Sreenivasulu & Fernie, 2022). Glycosides, characterized by sugar-coated molecules, exhibit diverse biological effects, including antioxidative and anti-inflammatory properties. Tannins, known for their astringency, have traditional medicinal applications. Resins, sticky substances, contribute to medicinal oil production with multifaceted biological effects. Polyphenols, an extensively studied compound group, encompass flavonoids, phenols, and tannins, showcasing antioxidant, anti-inflammatory, and antimicrobial properties. These secondary metabolites exert broad-ranging effects on human health, encompassing antioxidant, anti-inflammatory, antimicrobial, antihelminthic, anticoagulant, antidiabetic, and lipid-lowering properties. Some compounds are cytotoxic, impeding angiogenesis and tumor spread, while others safeguard nerve cells, promote skin protection, prevent bone calcium loss, and enhance fetal lung maturation. With over 25% of existing drugs originating from plant secondary metabolites, their pivotal role in pharmaceutical product development is underscored(Lal et al., 2023).

1.1.5 Analytical techniques for phytochemical analysis

To determine and measure the bioactive substances found in plants several analytical technical can be applied. The separation, identification, and quantification of phytochemicals are commonly achieved through the use of chromatographic techniques such as gas chromatography (GC), Optimum Performance Laminar Chromatography (OPLC), High-Performance Thin Layer Chromatography (HPTLC) or high-performance liquid chromatography (HPLC) (Sahira Banu & Cathrine, 2015a)

One of the most utilized is HPLC, which is able to separate a mixture of compounds. HPLC works by constantly passing a solvent (mobile phase) through a column under high pressures, causing the components of a mixture to separate from each other due to their different degrees of

interaction with the column's packing material (Bélanger et al., 1997).

Reversed high-performance liquid chromatography (HPLC) is a particular kind of HPLC that separates and analyzes mixtures of compounds using a mode of reversed polarity. The mobile phase in a typical HPLC is non-polar or less polar than the stationary phase, which is polar in nature. The mobile phase in reversed HPLC is polar, while the stationary phase is non-polar or less polar. Compounds that are difficult to separate using standard HPLC procedures can be separated thanks to this polarity reversal (Kazakevich & LoBrutto, 2007).

Likewise other Non-chromatographic Techniques can be applied to increase the accuracy of detection, for example immunoassay where low molecular weight compounds are separated using monoclonal antibodies based on the binding of an enzyme to a receptor, followed by additional analysis (Khare et al., 2018) .Another technique highly used is the Fourier-Transform Infrared Spectroscopy (FTIR). Fourier Transform Infrared (FTIR) spectroscopy is a technique employed to analyze the vibrational spectra of molecules. The fundamental principle of FTIR spectroscopy involves transforming the time-domain function of light into the frequency-domain function through Fourier transformation. In a simplified explanation, an infrared (IR) source emits IR rays, which are directed onto a sample through a slit, with mirrors aligning the rays and a prism dispersing the resulting light spectrum. As the light interacts with the sample, molecular vibrations occur, inducing changes in the electric and magnetic fields proportional to the vibration intensity. An IR detector converts these vibrations into an electrical signal, producing an FTIR spectrum displayed graphically with wavelength and intensity axes. This methodology, crucial in fields like chemistry, biology, and materials science, facilitates the study of molecular vibrational spectra and structural identification (Dutta, 2017)(Nikolić, 2011). These methods are quick, precise, and time-efficient for compound identification.

1.1.6 ELISA

An efficient, high-throughput quantitative immunoassay for the targeted detection of antigens is the enzyme-linked immunosorbant assay (ELISA), which was described initially for (Engvall & Perlmann, 1972). This is one of the most sensitive immunoassays. A test ELISA works using an antibody to detect and capture an antigen with a quantified substrate. Traditionally, in a microplate well (96-well polystyrene plates), the antigen is immobilized at the bottom of the solid surface of the plate where subsequently binds to an enzyme-linked antibody through passive absorption, then when a substrate is added a change color is visible or also detected for a signal light which is related with the amount of antigen present in the sample (Lin, 2015). This is known as Direct ELISA, which is the simplest one, but there are several types of ELISA depending on how the

antigen is detected and immobilized (Figure 1). The Indirect ELISA uses a primary antibody, specific for the antigen, that binds to the target, then a labeled secondary antibody binds to the primary antibody for its specificity. The antigen is immobilized to the surface of a multi-well plate. This method can also be used to detect specific antibodies in a serum sample by substituting the antigen with the antibody (Lin, 2015). The indirect ELISA has a higher sensitivity when compared to the direct ELISA, but the only major disadvantage of this type of ELISA is the risk of cross-reactivity(Shah & Maghsoudlou, 2016). The Sadwich ELISA uses two antibodies, firstly a primary antibody is immobilized in the plate with a specific antigen, then an enzyme-linked secondary antibody is added to "sandwich" the desired antigen. On the other hand, the Competitive ELISA, as its name suggests, it is based on the competition between a sample antigen with a reference antigen for binding to a specific amount of labeled antibody. The more antigen presents in the sample, the weaker the signal detected. One of the advantages of this technique is the high sensitivity to the different components found in a mixture of antigens (Gan & Patel, 2013).

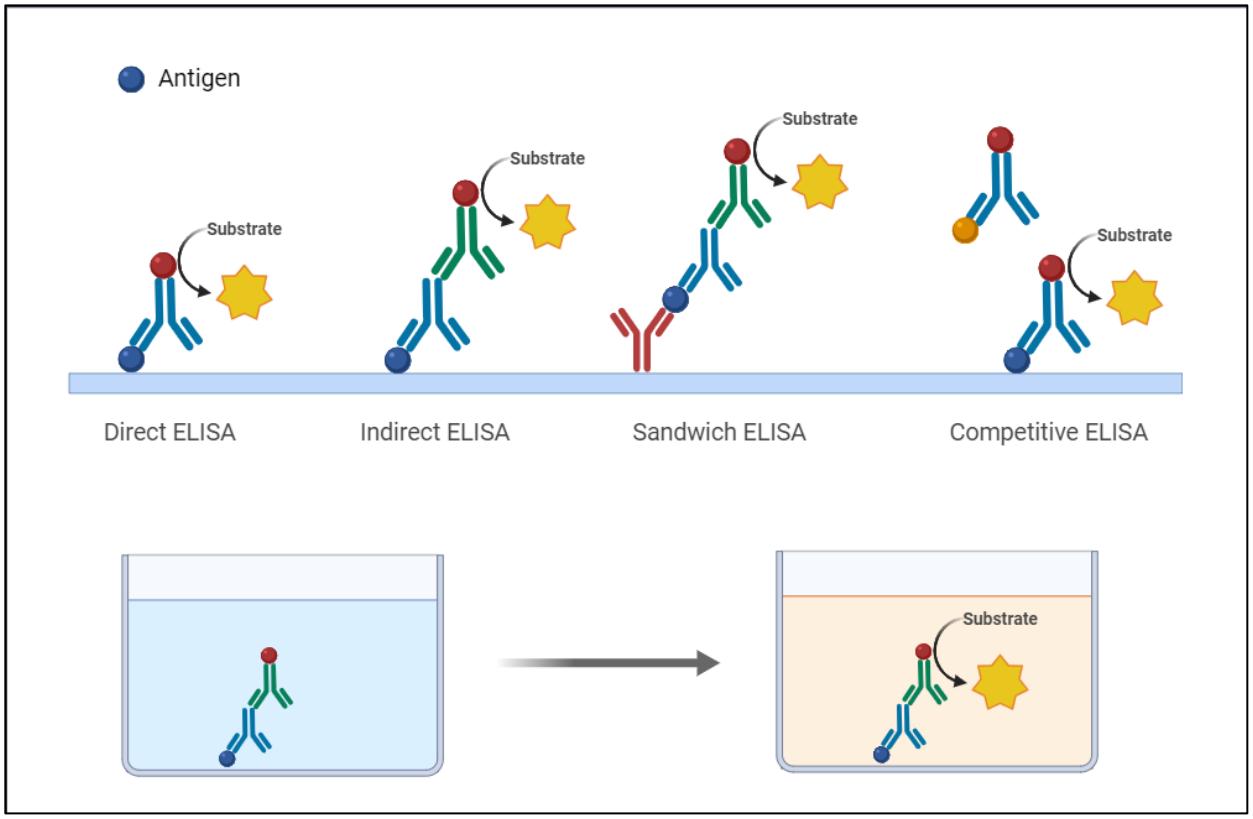


Figure 1. *Schematic representation of different types of ELISA tests*. (Created by María Belén Guerrero).

1.1.7 Cytotoxicity test

The cytotoxicity of substances, particularly those originating from plants, can be investigated using a variety of methods. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, which studies the cell viability, the LDH (Lactate Dehydrogenase) release assay, that evaluates the cell membrane integrity as a parameter for cell death, the neutral red uptake and the ATP content of treated cells, are a few of the frequently employed techniques. These assays are essential for determining possible drugs and evaluating the efficacy and safety of those medications (Weyermann et al., 2005).

One of the most utilized is the MTT calorimetric assay. This technique aims to determine the ability of viable cells evaluating the ability to convert a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] (MTT) into an insoluble formazan precipitate. The base of this technique is that enzymes NADH and NADPH, present in the mitochondria of living cells, or oxidized substrates release electrons which will be accepted by Tetrazolium salts (reduction) producing a change in the color form yellow to blue for the formation of formazan crystals by metabolically active cells. Some of the advantages of this assay over traditional techniques, include the versatility, rapidity and high reproductivity, helpful for large-scale experiments (Plumb, 2004; Supino, 1995) .

1.1.8 Antimicrobial activity

Evaluating new antimicrobial agents have gain interest in the current years. Several methods can be applied for this purpose, such as disk diffusion, well diffusion and broth or agar dilution, which are the most common bioassay used. Other methods such as flow cytofluorometric and bioluminescent methods are not highly used for the equipment requirement (Balouiri et al., 2016).

Kirby-Bauer disk diffusion is the standardized method for bacteria susceptibility which gain a great interest since 1961. The aim of this method is to test the susceptivity of bacteria (pathogenic aerobic and facultative anaerobic), growth commonly in Mueller Hinton agar, against antimicrobial compounds, thus to measure the ability to inhibit bacteria, the formation of an inhibition zone around the disk is evaluated. A paper disk inoculated with antibiotic is used as a positive control, once the antibiotic is absorbed the paper is located in the petri dish with the other substances to be tested. Various compounds can be tested at the same time. (Hudzicki, 2009) .

2. PROBLEM STATEMENT

Medicinal plants have been used even antient times, recently in the field of pharmacology it has gain interest for researchers due to its therapeutic potential. In Ecuador there are several plants which benefits are still unknown due to the lack of research, such us *Plantago major* and *Physalis Peruviana*. It is necessary that sample plants pass through a phytochemical analysis to discover what are the active compounds that they contain, so through this analysis we will be able to use them in a better manner.

2.1 HYPOTHESIS

Previous studies demonstrated that *Plantago major* and *Physalis peruviana* can be used in the field of medicine due to the compounds that are present in them. Thus, this research intent to demonstrated that these two medicinal plants can activate an immunogenic response reflected in the presence of antibodies in hyperimmune serum obtain from Balb/c mice. Likewise, the ethanolic extracts of *Physalis peruviana* and *Plantago major* can produce an antimicrobial activity. Also, it is important to demonstrated that those extract are not cytotoxic to their used in human beings.

3. OBJECTIVE

3.1 General Objectives

The aim of this project was the phytochemical analysis of ethanolic extracts of two plats used in Ecuador as medicinal, *Plantago major* and *Physalis peruviana* through spectroscopy techniques such as FT-IR and HPLC. Likewise, test the ability of each extract to activate the humoral immune response using hyperimmune serum obtained from BALB/c mice previously immunized with each compound. On the other hand, evaluate the cytotoxicity of the extracts, with SH-SY5Y Neuroblastoma cell line through MTT assay from Promega. Finally, perform an antimicrobial activity assay using disk diffusion technique with two bacteria strains (*S. aureus* ATCC 25922 and *E. coli* UITEY strains).

3.2 Specific objectives

- To perform ethanolic extraction of leaves and roots through two different methods, Soxhlet and maceration.
- To conduct a Qualitative Phytochemical Analysis to determine the secondary metabolites

of each extract.

- To use HPLC to confirm the presence of some compounds of each extract.
- Determine the main functional groups related to secondary metabolites present in each extract by Fourier transform infrared spectroscopy (FTIR).
- Standardization of indirect ELISA assay condition for each extract using hyperimmune sera from previously immunized BALB/c mice to determinate the humoral response.
- Perform cross-reaction assay between *Physalis peruviana* and *Plantago major* extracts by polyclonal antibody recognition through indirect ELISA assay using the compounds as antigens.
- To perform the cytotoxicity assay of ethanolic extracts of *Physalis peruviana* and *Plantago major* compounds through colorimetric MTT assay provided by Promega.
- To determine the antimicrobial activity of the compounds on *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ECBI-UITEY strains.

4. MATERIALS AND METHODS

4.1 Chemical analysis

4.1.1 Ethanolic extracts of *Physalis peruviana* and *Plantago major*.

To carry out the ethanolic extraction, each part of the plant (roots and leaves) were washed with distilled water and then dried in oven for at least 24h. Once the samples are complete dried, they were weighed. To be able to use the samples, they were cut into small pieces. Two methods of extraction were performed (Figure 2).

Maceration process

The samples previously dried (7,92 g of uvilla leaves; 30,06g of llantén leaves; 9,69g of uvilla roots and 1,24g of llantén roots) were taken in a 500ml beaker with ethanol at 70%, the solvent has to cover completely the sample. This mixture rest for 20 days in complete darkness. The liquid obtained was filtered through vacuum filtration and rotary evaporated. Finally, the extracts passed through the process of lyophilization to eliminate the rest of water.

Soxhlet process

The dried extract to be processed is placed in a filter paper bag which is inside of the extraction chamber. The round flask was filled with 190 ml of the solvent (ethanol). Once the solvent starts to boil, the system has to be repeated for 21 cycles. The sample passed through vacuum filtration and rotary evaporation to be refrigerated. Finally, lyophilization process was performed.

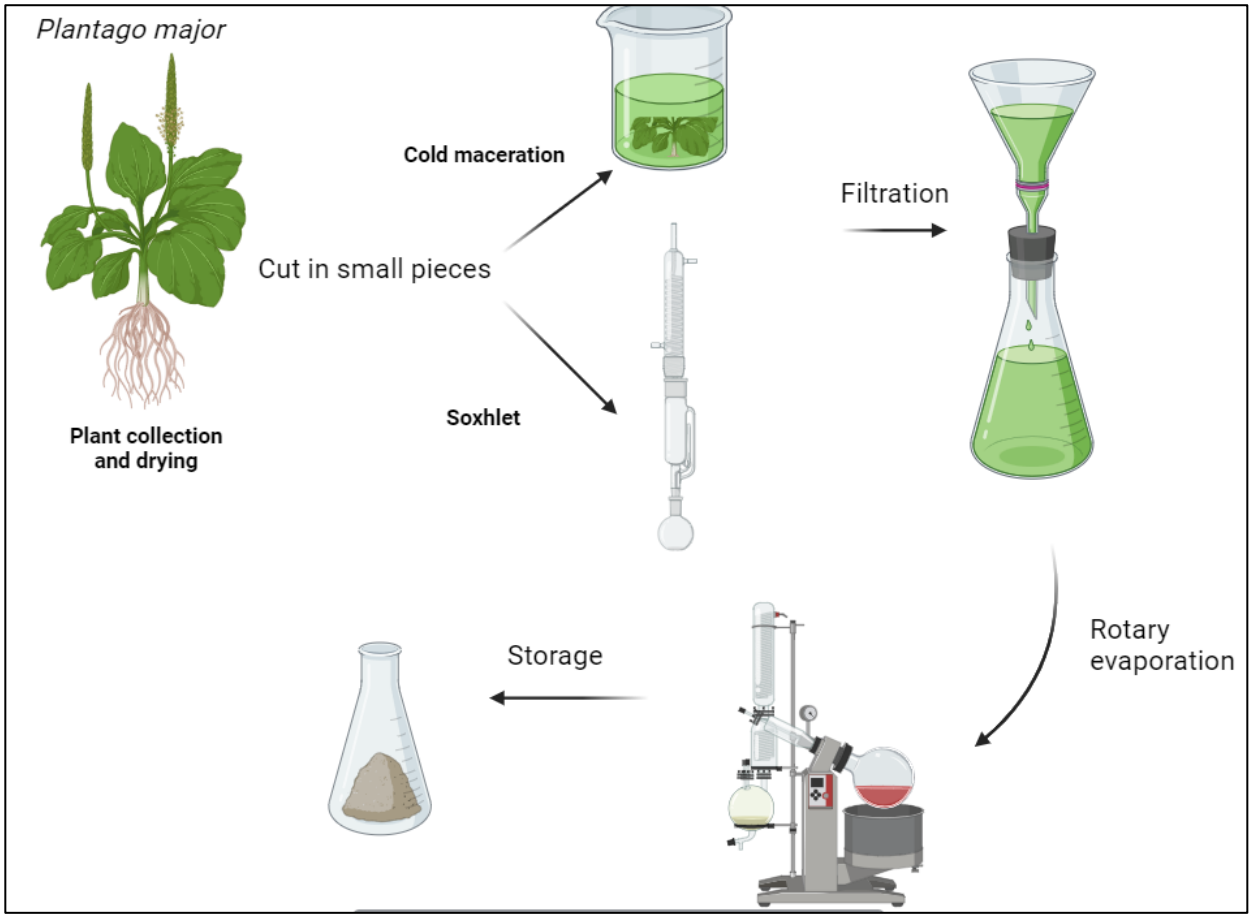


Figure 2. *Extraction process through maceration and Soxhlet techniques.* (Created by María Belén Guerrero).

4.1.2 Preliminary Qualitative Analysis

The presence of phytochemicals in the ethanolic extracts of *Physalis peruviana* and *Plantago major* leaves and roots was determined by applying some standard techniques, slightly modified, as follow:

4.1.2.1 Test for Alkaloids (Wagner test)

1 ml ethanolic extract of leaves and roots was acidified with 1 ml of 1% hydrochloric acid in a test tube, then heated gently. Then, 10 to 20 drops of Wagner reagent were added. The presence of alkaloids is indicated for the formation of a reddish-brown precipitate (Sahira Banu & Cathrine, 2015b)

4.1.2.2 Cardiac Glycosides (Keller-kiliani test)

1 ml of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 1 ml of glacial acetic acid containing two drops of 2% solution FeCl3 in a test tube. The mixture was poured into another test tube containing 1 ml of concentrated sulphuric acid. The presence of glycosides is indicated with the appearance of ring at the interphase or a blue coloration in the acetic acid layer and a red color in the two acids' interphase (Sheel et al., 2014)(Yadav & Agarwala, 2011).

4.1.2.3 Terpenoids (Salkowski test)

1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 2 mL of chloroform in a test tube. Then, 0,5 mL of concentrated sulphuric acid was added drop by drop and heated for 2 minutes. The presence of terpenoids is indicated by a color change of the mix to grayish (Sheel et al., 2014)(Abdel-Rahman et al., 2019)

4.1.2.4 Steroids (Libermann test)

1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 2 mL of chloroform and 2 mL of acetic acid in a test tube. The mixture was cooled in ice. Carefully, 0,5 mL of concentrated sulphuric acid was added drop by drop. The presence of steroidal molecules is indicated for a color change of the mix to violet, blue or green (NATH et al., 1946).

4.1.2.5 Saponins

1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 5mL of distilled water in a test tube, and it was shaken robustly. The determine the presence of saponins the formation of stable foam was observed (Sheel et al., 2014) .

4.1.2.6 Carbohydrates (Benedict test)

1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 1 mL of Benedict reagent in a test tube and then is boiled. The presence of carbohydrates is determined due to the formation of a reddish-brown precipitate in the test tube (Balamurugan et al., 2019).

4.1.2.7 Phenols and tannins

1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 1 mL of 2% solution of FeCl3 in a test tube. To determine the presence or absence of phenols and tannins a blue-green or black coloration is observed in the test tube (Sahira Banu &

Cathrine, 2015)

4.1.2.8 Flavonoids (Alkaline reagent test)

In a test tube, 1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with 1 mL of 2% NaOH solution. After, a few drops of weak hydrochloric acid were added, when the bright yellow color that has formed went colorless, it indicate the presence of flavonoids (Sheel et al., 2014) .

4.1.2.9 Flavonoids (Shinoda test)

In a test tube,1 mL of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* were mixed with ten drops of diluted hydrochloric acid, followed by 500 mg of magnesium. The presence of flavonoids is observed when Pink, Reddish Pink, or Brown color is produced (Yadav & Agarwala, 2011).

4.1.2.10 Proteins or amino acids (Ninhydrin test)

In a test tube, 1 ml of ethanolic extract of leaves and roots from *Physalis peruviana* and *Plantago major* and 1 mL of freshly prepared 0.2% ninhydrin reagent were mixed and heated to boiling for 1-2 min. The sample's blue or violet blue indicates the presence of proteins and amino acids (Yadav & Agarwala, 2011).

4.1.2 HPLC

Plantago major and *Physalis peruviana* leaves and roots ethanolic extracts were analyzed through HPLC Dionex UltiMate 3000 with a UV/Vis detector at 220nm wavelength and Chromeleon software for the data analysis. First of all, 1mg/ml of crude ethanolic extract and pure H_20 were prepared. The liquid was filtered to remove any particular matter. This process was repeated for each sample. The injection volume was 5 µl, and the flow rate was 1 ml/min. Using type 1 water and acetonitrile (from 0:100 to 100:0) as the mobile phases, the gradient elution was carried out for eight minutes at room temperature. Finally, the chromatogram obtained was analyzed for the number and retention times of peaks. The absorbance was presented in stands for milli-absorbance units (mAU).

4.1.3 Fourier transform infrared spectroscopy (FTIR)

The FTIR analysis of ethanolic plant extracts was carried out using an Agilent Cary 630 FT-IR spectrometer. The attenuated total reflection (ATR) method was employed in the 4000-400 cm−1 wavelength range. The spectrometer directs infrared beams at the sample and creates the spectrum by placing a small amount of each extract on the diamond crystal and adjusting the clamp. To capture an ideal FTIR spectrum, it was necessary to thoroughly clean the crystal and the diamond using pure ethanol isopropanol to prevent sample contamination and ensure that the rotary press is correctly attached to the solid extract. The peaks obtained in the infrared spectra were analyzed to recognize the metabolites of each sample.

4.2 Biological assays

4.2.1 Bradford test

The quantification of proteins was carried out using the colorimetric Bradford protein. This procedure uses Bradford Reagent (Sigma-Aldrich's) which produces a change dye's light absorption that is read to an absorbance of 465 nm to 595 nm. To make the calibration curve, Bovine Serum Albumin (BSA) dilutions were used from 1 to 0.06 mg/ml (1:1 to 1:16). Then, in a 96-well plate, 5 µl of protein standards were added individually in each well. The foliar extracts (unknown samples) were prepared with a concentration of approximately 1 to 0.06 mg/ml and added similarly to BSA dilutions, and adding 250 µl of Bradford reagent to each well, the mixture was shaken for about 30 seconds, after that the 96-well plate was incubated at room temperature for 5 to 45 minutes. The absorbance was read at 595 nm. The protein-dye complex was stable for up to 60 minutes. Finally, the absorbance vs concentration of BSA dilutions were plotted to graph the standardized curve, thus extrapolating the data the concentration of unknown samples concentration was calculated.

4.2.1 Protocol of BALB/c mice immunization to obtain the pre-immune and hyperimmune sera.

Firstly, to obtain pre-immune serum, blood samples from healthy mice were poured into Eppendorf tubes and subsequently were centrifuged for 8 minutes at 4500 rpm. The supernatant was collected. To begin with the experiment, the initial immunization was performed by injecting an emulsion made of 100µl of Freund's complete adjuvant and 100µl of foliar extract as antigen to each mouse. The emulsion was administered intraperitoneally. After that, three immunizations

were administered intraperitoneally using an emulsion that contained 200µl/mouse (100µl of incomplete Freund's adjuvant plus 100µl of foliar extract). After seven days, the last immunization was performed and hyper-immune sera could be obtained by sacrificing the mice and drawing blood from their left ventricle. Avoiding hemolysis, each blood sample was placed into Eppendorf tubes. After allowing the blood sample to settle for 20 minutes, they were centrifuged for 8 minutes at 4500 rpm to extract the serum from the clot.

4.2.3 **ELISA indirect assay using extracts from *Physalis peruviana* and *Plantago major* as antigens (Standardization).**

After obtaining the sera from BALB/c mice, the humoral immune response of the foliar macerated extracts is assessed using an indirect ELISA test. This technique was performed by Voller et al., (1976) protocol, which aims to determine the ideal test conditions, including antigen concentration, blood serum dilution, and reading time.

The following protocol was applied: uvilla and llantén foliar macerated extracts (antigens), were diluted at the final concentrations of 5 µg/ml, 10 µg/ml, and 20 µg/ml in a Carbonate/Bicarbonate buffer (pH 9.6). The antigen dilutions were evenly distributed in 100 µl wells of a 96-well ELISA microplate (Nunc TM), following the layout shown in Figure 1. The microplate was kept overnight at 4°C in a humid chamber. After discarding the coating solution, 200 µl of PBS + 0.005% Tween 20 washing solution was used to wash the plate three times for three minutes. After that, 100 µl of diluted 6% bovine serum albumin (BSA) in PBS was added to each well of the microplates to block them. The plate was then incubated for one hour at 37°C in a humid chamber. The blocking agent was thrown away.

To wash the microplate, 200 µl of washing solution was added to each wall for three times, three minutes each time. It was kept for the night at 4°C. Pre-immune and hyper-immune sera were diluted in two concentrations, 1:100 and 1:200 in PBS for the primary antibody incubation. The negative control used was PBS. Thus, the negative control and the diluted sera were arranged according to the diagram in Figure 3. For an hour, the microplate was incubated at 37°C in the humid chamber. The sera were taken out. The wash solution was used three times for three minutes to wash the microplate. The microplate was incubated for one hour at 37°C in a humid chamber with 100µl of conjugated secondary antibody (Anti-Mouse IgG (Fc) - horse radical peroxidase (HRP) antibody produced in rabbit by SIGMA) diluted 1:1000. The microplate was cleaned using the washing solution following incubation. All wells were filled with 100 µl of a solution made up ABTS substrate 2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) by Sigma plus and 10 µl of 30% H2O2.

The ELISA microplate reader was used to identify the reaction. Over the course of an hour, the samples' absorbance values at 405 nm were noted at 5-minute intervals. 100 µl of diluted 6% bovine serum albumin (BSA) in PBS was added to each well of the microplates to block them. The plate was then incubated for one hour at 37°C in a humid chamber.

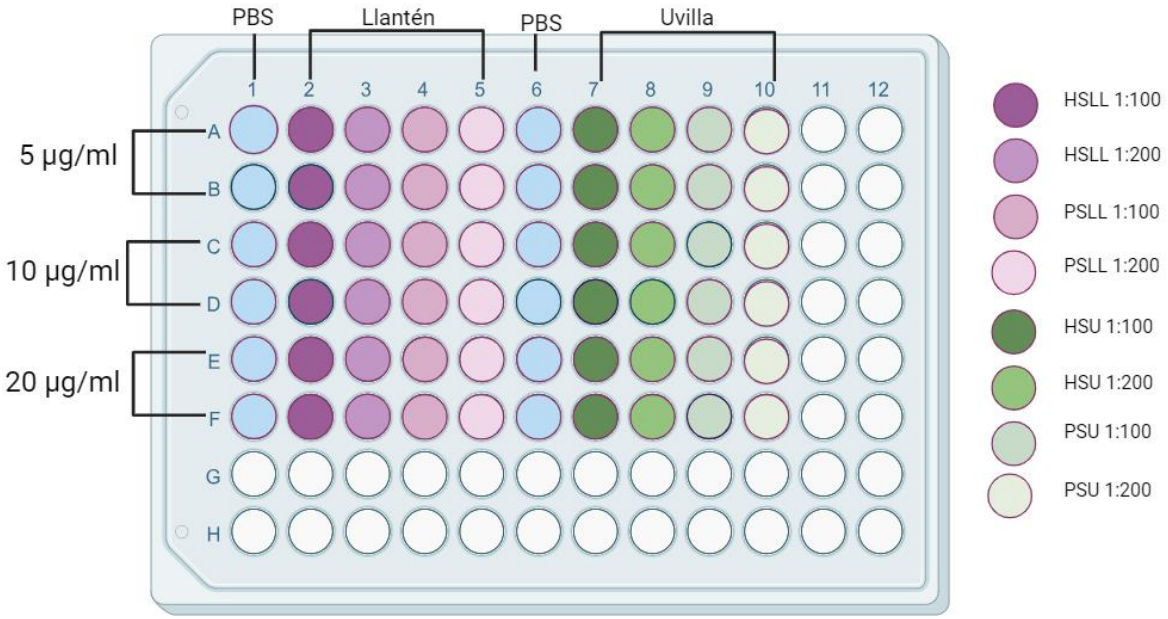


Figure 3*. Localization of sera and antigens on 96-well ELISA microplate. HSLL: Hyper immune serum of llantén leaves; PSLL: Pre-immune serum of llantén leaves: HSU: Hyper-immune serum of uvilla leaves; PSU: Pre-immune serum of uvilla leaves.* (Created by María Belén Guerrero).

4.2.4 Indirect ELISA assay for analysis of cross activity

The procedure used to the experiment is the same as that described in section 7.2, however, all hyperimmune sera were assessed using a cross-reactivity test against the two antigens (Figure 2). Uvilla hyperimmune sera (HSU) was diluted 1:200, and llantén hyperimmune sera (HSLL) was diluted 1:100. Uvilla and llantén extracts were kept at a concentration of 5 µg/ml. The different dilutions of hyperimmune serum were evaluated against its antigen and the antigen of the other plant as follows: HSU vs uvilla extract, HSU vs llantén extract, HSLL vs uvilla extract, HSLL vs llantén extract.

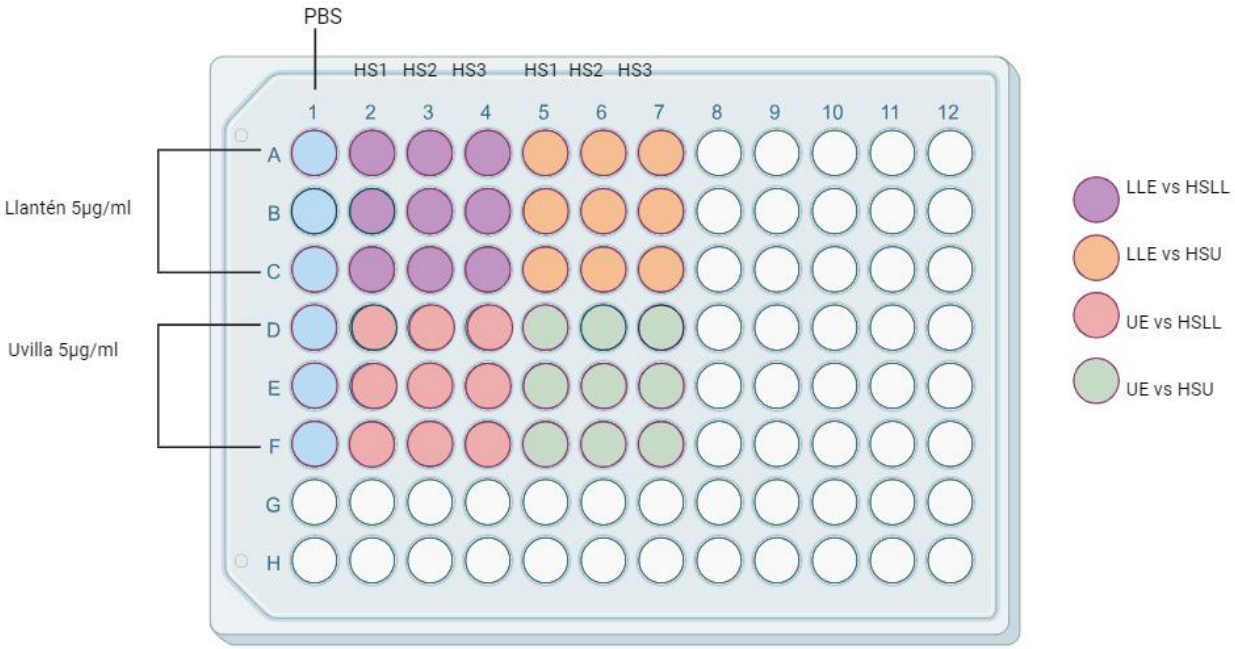


Figure 4*. Localization of sera and antigens in 96-well ELISA microplate. PBS was used as negative control. LLE: llantén leaves extract; HSLL: Hyper-immune serum of llantén leaves; UE: uvilla leaves extract; HSU: Hyper-immune serum of uvilla leaves.* (Created by María Belén Guerrero).

4.2.5 Cytotoxicity experiment

Physalis peruviana and *Plantago major* ethanolic leaf extracts were tested for cytotoxicity using the standard protocol of Promega Corporation (2012) for CellTiter 96 ® Aqueous One Solution cell proliferation assays (MTT). To perform this procedure, the leaf extracts were diluted to a concentration of 5µg/ml and 10µg/ml, and the SH-SY5Y neuroblastoma cell culture must be prepared beforehand.

The SH-SY5Y neuroblastoma cell line was harvested with a rake to detach them.

A Neubauer chamber was used to count the number of cells. Based on the related computations, the findings indicate that there are roughly 10^5 cells in every 50 µl of the solution.

For this procedure, Gibco Dulbecco's modified Eagle medium (DMEM) supplemented with 1% antibiotic was used. After that, the cells were seeded in each well, as is shown in Figure 5, adding 50 µl of medium (1x), 50 µl of medium (4x), 50 µl of diluted compounds, and 50 µl of cells in that order. At this stage, the positive control will be medium plus cells, and the negative control will be medium without cells.

Incubate the plate at 37 ºC for 72 hours with 5% CO2.

Once 20 µl of MTT labeling reagent was added to each well, it was then incubated for 4 hours at 37 °C in a humidified chamber. At 0,1,2, and 3 hours into the incubation, the absorbance was

measured at 490 nm using an ELISA reader.

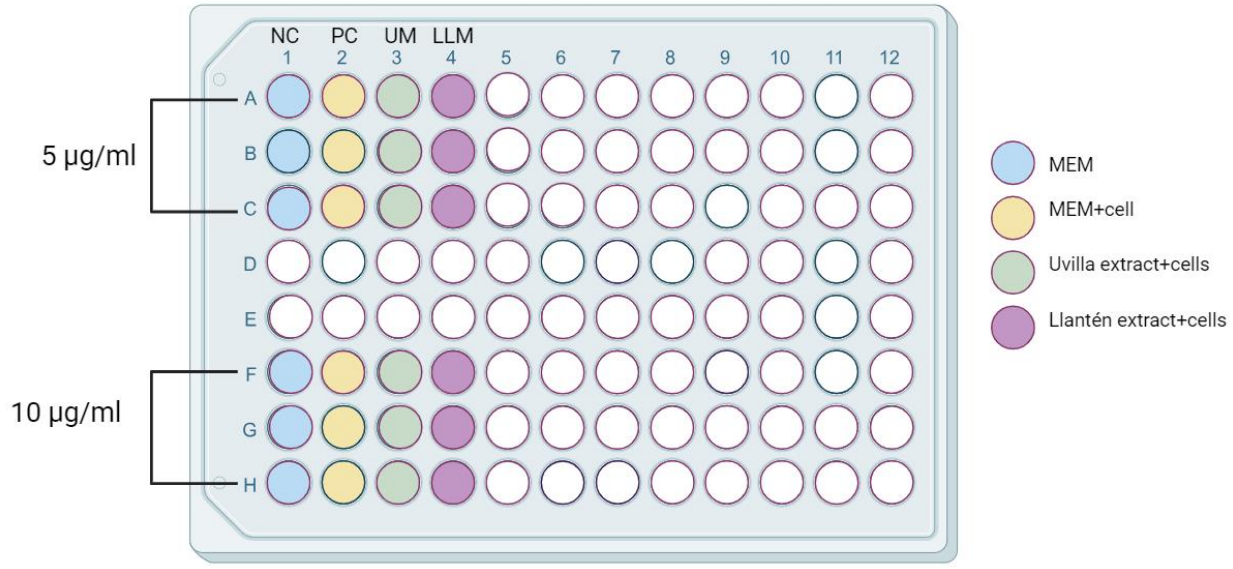


Figure 5. *Schematic representation of the localization of neuroblastoma cells and antigens. NC: Negative control (MEM without cells); PC: Positive control (MEM + cells); UM: uvilla macerated extract; LLM: llantén macerated extract.* (Created by María Belén Guerrero).

4.2.6 Antimicrobial Test

The antimicrobial activity of *Physalis peruviana* and *Plantago major* ethanolic plant extracts against *E. coli* ATCC 25922 and *S. aureus* UITEY-Sa strains was assessed using the Kirby-Bauer disk diffusion susceptibility test on Mueller-Hinton (MH) agar medium(Hudzicki, 2009). The extracts were previously filtered to remove impurities, and their concentrations were 1 mg/ml and 2 mg/ml. Ampicillin at a concentration of 1 mg/ml was the antibiotic used.

To prepare the inoculum, 20µl of each bacteria strain was added to 2 milliliters of sterile Culture Broth, and the mixture was shaken at 120 revolutions per minute for 12 hours at 37°C.

The growing inoculum's turbidity was adjusted to reach the 0.5 McFarland standard, which corresponds to an absorbance of 0.08–0.1 in a Nanodrop at 625 nm. Within fifteen minutes of preparation, this suspension was put to use.

Using a sterile swab, a sample of the modified inoculum was taken and placed on top of the MH agar in each Petri dish for bacterial seeding. Next, as is shown in Figure 6, 10µl of each solution was added to Whatman No. 1 filter paper disk that had been previously sterilized. The disks were then carefully placed on the agar surface in the Petri dishes and incubated at 35°C for 16-18 hours.

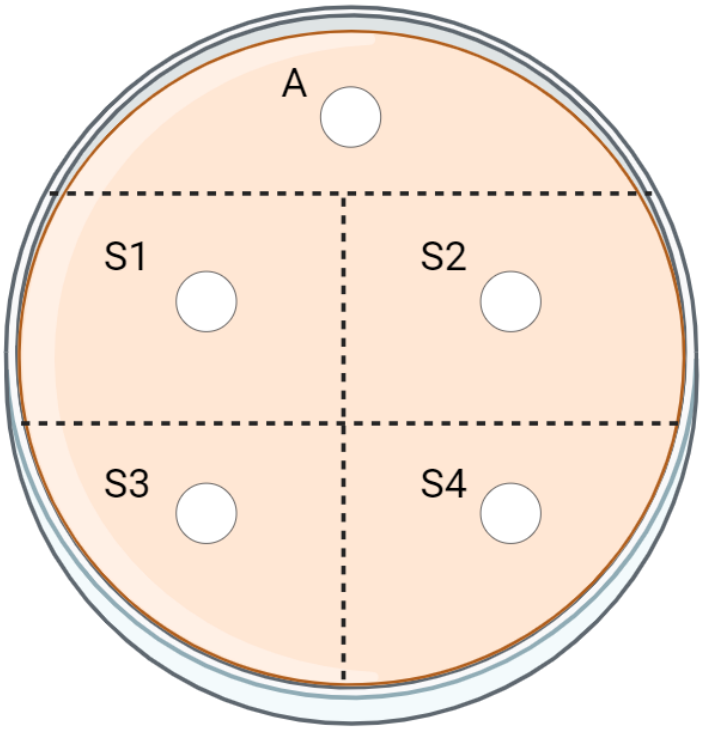


Figure 6. *Scheme of the location of the compounds and antibiotic for the antimicrobial activity test on the plate. The same scheme was s followed with E. coli and S. aureus strains. S1=Sample 1 (uvilla leaves); S2=Sample 2 (uvilla roots); S3= Sample 3 (llantén leaves); S4= Sample 4 (llantén roots); A= Ampicilin.* (Created by María Belén Guerrero).

5. RESULTS AND DISCUSSION

For the development of the research project, we analyze the plant extracts using FTIR, HPLC and qualitative phytochemical tests. After that, we standardized the indirect ELISA test values, in order to establish the correct reagent concentration and reading time. From these values, we performed cross-reactivity experiments using the macerated foliar extract of *Physalis Peruviana* and *Plantago major*, to then analyze the antibody recognition. Finally, the cytotoxicity and antimicrobial activity of each compound were evaluated.

5.1 Ethanolic extraction

The crude extracts, which were obtained after the process of lyophilization, were weighted and the percentage yield (w/w) was calculated, as follows:

$$\%\ yield\ \left(\frac{w}{w}\right) = \frac{Actual\ yield}{Theorical\ yield} x100\%$$

The theorical yield, corresponds to the weight of plant material (Wi), while the actual yield is related to the to the weight of the crude extract (CE), in grams. The results are presented in Table 1.

The two species of plants, *Physalis peruviana* and *Plantago major*, have shown variations in the percentage yield for both extraction process (i.e., Maceration and Soxhlet).

The highest yield obtained for *Physalis peruviana* roots and leaves were reached through the process of maceration. For the leaves we obtained 48% of yield and for roots 41.7%. On the other hand, the leaves and roots of *Plantago major*, reached a yield of 36.7% and 34.3%, respectively. Nonetheless, unlike to *Physalis peruviana,* the samples of llantén obtained the highest values with the process of Soxhlet. Finally, comparing both processes, Maceration presented the best results.

Table 1. *Percentage yield Physalis peruviana and Plantago major extracts.*

Wi= initial weight, UH = leaves of Physalis peruviana, UR = roots of Physalis peruviana, LLH = leaves of Plantago major, LLR = roots of Physalis peruviana, CE=Crude extract.

Method	**Sample**	**Wi (g)**	**CE (g)**	**% Yield (W/W)**
Maceration	U_H	7.92	3.81	48.1
	U_R	9.69	4.04	41.7
	LL_H	3.06	0.71	23.2
	LL_R	1.24	0.26	21.0
Soxhlet	U_H	5.45	0.74	13.6
	U_R	9.01	1.78	19.8
	LL_H	4.69	1.72	36.7
	LL_R	2.68	0.92	34.3

5.2 Qualitative phytochemical analysis

The results of qualitative phytochemical analysis *Plantago major* and *Physalis peruviana* extracts are presented in Table 3. *Physalis peruviana* macerated leaves extracts presented phytochemicals such as: phenols and tannins, flavonoids, proteins, saponins, cardiac glycosides and alkaloids, as was reported by Njoroge et al. (2023). However, in this research we unveiled that the extracts also contain carbohydrates, steroids and terpenoids. On the other hand, in *Physalis peruviana* extracts through Soxhlet process just alkaloids and saponins were confirm, Nevertheless, the possible presence of phenols, carbohydrates, flavonoids, steroids and cardiac glycosides was observed. Macerated extracts of *Plantago major* revealed the presence of flavonoids, phenols, saponins, carbohydrates, and cardiac glycosides while the Soxhlet extracts revealed the presence of flavonoids and steroids. Macerated extracts presented a higher number of secondary metabolites rather than Soxhlet. One of the disadvantages of using Soxhlet as extraction process is the exposure to high temperature that combine with a long extraction time can lead to a thermal

degradation (Q. W. Zhang et al., 2018). Roots samples in comparison to leaves contain fewer secondary metabolites.

It was observed that leaves contained a higher abundance of phytochemicals. Various biological activities, including antibacterial, anti-inflammatory, antioxidant, and anticancer effects, have been linked to the wide variety of phytochemicals present in plants. To utilize the medicinal potential of plants in modern pharmacology, it is imperative to comprehend the qualitative characteristics of these bioactive chemicals.

Table 2. *Physalis peruviana and Plantago major phytochemical screening*

Presence= (+), Absence= (-), Possible presence= (*). UH = leaves of *Physalis peruviana* (uvilla), UR = roots of *Physalis peruviana* (uvilla), LLH : leaves of *Plantago major* (llantén), LLR= roots of Plantago major (llantén), ME: Macerated extract, SE: Soxhlet extract.

	Qualitative Phytochemical Screening								
		Results							
Secondary Metabolite	**Performed test**	**U_H**		**U_R**		**LL_H**		**LL_R**	
		ME	**SE**	**ME**	**SE**	**ME**	**SE**	**ME**	**SE**
Phenols andTannins	Phenols and Tannins' tests	+	*	+	*	+	*	+	+
Flavonoids	Alkaline Reagent Test	+	-	+	+	+	+	-	-
	Shinoda Test	+	*	-	+	*	+	-	+
Proteins/Amino Acids	Ninhydrin Test	*	*	-	-	*	-	-	-
Saponins	Saponins' Test	+	+	+	*	+	*	*	-
Carbohydrates	Benedict Test	+	*	-	+	+	-	+	+
Steroids	Libermann Test	+	*	-	*	*	+	-	-
Terpenoids	Salkowski test	*	-	*	*	*	-	*	+
Cardiac Glycosides	Keller-kilani Test	+	*	+	+	+	*	*	+

Alkaloids	Wegner Test	+	+	*	+	*	*	*	*

5.3 FTIR (Fourier Transform Infrared)

The spectroscopy FTIR analysis help as to confirm the findings of the phytochemical studies by identifying the predominant functional groups present in the extracts. In the macerated ethanolic extract of *Plantago major* (Figure 7 and 8), at least six primary peaks were identified. The first peak, observed in the 3230-3550 cm-1 range, corresponds to the stretching vibration of the O-H bond, which is indicative of phenols and alcohols. The second peak, found in the 2850-300 cm-1 range, is associated with the C-H stretching region, specifically sp3-1s C-H (Alkyl group). The next peak C=C, with a range from 1600-1680 cm-1, corresponds to alkenes(Abidi, 2022; Hayat et al., 2017).

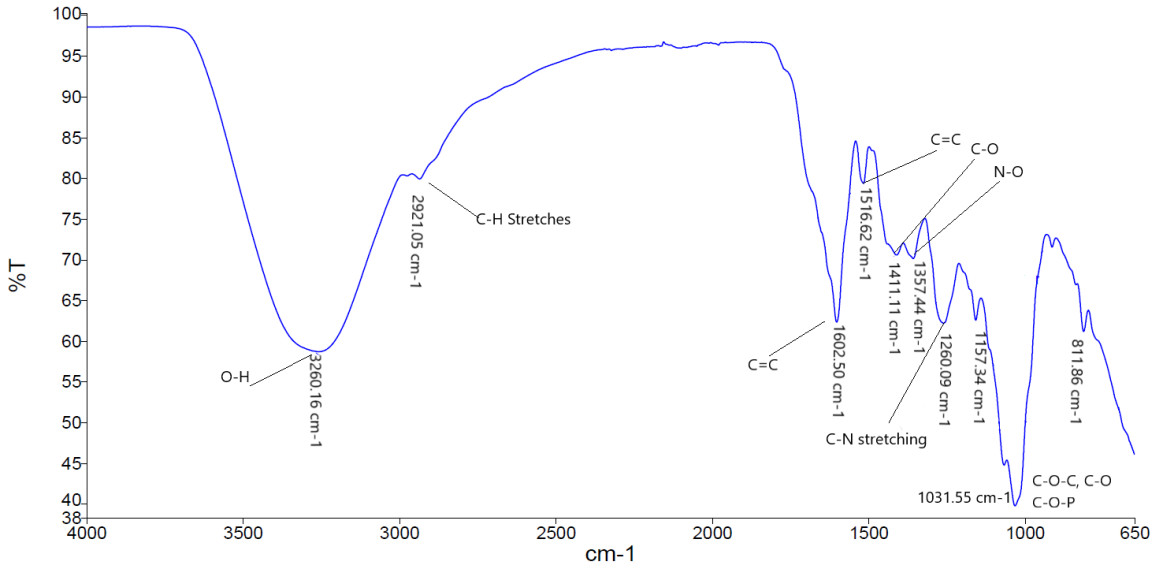


Figure 7*. FTIR spectra of ethanolic macerated extract of Plantago major leaves.*

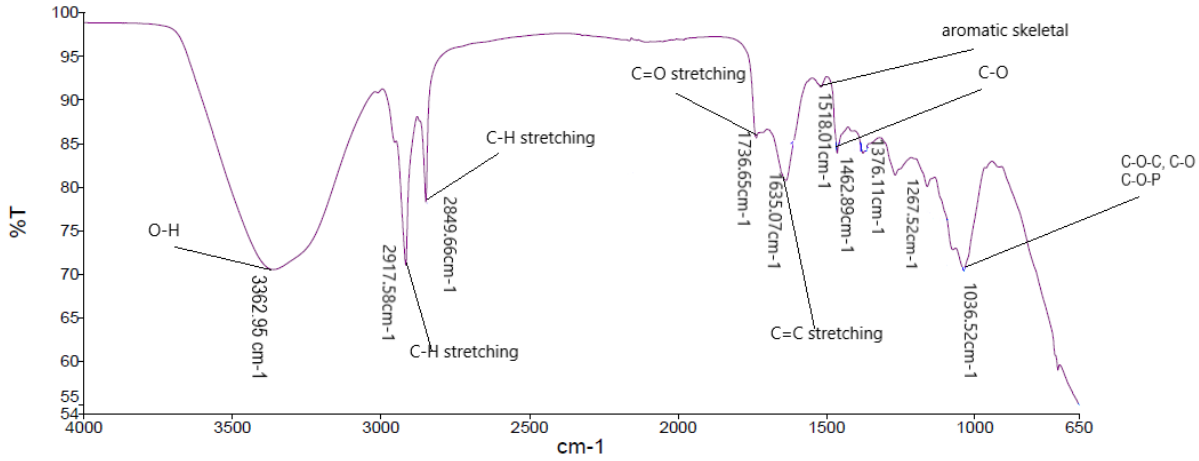


Figure 8. *FTIR spectra of ethanolic macerated extract of Plantago major roots.*

The fourth peak, with absorption between 2850-300 cm-1, corresponds to the C-H functional group, while the fifth peak, with absorption between 1680-1750 cm-1, signifies the presence of the C=O group, suggesting the existence of aldehydes. Furthermore, the sixth peak, located between 1000-1300 cm-1, is attributed to the C-O group, characteristic of alcohols (Sravan Kumar et al., 2015). As we can observe, the IR spectra of *Plantago major* extracts, also present similar peaks, these findings indicate that the ethanolic extract of *Physalis peruviana* and *Plantago major* contains functional groups commonly associated with tannins, saponins, flavonoids, alkaloids, proteins, and carbohydrates. Overall, the IR analysis provides valuable insights into the chemical composition of the extracts, aligning with and enhancing the understanding derived from phytochemical studies.

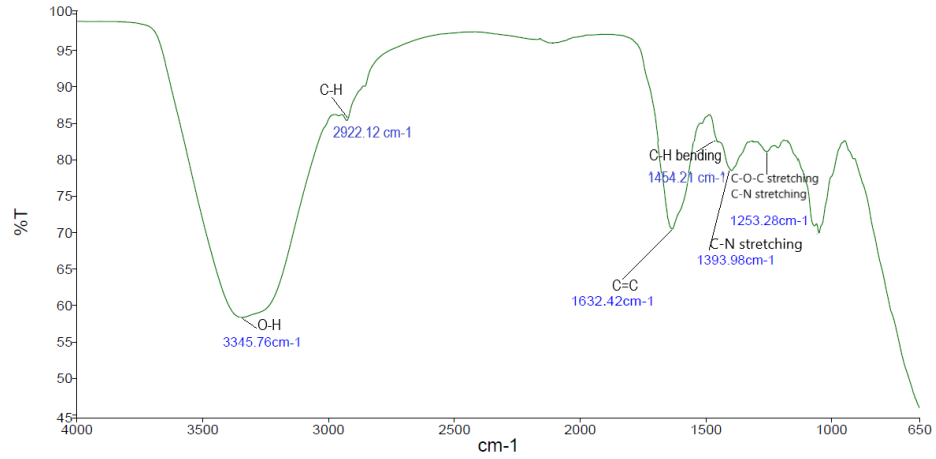


Figure 9. *FTIR spectra of ethanolic macerated extract of Physalis peruviana leaves.*

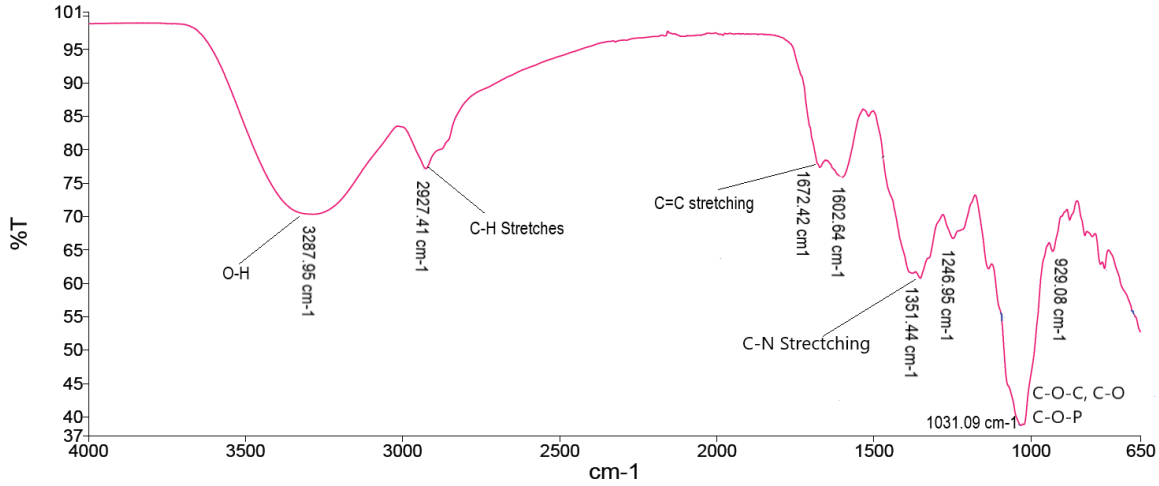


Figure 10. *FTIR spectra of ethanolic macerated extract of Physalis peruviana roots.*

5.4 HPLC

According to High-Performance Liquid Chromatography analytical technique several peaks are observed related to the presence of bioactive compounds. Macerated extracts of *Plantago major* presented peaks at the injection time of: 1.613, 1.743, 1.917,2.980. The first peak represents the 89% of the sample. Furthermore, *Physalis peruviana* leaves, presented some important peaks at: 1.583, 1.623, and 1.977, which represent the 36.13%, 29.41% and 27.42% of the sample, respectively. Thus, we observe that are many compounds present in both samples, as it is shown in Figure 11 and 12.

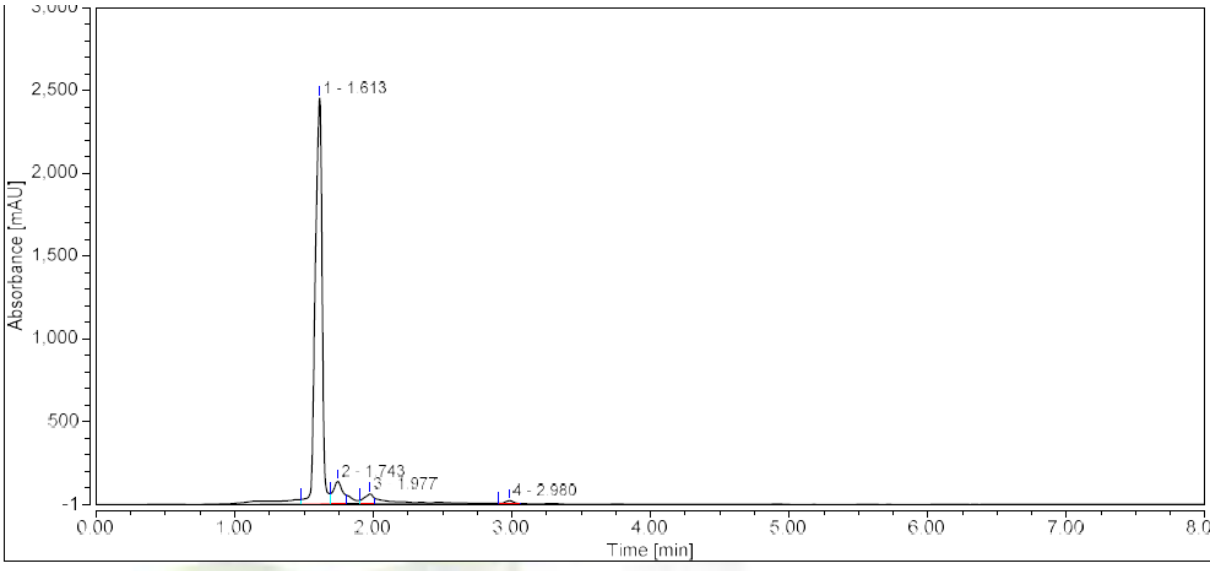


Figure 11. *HPLC Chromatogram graph of macerated extract of Plantago major leaves.*

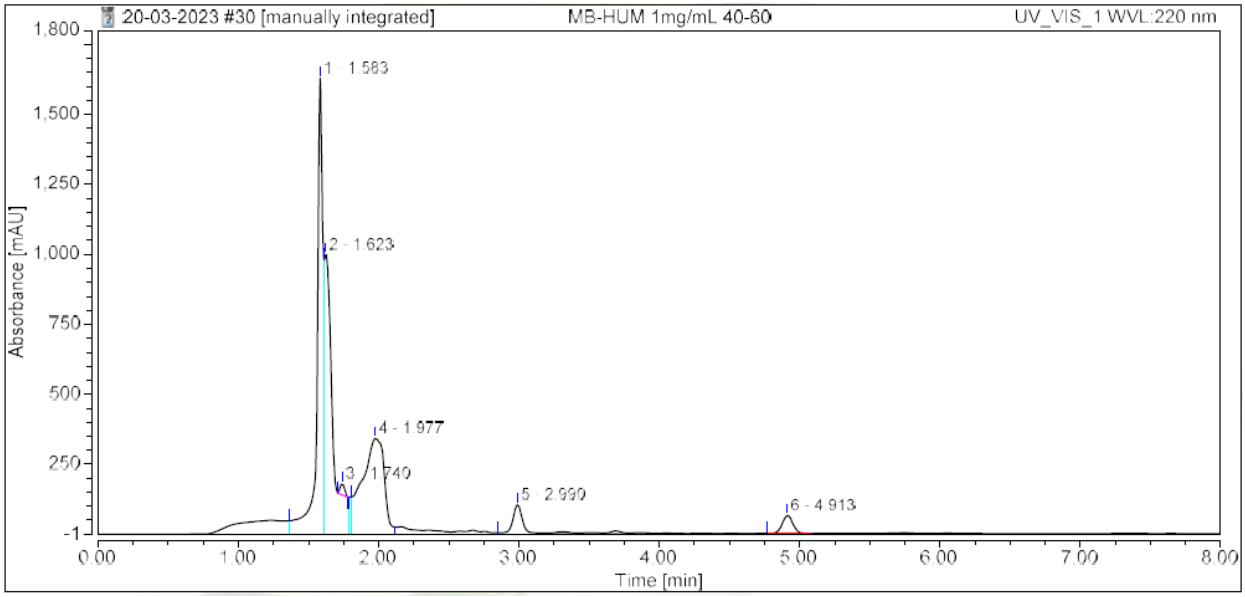


Figure 12. *HPLC Chromatogram graph of macerated extract of Physalis peruviana leaves.*

On the other hand, due to the complications of obtaining the peaks, the roots samples were analyzed at a smaller scale of absorbance; by 20 of mAu for uvilla roots and 25 mAu for llantén roots. As we can see in Figure 13, some representative peaks appear at the injection time of: 1.897, 5.200, 5.500, 6.443, these peaks represent the 44.79%,2.83%, 25.73% and 21.10% of the sample, respectively. Likewise, the peaks observed in llantén roots, sample are at: 1.833, 2.937, 3.517, 5.210, 5.33, 5.45, which depict the12.31%, 10.03%, 16.46%, 8.26%, 14.67%, and 9.20% in that order (Figure 14).

We are using an HPLC of reverse phase, so, the least polar compounds will appear first followed by polar compounds. As we can see, the majority of the peaks appear since minute 4, so we can infer that the compounds found in the samples are polar, some examples of polar secondary metabolites include: phenols, flavonoids, alkaloids and saponins.

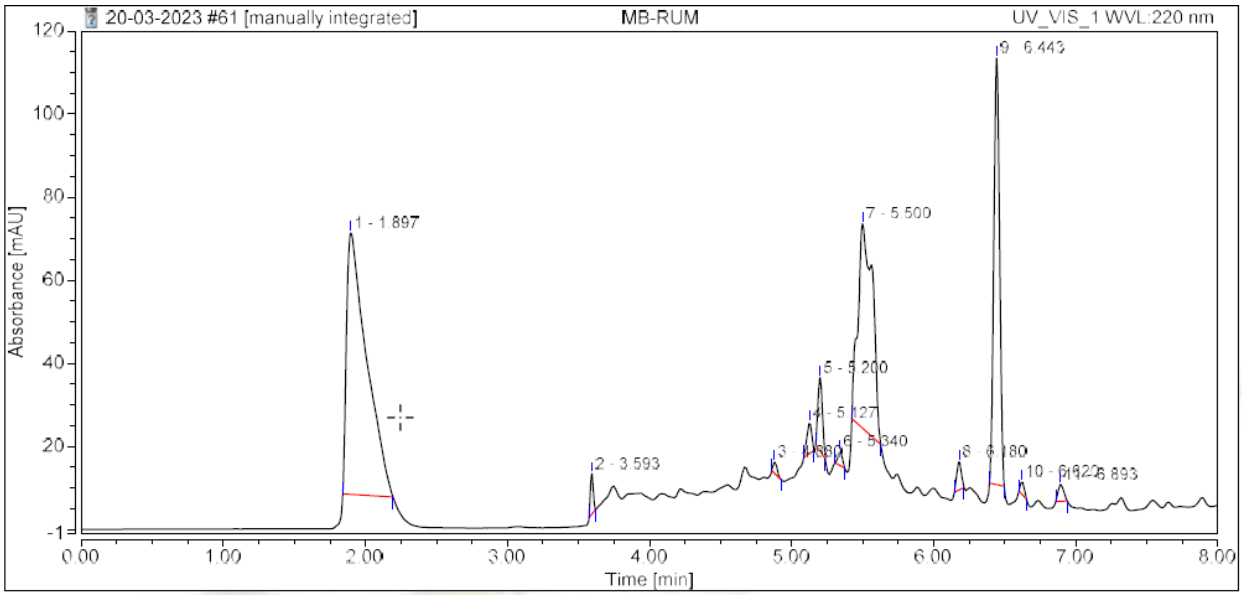


Figure 13. *HPLC Chromatogram graph of macerated extract of Physalis peruviana roots.*

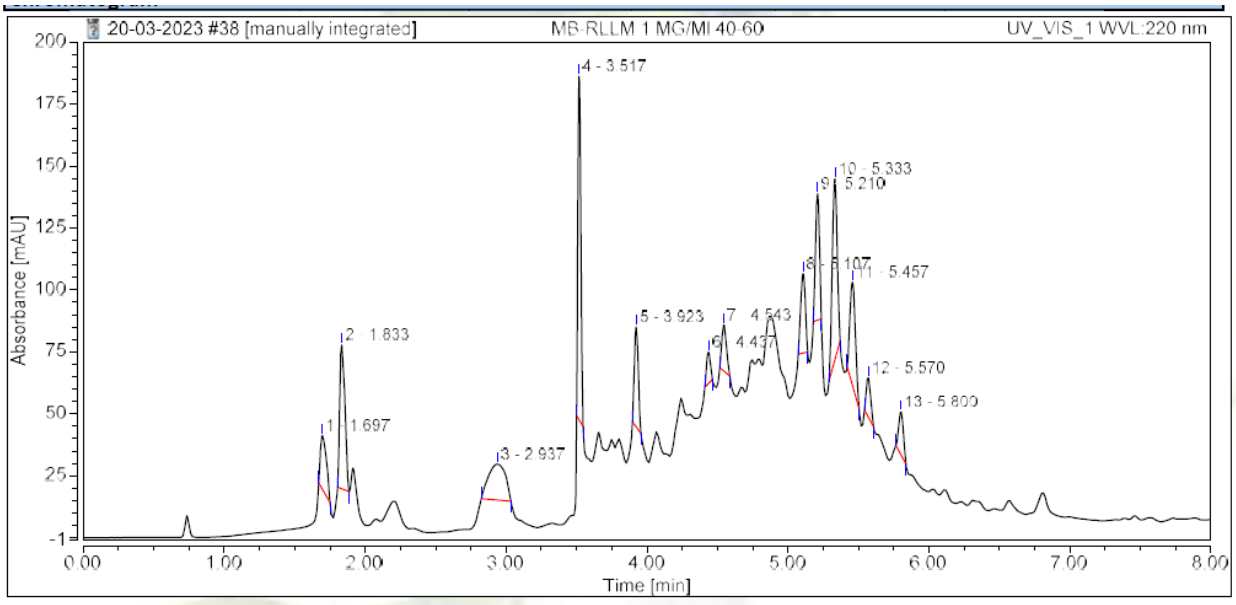


Figure 14. *HPLC Chromatogram graph of macerated extract of Plantago major roots.*

5.5 Indirect ELISA

Conforming to the Mini-Bradford test, we started with a protein concentration of 2.95 mg/ml and 7.6 mg/ml for *Physalis peruviana* and *Plantago major* foliar extracts, respectively. Indirect

ELISA experiments were performed with three concentrations 5 µg/ml, 10 µg/ml, and 20 µg/ml of protein. For each concentration two dilutions of hyperimmune serum obtained from Balb/c mice were tested, 1:100 and 1:200. PBS and normal serum from healthy mice with the same dilutions used for hyperimmune serum were considered as negative control, at a reading time of 5 minutes and an absorbance of 405nm.

The results of humoral response for *Plantago major* and *Physalis peruviana* antigen after 50 min are shown in Figures 15 and 16 respectively. According to the outcomes, there is a clear humoral response activated by the antigens due the bioactive compounds that possess. For example, alkaloids from *Galipea longiflora* have been reported to contribute to the activation of human dendritic cells (Calla-Magariños et al., 2013).

The best concentration for both antigens was 5 µg/ml. In the case of *Physalis peruviana* antigen, the best hyperimmune serum to be used was at a concentration of 1:200 at 40 min However, for *Plantago major* antigen, the best condition was the hyperimmune serum at 1:100 at the same time reading.

Based on these results, we can conclude that there is a clear humoral response against the foliar extracts of both plants. These insights align with the literature that highlight the capability of the family *Solanaceae* and *Plantagiceae* to improve the humoral response (Kumari et al., 2020; Rezaeipoor et al., 2000).

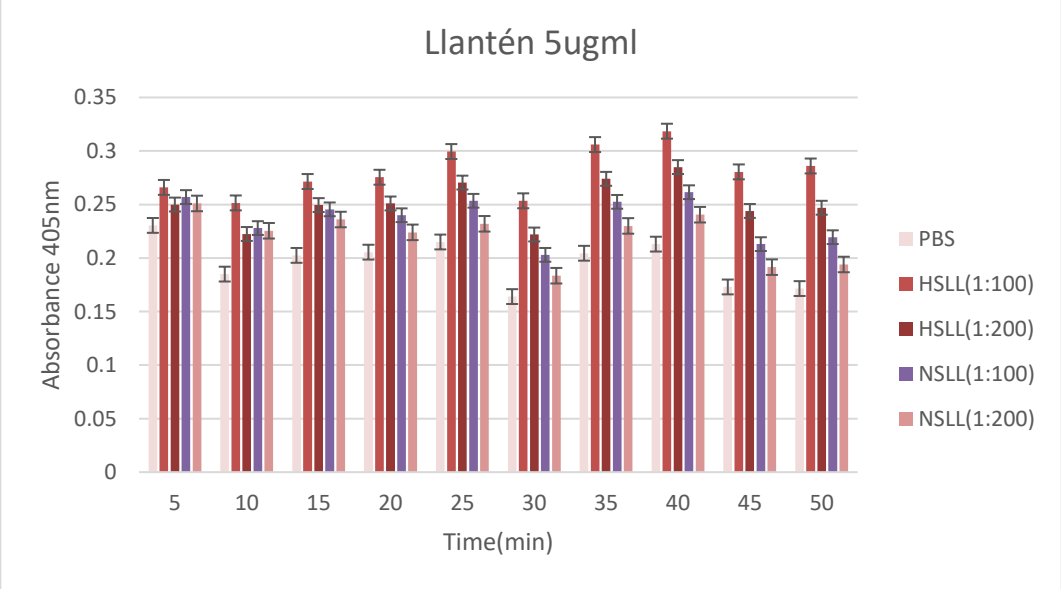


Figure 15. *Results of an indirect ELISA demonstrating antigen-specific antibody interaction using a foliar macerated extract of Plantago major (llantén) at a concentration of 5 µg/ml. The absorbance at 405 nm is represented by the Y-axis, while the X-axis shows the reaction time. The*

standard error is represented by a black line on the bars. Sera hyperimmune (HS) and Normal pre-immune serum (NS).

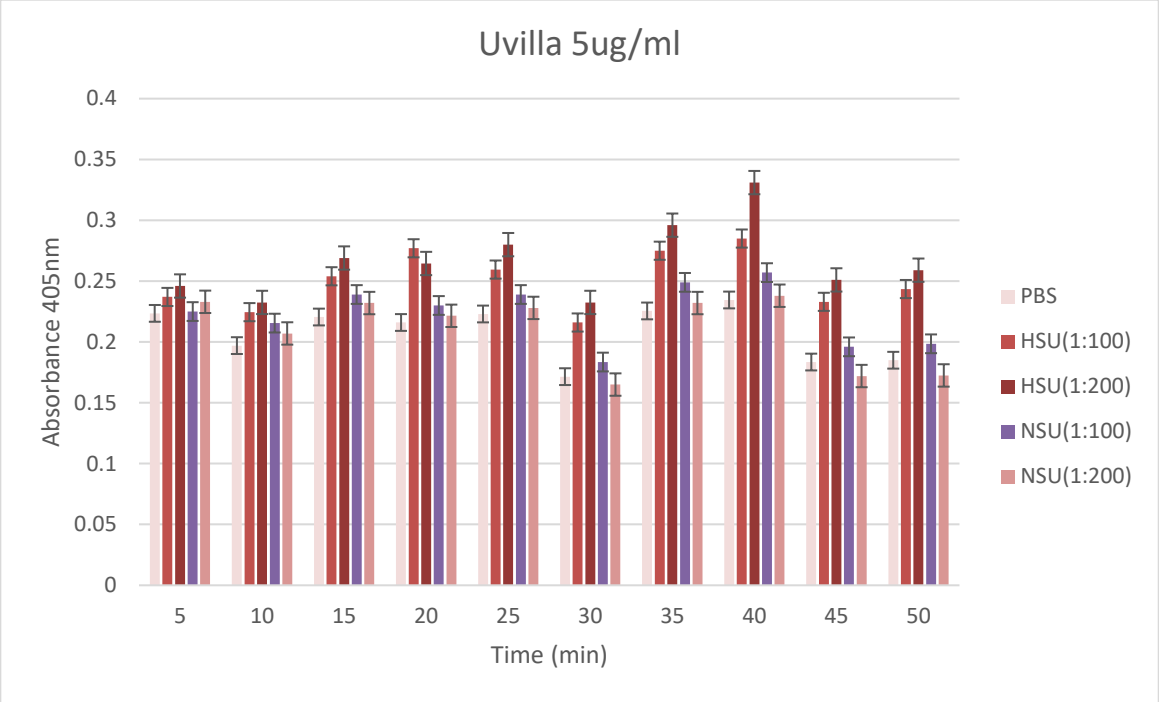


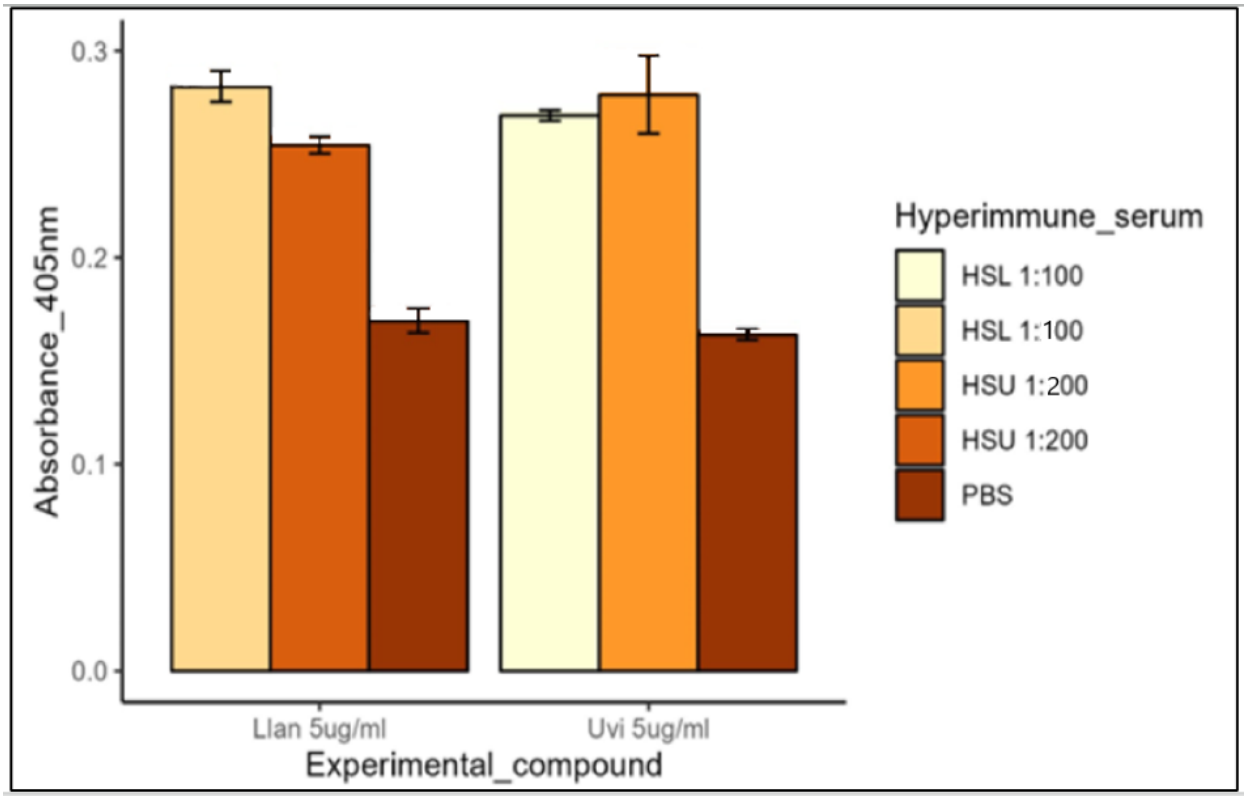
Figure 16. *Results of an indirect ELISA demonstrating antigen-specific antibody interaction using a foliar macerated extract of Physalis peruviana (Uvilla) at a concentration of 5 µg/ml. The absorbance at 405 nm is represented by the Y-axis, while the X-axis shows the reaction time. The standard error is represented by a black line on the bars. Sera hyperimmune (HS) and Normal pre-immune serum (NS).*

5.6 Cross-reactivity using the two foliar extracts from *Physalis peruviana* and *Plantago major*

The assessment of cross-reactivity between the foliar extracts of *Plantago major* and *Physalis peruviana* was conducted using the indirect ELISA serological test.

The normality of data was confirmed using a Shapiro-Wilks test. According to the non-parametric test Krustal Wallis (p-value=0.3 >0.05) there is no significative difference between the antigen used. It was observed the same humoral response for *Plantago major* and *Physalis peruviana*, it is because the secondary metabolites found in each extract are similar. Although the cross-reactivity of these two plants is not reported in literature, several cases of IgE cross-reactivity produced by different plants is reported. For example, exposure to various plant profilins can result in broad cross-reactivity, and binding of monoclonal antibodies to these profilins can cause IgE-cross-reactivity(Terán et al., 2023). Likewise, the benefits of cross-reactivity related to the

pharmacological field can be associated to synthesis of antigen conjugates (Yan et al., 2017).



Figurure 17. *Results of indirect ELISA cross-reactivity assay of Physalis peruviana (uvilla) and Plantago major (llantén). On the X-axis is represented the reaction time vs the absorbance at 405 nm on the Y-axis. The standard error is shown as a black line on the bars.*

5.7 MTT cytotoxicity assay

The cytotoxicity of leaves macerated extracts of *Physalis peruviana* and *Plantago major* was evaluated through Promega colorimetric MTT assay using the SH-SY5Y neuroblastoma cell line. All compounds were assessed at concentrations of 5 µg/ml and 10 µg/ml. Our findings are displayed in Figures 4 and 5. The ideal reading time to evaluate the results was two hours.

To test the normality of data a Shapiro-Wilks test was performed. In the case of the experiment carried out with 5ug/ml, the p-value obtained was 0.001181, thus, we can reject the null hypothesis showing that data do not follow a normal distribution. Due to this fact, the non-parametric Krustal-Wallis test was conducted, obtaining a p-value= 0.36 > 0.05, which means that the samples don't present significative differences. Therefore, we can conclude that both extracts at a concentration of 5ug/ml are not cytotoxic.

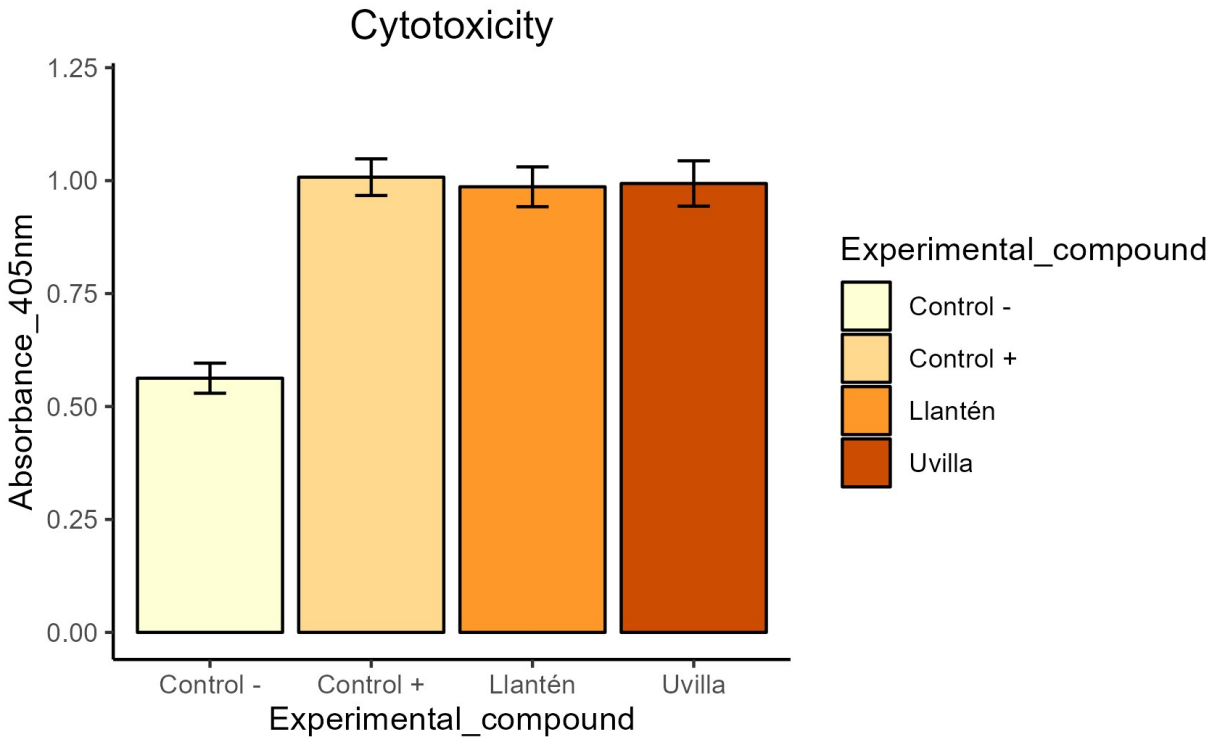


Figure 18. *Cytotoxicity test performed with neuroblastoma cells in a Dulbecco's Modified Eagle's Medium (D-MEM), with a concentration of 5µg/ml of Physalis peruviana (uvilla) and Plantago major (llantén) foliar extracts. Lectures were read every hour and measured with the spectrophotometer at 490nm.*

On the other hand, for the concentration of 10 ug/ml, the results of Shapiro Wilks (p- value =0.01673 < 0.05) showed that data do not follow normal distribution. Besides the Krustal Wallis test, we perform the Wilcoxon_Mann in order to validate the results. The p-value obtained through Krustal Wallis was 0.36 > 0.05, thus, we can infer that there is no significant difference between the positive control and the experiments using the antigens.

According to Wilcoxon-Mann-Whitney, a p-value of 0.07>0.05 was obtained, suggesting that there is not enough significative evidence to reject the null hypothesis. In that way, we can infer the same result. In this case, it cannot be concluded that there is a significant difference between the control and the antigens. Finally, it is possible to deduce that any extract is cytotoxic to a concentration of 10 ug/ml. According to (Whelan & Ryan, 2003) *Plantago major* did not exhibit cytotoxic activity against cell lines from human epidermoid carcinoma of the larynx HEp-2 and human lung carcinoma A549. However, other species like *Plantago arenaria* has the capability to inhibit human tumor cells MCF-7. Furthermore, *Physalis peruviana* reveal cytotoxic activity at a concentration of 25µg/ml against Hepatocellular carcinoma cells (HepG2) and against HT-29,

Hep3B, SaOS-2 and SH-SY5Ycell lines since a concentration of 15 µg/ml (Abou Baker & Rady, 2020; Tuğçe, 2014).

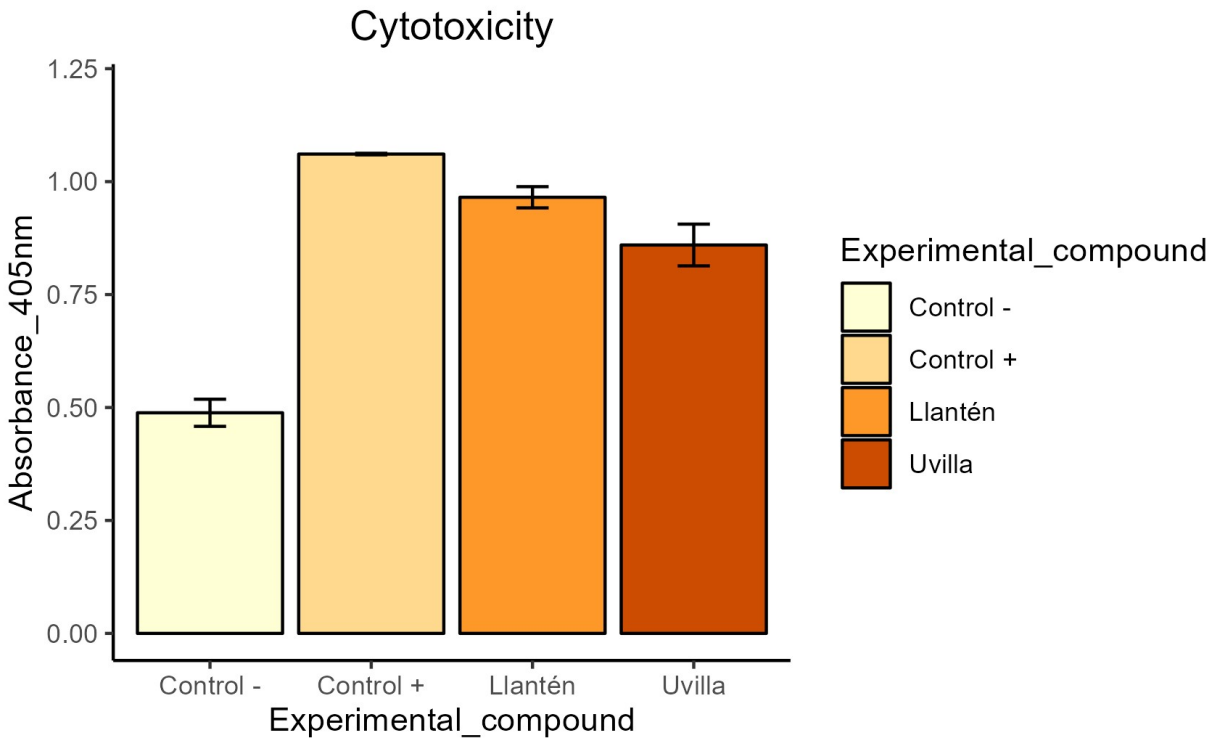


Figure 19*. Cytotoxicity test performed with neuroblastoma cells in a Dulbecco's Modified Eagle's Medium (D-MEM), with a concentration of 10 µg/ml of Physalis peruviana (uvilla) and Plantago major (llantén) foliar extracts. Lectures were read every hour and measured with the spectrophotometer at 490nm.*

5.8 Antimicrobial activity assay.

To evaluate antimicrobial activity a Kirby-Bauer Disk Diffusion Susceptibility Test was performed, at two concentrations of *Plantago major* and *Physalis peruviana* antigens, 1mg/ml and 2mg/ml. Evaluating the extracts against S. *aureus,* it was possible to observe a zone of halo inhibition around the disk with the samples of roots and leaves of uvilla and llantén antigens in the petri dish, so we can confirm the antimicrobial activity of those compounds. On the other hand, for the other bacteria (*E. coli)* it was not possible to detect any inhibition halo, even comparing the antibiotic, we can observe a better antimicrobial activity against *S. aureus* than *E. coli.*

Several studies confirm the antimicrobial activity of *Plantago major*. According to Stanisavljević et al., (2008)macerated extract are able to inhibit bacteria such as: B*acillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and a better antimicrobial activity against yeast such as *Saccharomyces cerevisiae* and *Candida albicans*. Likewise, *Physalis peruviana* extracts were tested to inhibit *E. coli* and *S. aureus* since a concentration of 6.25µg/ml (Njoroge et al., 2023b).

The morphological differences between Gram-positive (*S. aureus)* and Gram-negative (*E. coli*) bacteria may explain why they exhibit varying sensitivity to plant extracts. Gram-negative bacteria possess an outer phospholipidic membrane with lipopolysaccharide components, such as lipid A, and O-antigen making their cell wall resistant to lipophilic solutes. Additionally, porins act as a selective barrier, preventing hydrophilic solutes with a limit of around 600 Da from entering. In contrast, Gram-positive bacteria, with only an outer peptidoglycan layer lacking effective permeability, are expected to be more susceptible (Prajapati et al., 2021).

Furthermore, it is important to mention that the strain of *E. coli* used to this research was isolated from a clinical patient, what could have contributed to being more resistant. The diameter of the inhibition halo of each sample is presented in Table 3.

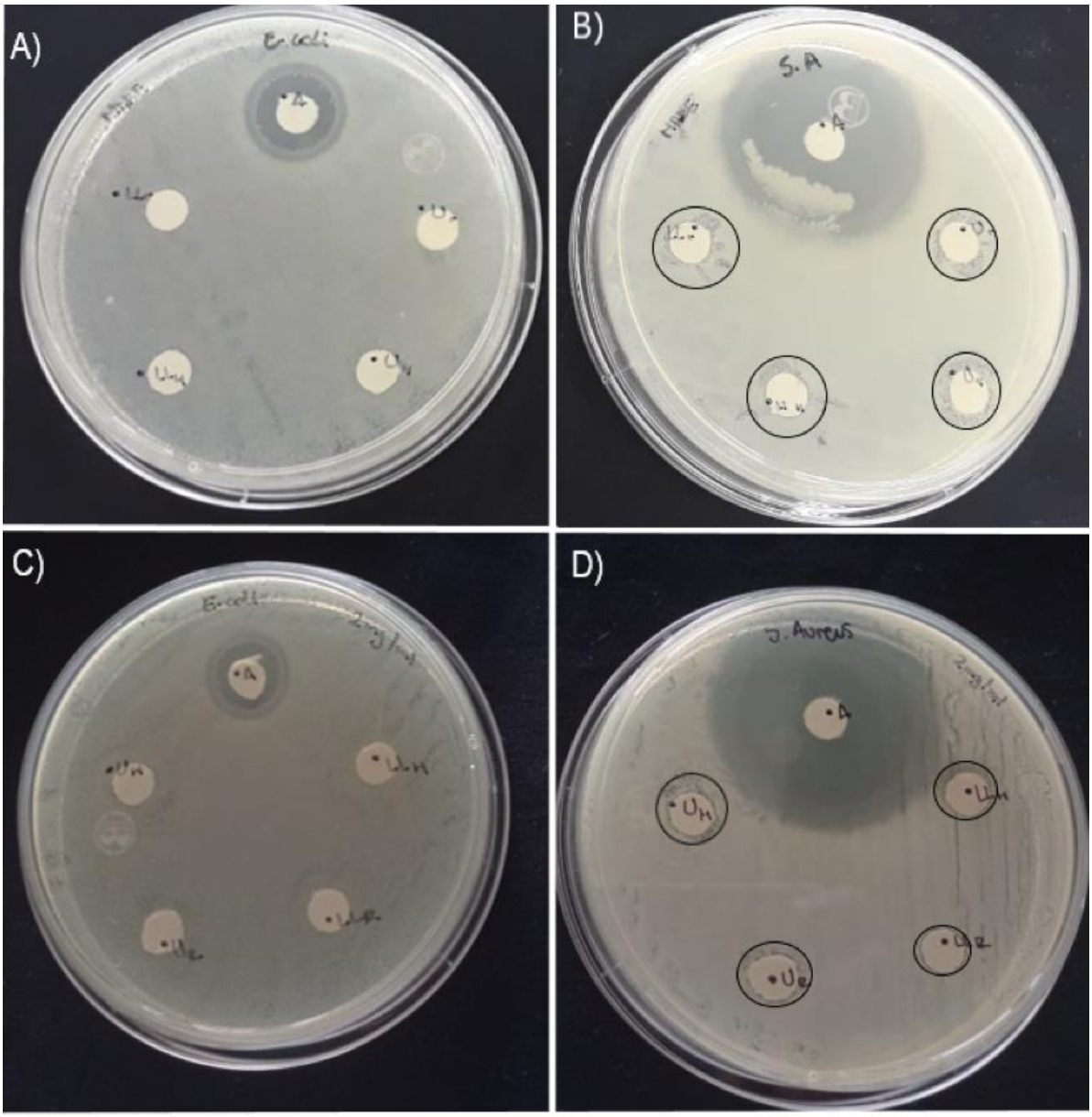


Figure 20. *Antimicrobial activity results obtained with leaves and roots samples of Physalis Peruviana and Plantago major. A) Evaluation of inhibition of the samples at the concentration of 1mg/ml against E. coli. B) Evaluation of inhibition of the samples at the concentration of 1mg/ml against S. aureus. C) Evaluation of inhibition of foliar and roots samples at the concentration of 2 mg/ml against E. coli. D) Evaluation of inhibition of foliar and roots samples at the concentration of 2 mg/ml against S. aureus.* LL_H*= llantén leaves ,* LL_R*= llantén roots,* U_H*= uvilla leaves,* U_R*=uvilla roots.*

Table 3*. Resulting inhibition halo of leaves and roots extracts against E. coli and S. aureus bacteria.*

Strain	Experimental compound							
	Concentration: 1mg/ml				Concentration: 2mg/ml			
	UE leaves	UE roots	LLE leaves	LLE roots	UE leaves	UE roots	LLE leaves	LLE roots
E. coli ATCC 25922	-	-	-	-	-	-	-	-
S. aureus UITEY-Sa	11,36 mm	9,66mm	10,21 mm	10,52 mm	10,55 mm	8,75 mm	8,04 mm	6,88 mm

6. CONCLUSIONS

To conclude, the qualitative phytochemical analysis of ethanolic macerated extracts from *Plantago major* and *Physalis peruviana* revealed a higher detection of secondary metabolites compared to the ethanolic extracts obtained through the soxhlet method. However, according to the FTIR and HPLC spectra, it was found that there are no significant differences between macerated and Soxhlet extracts.

Plantago major and *Physalis peruviana* presented almost the same bioactive compounds according the qualitative phytochemical analysis, such as: flavonoids, phenols, saponins, carbohydrates, and cardiac glycosides. Likewise, *Physalis peruviana* extracts contained proteins, steroids, terpenoids and alkaloids. Another important insight observed is that leaves presented more bioactive compounds than roots.

The results from the indirect ELISA test demonstrated that the macerated extracts of *Plantago major* and *Physalis peruviana* elicited antigenicity by stimulating an antibody response in the immune system of BALB/c mice. Furthermore, cross-reactivity assessment confirmed antibody recognition between uvilla and llantén, possibly due to chemical structure similarities as indicated by FT-IR analysis and qualitative phytochemical analysis.

According to the MTT technique, the macerated ethanolic foliar extracts of *Plantago major* and *Physalis peruviana* showed to be not cytotoxicity at concentrations of 5µg/ml and 10µg/ml, tested on the SH-SY5Y cell line.

None of the evaluated extracts exhibited inhibitory activity against the Gram-negative bacteria *E. coli* ATCC 25922 at the concentration of 1mg/ml and 2mg/ml. However, leaves and roots extracts of *Plantago major* and *Physalis peruviana* demonstrated antimicrobial activity against the Gram-positive bacteria *S. aureus* UITEY-Sa at the concentrations mentioned.

Future research on *Physalis peruviana a*nd *Plantago major* should prioritize analyzing the cross-activity of both leaf extracts. This will enable the determination of potential synergistic or antagonistic effect. Likewise, more research in needed to study the metabolites separately, to perform a test more specific, including also bioinformatic tools.

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8. ANNEXES

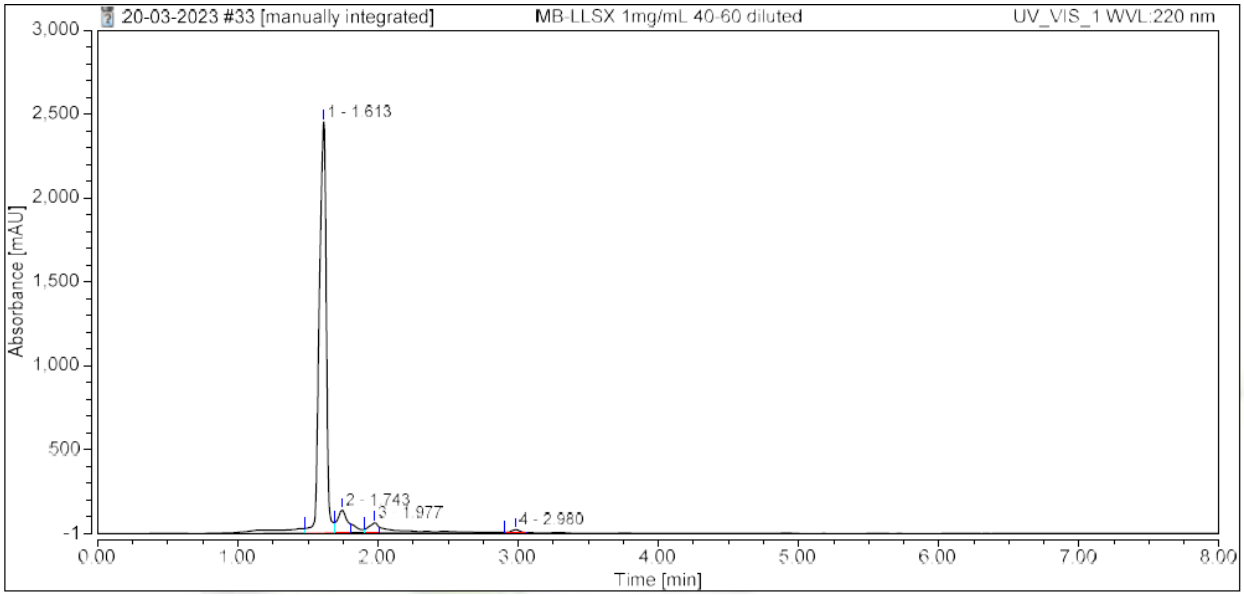

Figure 21. *HPLC Chromatogram graph of Soxhlet of Plantago major leaves.*

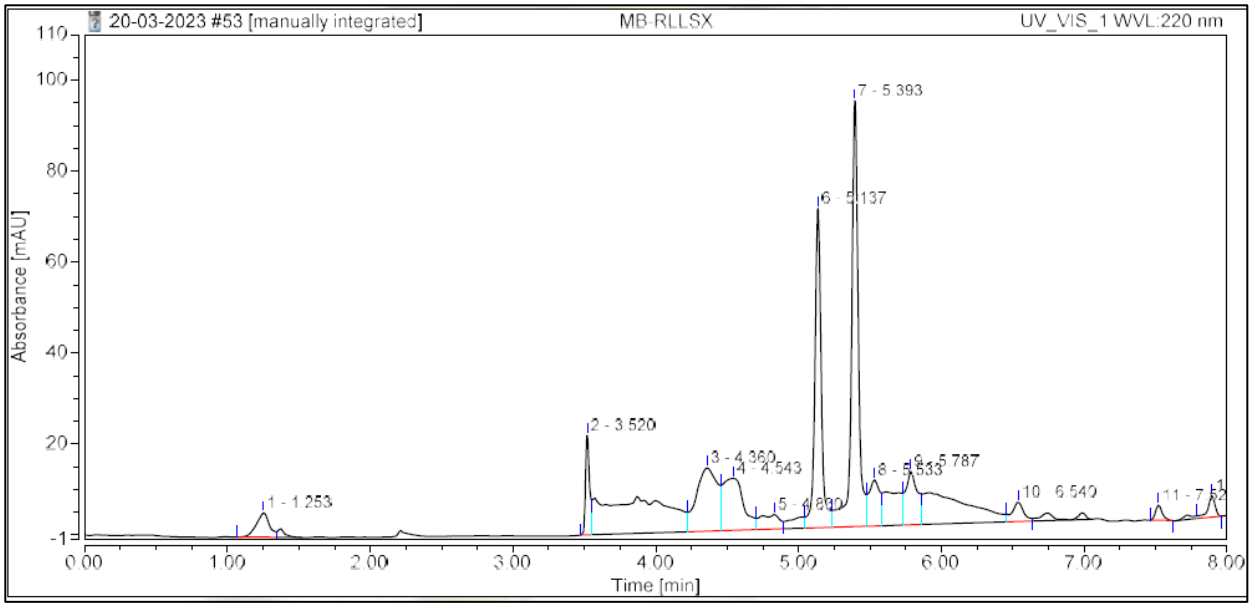

Figure 22. *HPLC Chromatogram graph of Soxhlet of Plantago major roots.*

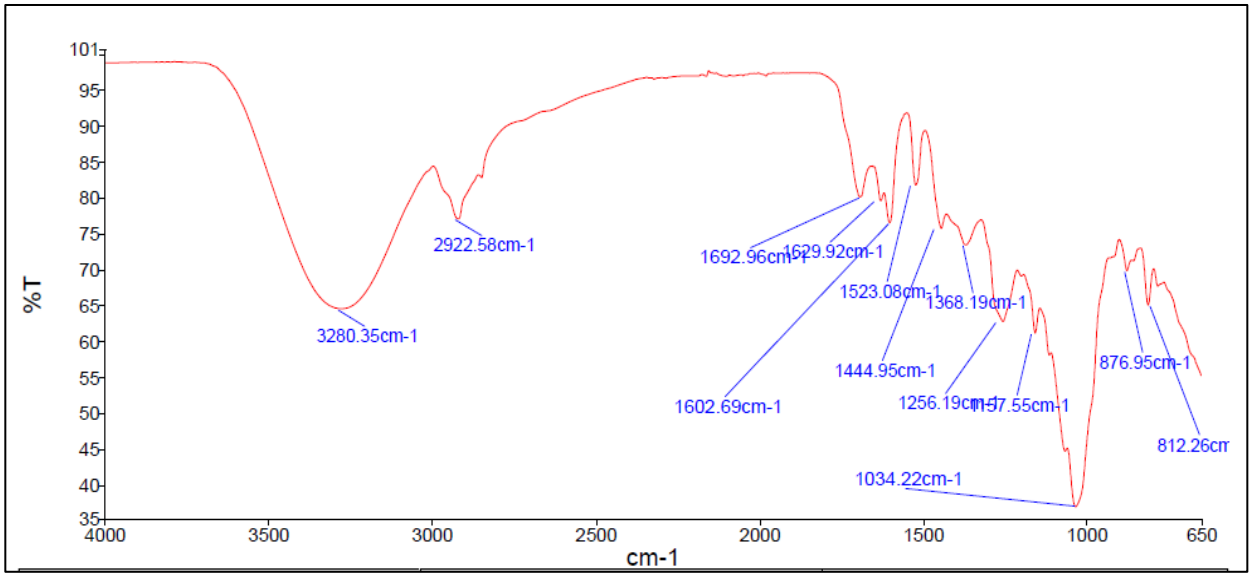

Figure 23. *FTIR spectra of ethanolic Soxhlet extract of Plantago major leaves.*

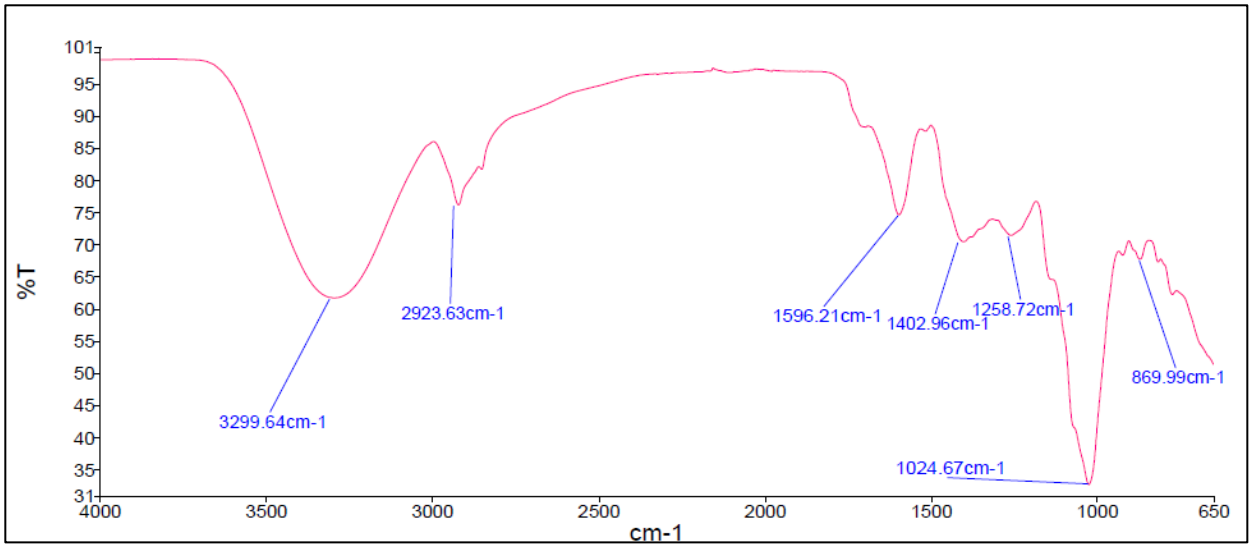

Figure 24. *FTIR spectra of ethanolic Soxhlet extract of Plantago major roots.*

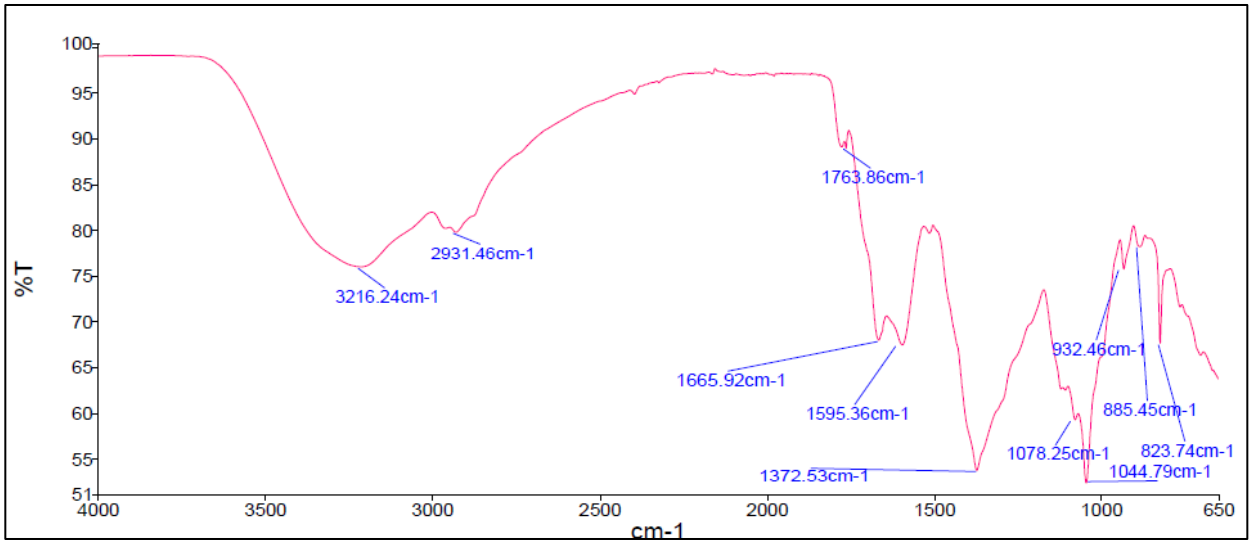

Figure 25. *FTIR spectra of ethanolic Soxhlet extract of Physalis peruviana leaves.*

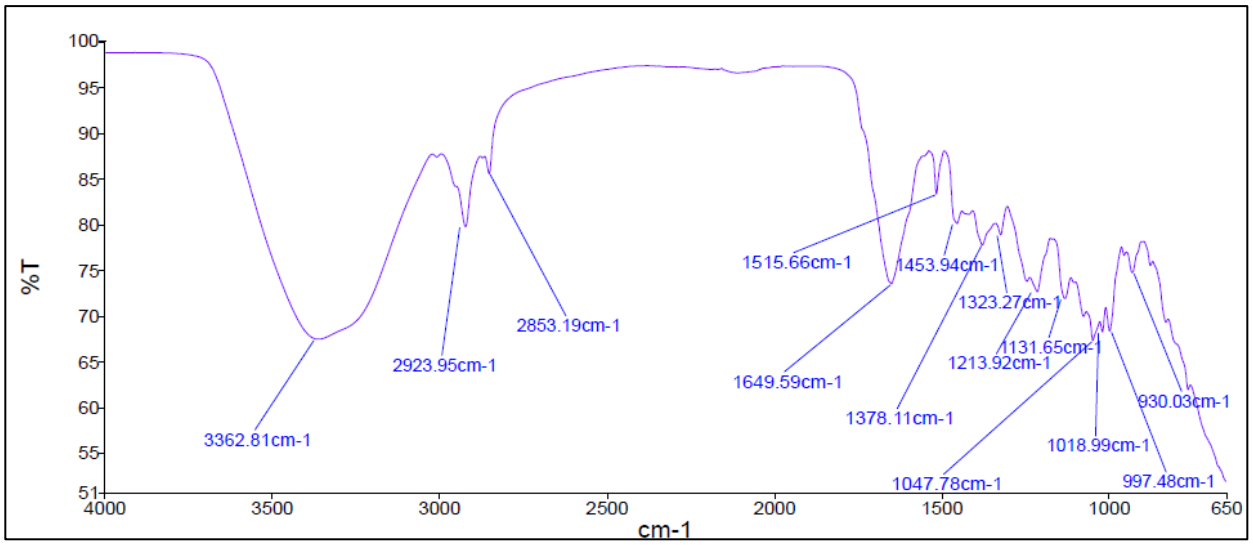

Figure 26. *FTIR spectra of ethanolic Soxhlet extract of Physalis peruviana roots.*

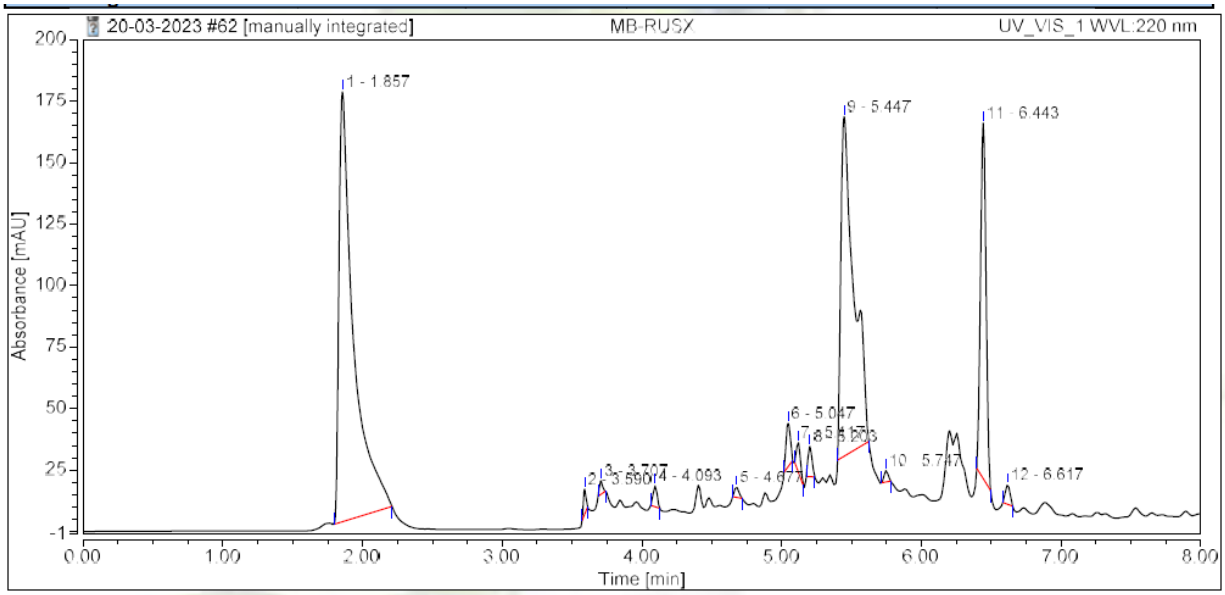

Figure 27. *HPLC Chromatogram graph of Soxhlet of Physalis peruviana leaves.*

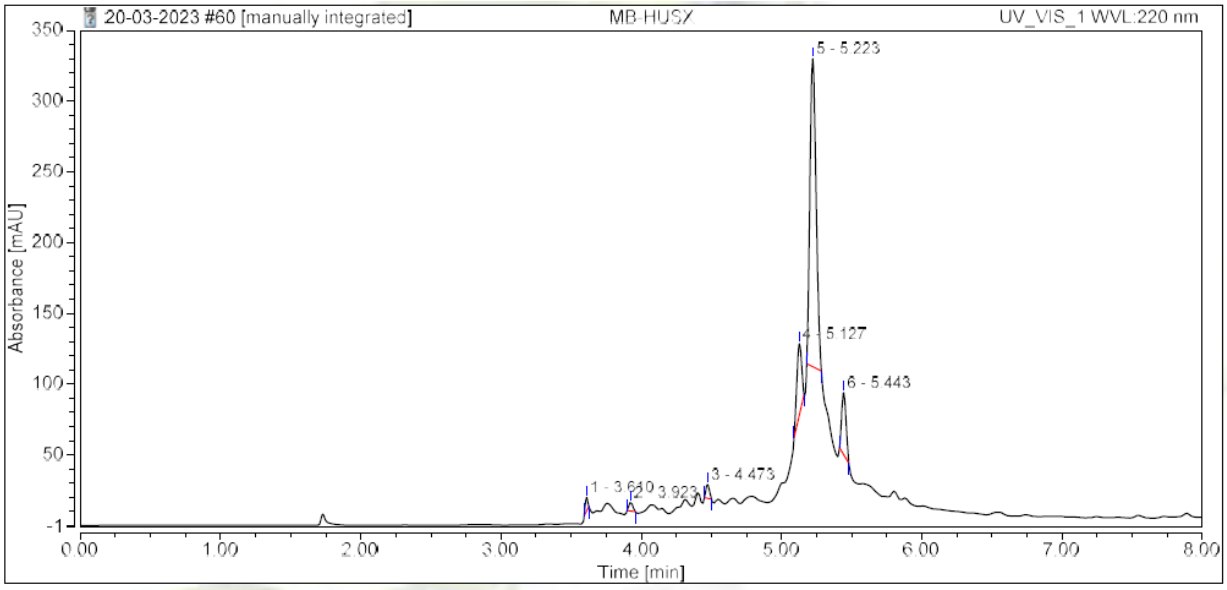

Figure 28. *HPLC Chromatogram graph of Soxhlet of Physalis peruviana roots.*