UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Determination of the anti-inflammatory activity of Ecuador´s natural products

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

Autor:

Dario Félix Castro Izurieta

Tutor:

PhD, Santiago Vispo Nelson

Urcuquí, octubre 2021.

Urcuquí, 19 de enero de 2022

SECRETARÍA GENERAL
(Vicerrectorado Académico/Cancillería)
ESCUELA DE CIENCIAS BIOLÓGICAS E INGENIERÍA
CARRERA DE BIOLOGÍA
ACTA DE DEFENSA No. UITEY-BIO-2022-00006-AD

A los 19 días del mes de enero de 2022, a las 10:00 horas, de manera virtual mediante videoconferencia, y ante el Tribunal Calificador, integrado por los docentes:

Presidente Tribunal de Defensa	Mgs. VILLALBA MENESES, GANDHI FERNANDO
Miembro No Tutor	Dr. GUDIÑO GOMEZJURADO, MARCO ESTEBAN , Ph.D.
Tutor	Dr. SANTIAGO VISPO, NELSON FRANCISCO , Ph.D.

El(la) señor(ita) estudiante **CASTRO IZURIETA, DARIO FELIX**, con cédula de identidad No. **1714680723**, de la **ESCUELA DE CIENCIAS BIOLÓGICAS E INGENIERÍA**, de la Carrera de **BIOLOGÍA**, aprobada por el Consejo de Educación Superior (CES), mediante Resolución **RPC-SO-37-No.438-2014**, realiza a través de videoconferencia, la sustentación de su trabajo de titulación denominado: **DETERMINATION OF ANTIINFLAMATORY ACTIVITY OF ECUADOR´S NATURAL PRODUCTS.**, previa a la obtención del título de **BIÓLOGO/A**.

El citado trabajo de titulación, fue debidamente aprobado por el(los) docente(s):

Tutor	Dr. SANTIAGO VISPO, NELSON FRANCISCO , Ph.D.

Y recibió las observaciones de los otros miembros del Tribunal Calificador, las mismas que han sido incorporadas por el(la) estudiante.

Previamente cumplidos los requisitos legales y reglamentarios, el trabajo de titulación fue sustentado por el(la) estudiante y examinado por los miembros del Tribunal Calificador. Escuchada la sustentación del trabajo de titulación a través de videoconferencia, que integró la exposición de el(la) estudiante sobre el contenido de la misma y las preguntas formuladas por los miembros del Tribunal, se califica la sustentación del trabajo de titulación con las siguientes calificaciones:

Tipo	Docente	Calificación
Tutor	Dr. SANTIAGO VISPO, NELSON FRANCISCO , Ph.D.	10,0
Miembro Tribunal De Defensa	Dr. GUDIÑO GOMEZJURADO, MARCO ESTEBAN , Ph.D.	10,0
Presidente Tribunal De Defensa	Mgs. VILLALBA MENESES, GANDHI FERNANDO	10,0

Lo que da un promedio de: **10 (Diez punto Cero)**, sobre 10 (diez), equivalente a: **APROBADO**

Para constancia de lo actuado, firman los miembros del Tribunal Calificador, el/la estudiante y el/la secretario ad-hoc.

Certifico que *en cumplimiento del Decreto Ejecutivo 1017 de 16 de marzo de 2020, la defensa de trabajo de titulación (o examen de grado modalidad teórico práctica) se realizó vía virtual, por lo que las firmas de los miembros del Tribunal de Defensa de Grado, constan en forma digital.*

CASTRO IZURIETA, DARIO FELIX
Estudiante

Firmado electrónicamente por:
GANDHI FERNANDO
VILLALBA MENESES

Mgs. VILLALBA MENESES, GANDHI FERNANDO
Presidente Tribunal de Defensa

Firmado electrónicamente por:
NELSON
FRANCISCO
SANTIAGO VISPO

Dr. SANTIAGO VISPO, NELSON FRANCISCO , Ph.D.
Tutor

UNIVERSIDAD YACHAY TECH

MARCO ESTEBAN GUDINO GOMEZJURADO

Firmado digitalmente por MARCO ESTEBAN GUDINO GOMEZJURADO
Fecha: 2022.01.19 13:58:18 -05'00'

Dr. GUDIÑO GOMEZJURADO, MARCO ESTEBAN , Ph.D.
Miembro No Tutor

Firmado electrónicamente por:
KARLA ESTEFANIA ALARCON FELIX

ALARCON FELIX, KARLA ESTEFANIA
Secretario Ad-hoc

AUTORÍA

Yo, **Darío Félix Castro Izurieta**, con cédula de identidad 1714680723, declaro que las ideas, juicios, valoraciones, interpretaciones, consultas bibliográficas, definiciones y conceptualizaciones expuestas en el presente trabajo; así cómo, los procedimientos y herramientas utilizadas en la investigación, son de absoluta responsabilidad de el/la autora (a) del trabajo de integración curricular. Así mismo, me acojo a los reglamentos internos de la Universidad de Investigación de Tecnología Experimental Yachay.

Urcuquí, octubre 2021

Firmado electrónicamente por:
DARIO FELIX
CASTRO
IZURIETA

Dario Félix Castro Izurieta

C.I. 1714680723

AUTORIZACIÓN DE PUBLICACIÓN

Yo, Darío Félix Castro Izurieta, con cédula de identidad 1714680723, cedo a la Universidad de Tecnología Experimental Yachay, los derechos de publicación de la presente obra, sin que deba haber un reconocimiento económico por este concepto. Declaro además que el texto del presente trabajo de titulación no podrá ser cedido a ninguna empresa editorial para su publicación u otros fines, sin contar previamente con la autorización escrita de la Universidad.

Asimismo, autorizo a la Universidad que realice la digitalización y publicación de este trabajo de integración curricular en el repositorio virtual, de conformidad a lo dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior.

Urcuquí, octubre 2021.

Firmado electrónicamente por:
DARIO FELIX
CASTRO
IZURIETA

Darío Félix Castro Izurieta

C.I. 1714680723

Agradecimientos

Quiero extender mis agradecimientos a:

Nelson Santiago Vispo PhD quien a más de ser mi tutor y docente se convirtió en un amigo y modelo a seguir tanto dentro como fuera de las aulas.

Hortensia Rodriguez PhD quien siempre me ha bridando todo su apoyo y siempre ha estado presta ayudarme en los momentos más críticos en la realización de este trabajo de titulación.

Lola De Lima Msc por todo el apoyo y guía técnica la cual fue clave en este trabajo.

Todas las técnicas de laboratorio de la Escuela de Ciencias Biológicas e Ingeniería, por su aliento y apoyo durante los largos meses de trabajo incansable en el laboratorio.

A todos los docentes que conforman la Escuela de Ciencias Biológicas e Ingeniería por su dedicación y aporte en conocimiento que me ha permitido llegar hasta este punto en mi vida académica y personal

A todos aquellos que forman parte de la Universidad Yachay Tech, por el incansable trabajo que ha supuesto el levantar a una universidad como la nuestra y convertirla en un pilar de investigación en nuestro país.

Darío Castro I.

Resumen

El proceso de inflamación es una respuesta natural inmunitaria ante estímulos dañinos del exterior donde se genera una cascada de señalizaciones liberando sustancias proinflamatorias como citoquinas con la finalidad de activar más células inmunitarias para contrarrestar y eliminar el estímulo causante de la inflamación. El problema con este proceso es cuando se liberan en exceso estas sustancias generando malestar con síntomas como enrojecimiento, dolor e incluso fiebre. Es por esto que la comunidad científica en la actualidad se esfuerza en desarrollar nuevas soluciones para disminuir o desaparecer la inflamación, la cual es recurrente en ciertas patologías como la artritis. Para poder evaluar en el laboratorio la actividad antiinflamatoria de nuevas moléculas ya sean sintéticas o naturales se han desarrollado protocolos estandarizados *in vitro* basados en la estabilización de la membrana del eritrocito, la cual es similar a la membrana del lisosoma que en procesos inflamatorios se rompe liberando su contenido y contribuyendo a la inflamación. Actualmente se está tratando de comprobar el potencial antiinflamatorio de plantas conocidas siendo estas alternativas que contribuyan a disminuir o eliminar la inflamación. Ecuador al ser un país mega diverso posee especies de plantas con un gran potencial medicinal y antiinflamatorio, es por ello que se analizó *in vitro* el potencial antiinflamatorio de plantas existentes en el Ecuador obteniendo resultados positivos y alentadores.

Palabras claves: Inflamación, antiinflamatorio, estabilización de membrana, natural.

Abstract

The inflammation process is a natural immune response to harmful stimuli from the outside. Therefore, a signaling cascade is generated, releasing pro-inflammatory substances such as cytokines to activate more immune cells to counteract and eliminate the stimulus causing the inflammation. The problem with this process is when these substances are released in excess, generating discomfort with symptoms such as redness, pain, and even fever. The scientific community is currently developing new solutions to reduce or eliminate inflammation, which is recurrent in certain pathologies such as arthritis. To evaluate the anti-inflammatory activity of new synthetic or natural molecules in the laboratory, standardized in vitro protocols have been developed based on the stabilization of the erythrocyte membrane, which is similar to the lysosome membrane that is in inflammatory processes ruptures, releasing its contents and contributing to inflammation. Currently, researchers are trying to prove the anti-inflammatory potential of known plants as these alternatives that reduce or eliminate inflammation. As a mega-diverse country, Ecuador has plant species with great medicinal and anti-inflammatory potential; that is why the anti-inflammatory potential of existing plants in Ecuador was analyzed in vitro, obtaining positive and encouraging results.

Keywords: Inflammation, anti-inflammatory, membrane stabilization, natural.

Index

1. Title 1

2. Introduction 1

2.1 Inflammation process 2

2.2 Anti-inflammatory essays for new molecules 3

2.2.1 Erythrocyte Membrane stabilization 3

2.2.2 Paw edema 5

2.3 Plants as anti-inflammatory drugs 6

2.4 Extraction methods 7

3. Problem statement 8

4. Objectives 9

4.1 General objective 9

4.2 Specific objectives 9

5. Methodology 9

5.1 Extraction process 9

5.2 Preparation of the extracts 9

5.3 Blood extraction. 9

5.4 Anti-inflammatory activity by heat 10

5.5 Anti-inflammatory activity by hypotonicity 10

6. Results and discussion 11

6.1 Comparison between heat and hypotonicity. 11

6.2 Comparison between aspirin and diclofenac (hypotonicity protocol) 12

6.3 Comparison between plant extract and positive controls. 13

7. Conclusions and Recommendations 15

7.1 Conclusions 15

7.2 Recommendations. 16

References 17

Annex 22

List of figures

Figure 1. Heat-induced hemolysis. 4
Figure 2 Hypotonic induced hemolysis. 5
Figure 3 Paw edema by carrageenan.. 6

List of tables

Table 1 Some examples of plants species with chemical compounds that have anti-inflammatory activity 7
Table 2 Most common secondary metabolites in plants with an essential and relevant biological activity 7
Table 3 Solvents used for separation of different secondary metabolites 8

1. Title

Determination of the anti-inflammatory activity of Ecuador´s natural products

2. Introduction

Inflammation is a defense mechanism of the organism against dangerous stimuli such as pathogens, cell damage, and toxic elements. The immune system mediates this mechanism and cells not belonging to it to eliminate the stimulus that causes damage and begin the process of recovery and repair[1, 2, 3].

Even though inflammation is a natural process that causes redness, pain, and swelling, the damage is generated in tissues and can lead to the loss of its function; in addition, there are changes in the tissue's microcirculation due to a change in the permeability of the blood vessels. That is why decreasing the effects of this process helps to recover tissue homeostasis and prevent, in some cases that inflammation from becoming a chronic disease[1, 4].

In response to this problem, drugs with anti-inflammatory capabilities have been developed whose origin dates back to the ancient cultures of China, India, among others, which through the use of plants such as the different species of *Salix*, treated pain and inflammation[5].

In our current century, interest in pharmacological products of plant origin has increased. The use of medicinal plants is considered the future for health care management[6]. According to the World Health Organization (WHO), it is estimated that 80% of the world's population sees traditional medicine as the first choice for their health care[7].

Additionally, this international organization encourages developing countries to use non-toxic plants to support traditional medicine[8]. Because according to their data, about 25% of modern medicines come from medicinal plants, and many other drugs are synthetic analogs of compounds isolated from plants[6].

This new interest in medicinal plants is considered the "Back to Natural." These plants beneficial to humans have been employed for millennia to prevent disease and cure

ailments by ancestral peoples[6]. In Latin America, these practices are a standard in indigenous groups which transmit this knowledge through different generations[9, 10].

There is a great diversity in the flora of Ecuador being quite recognized and studied, having up to 17 000 species of plants indexed in the country. Being part of the top ten most diverse countries worldwide, this country has great potential in various useful plants, including medicinal ones, documented more than 3000 species used to treat different diseases[11]. In Ecuador, it is estimated that 80% of the population uses and depends on herbal medicine as the primary mechanism to treat different diseases[12, 13].

2.1 Inflammation process

The inflammation process is due to regulatory pathways in cells at the tissue level and in inflammatory cells in the blood. Inflammation is a common symptom in chronic diseases such as cardiovascular problems, diabetes, arthritis, and cancer. This inflammatory response depends on the stimulus received and where it was received[1].

Immune cells such as neutrophils, eosinophils, and macrophages are activated and travel to the damaged area with the mission of suppressing the stimulus causing the inflammation, however in the process, pro-inflammatory molecules such as cytokines are released: tumor necrosis factor (TNF) and interleukin-1β which activate more neutrophils and macrophages[14, 15, 16].

When cytokines are released in large quantities into the bloodstream due to the inflammatory process, they can generate an endocrine effect and induce specific physiological responses such as platelet activation, fever, fatigue, among others. Another function of cytokines is to facilitate the entry of immune cells by activating endothelial cells; however, this can also generate adverse effects such as vasodilatation, hypotension. In addition, the activated immune cells in the bloodstream and the release of cytokines in the body also start the production of prostaglandins molecules that play an essential role in inflammation and are responsible for some of the symptoms that this process entails[17].

Prostaglandins are generated by a family of enzymes called Cyclooxygenases (COX), having two main isoforms, which are COX-1 and COX-2, being this one quite studied nowadays[18, 19]. Knowing the metabolic pathway that synthesizes an influential group of

molecules responsible for inflammation and subsequent symptoms have allowed us to develop drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the COX enzyme, relieving symptoms and contributing to worsening the inflammatory episode[20, 21].

2.2 Anti-inflammatory essays for new molecules

2.2.1 Erythrocyte Membrane stabilization

The stabilization of the erythrocyte membrane is based on the similarity with the lysosome membrane. Therefore, there is cellular damage in inflammation whereby lysosomes rupture and spill their contents, promoting cellular damage and inflammation[19, 22].

For testing the anti-inflammatory potential of different natural or synthetic compounds, erythrocytes exposed to situations that promote their lysis, either a hypotonic medium or heat, are used; these models have been widely used in various preliminary studies[23].

Inducing hemolysis by heat (Figure 1), the samples will be incubated at temperatures ranging from 60 °C to 54 °C for half an hour; this is how the erythrocytes will be broken, releasing hemoglobin which spectrophotometric techniques will measure.

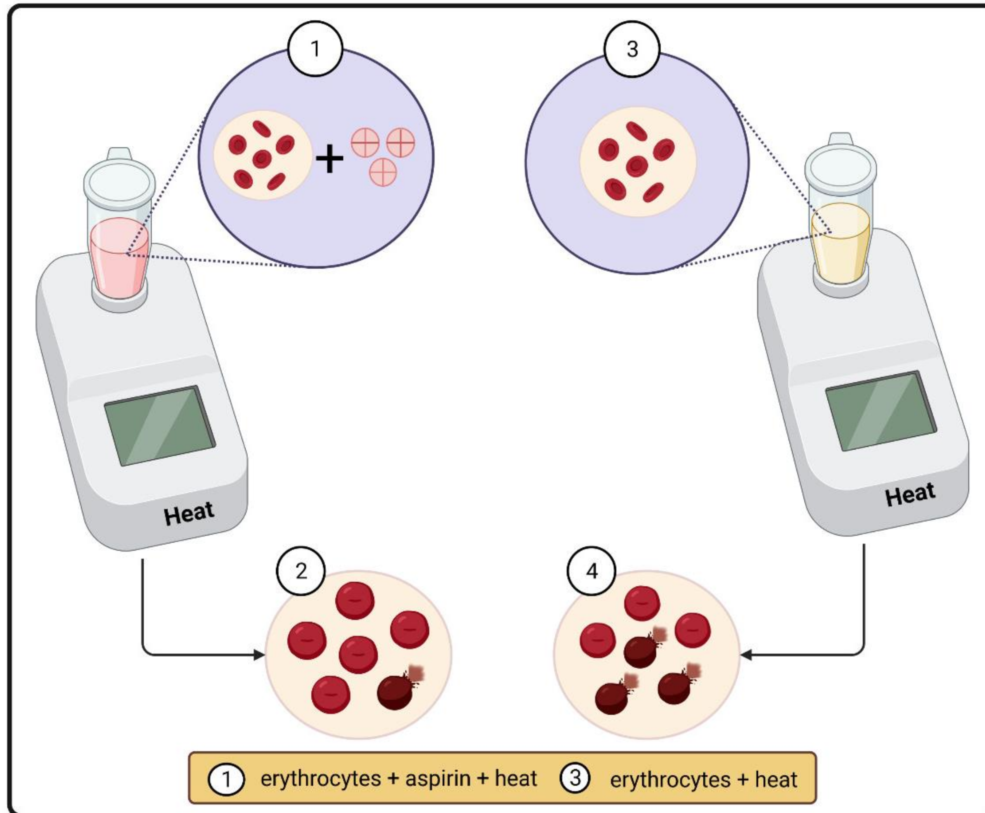


Figure 1. Heat-induced hemolysis. 1.- Erythrocyte suspension with anti-inflammatory or the substance to be tested in the heating plate, 2.- The erythrocytes are hemolyzed releasing hemoglobin, having the anti-inflammatory inhibits hemolysis and maintains the integrity of most of the erythrocytes. 3.- The erythrocyte solution is heated in the plate. 4.- Since there is no compound that maintains the stability of the membrane, the erythrocytes are hemolyzed.

In the case of a hypotonic medium (Figure 2), it is possible to use different solutions such as hypo-saline solution or distilled water, which enter into reaction with the erythrocytes producing their lysis, releasing the hemoglobin and being able to quantify it by spectroscopy[24].

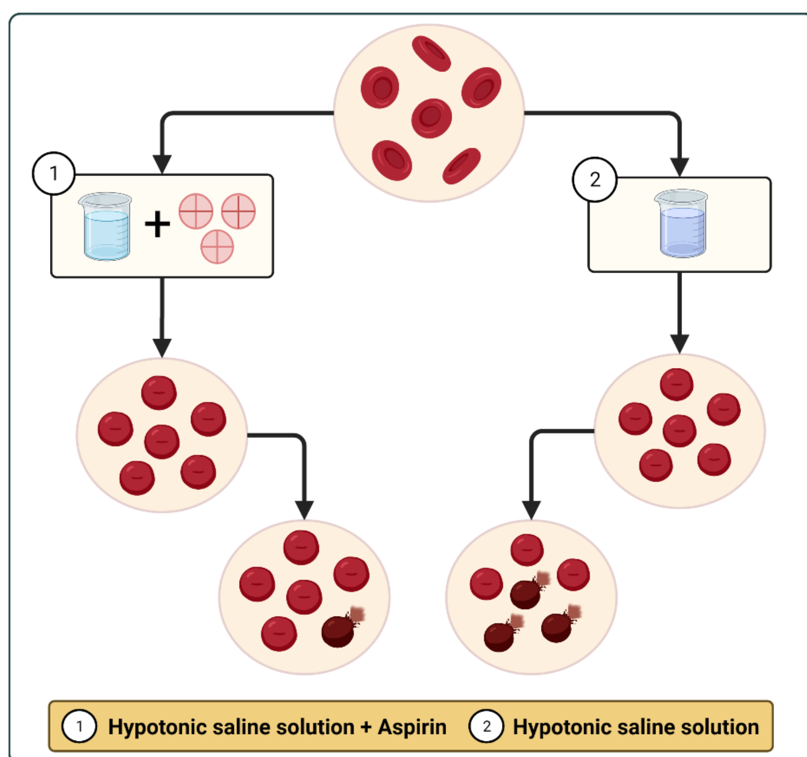


Figure 2 Hypotonic induced hemolysis. In this method, we have 2 ways of treatment for the erythrocytes, 1.- The red blood cells are exposed to a Hypotonic media with an anti-inflammatory or the compound to be tested, swell due to the action of the hypotonic medium until they are lysed; however, due to the action of the anti-inflammatory, the percentage of hemolysis is low because of the stability that this provides to the membrane. Erythrocytes are exposed to a hypotonic solution without any other compound, so they swell until their lysis, having a significant number of broken erythrocytes in comparison to the medium with anti-inflammatory; this is due to the fact that there is no stabilizer in the membrane

For this type of research, it is necessary to use a positive control, which is an already known anti-inflammatory drug. In this case, one of the most used is Aspirin or Diclofenac; because they are considered drugs of the first order and have an acceptable tolerance and become an example and mold for the rest of non-steroidal anti-inflammatory drugs[25, 26].

2.2.2 Paw edema

Another method to corroborate the anti-inflammatory activity of new compounds *in vivo* is through the carrageenan-induced edema test (Figure 3). This method is used as an experimental animal model to corroborate the anti-inflammatory potential in acute inflammations. This method is based on two phases, the first one where pro-inflammatory agents such as cytokines are released due to the injection of carrageenan and a second

phase where prostaglandins and lysosomal content are released, in this phase is where the best results are obtained in the test of anti-inflammatory compounds[27, 28].

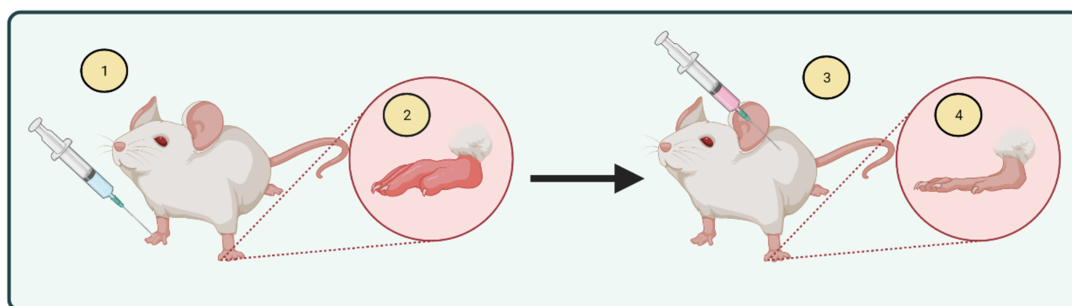


Figure 3 Paw edema by carrageenan. 1.- The mice get an injection of the carrageenan, 2.- the inflammation starts in the paw of the mice, 3.- The compound to be tested is injected in the rat, 4.- The inflammation in the paw of the mice is gone.

2.3 Plants as anti-inflammatory drugs

Products of natural origin have attracted the scientific community's attention due to the few side effects they represent. These products are employed for a wide field of diseases, including anxiety, depression, bacterial infections, fungal infections, and anti-inflammatory drugs[29].

Many medicinal plants are still currently employed as the primary source of biomolecules (Table 1), as is the case of Aspirin with its anti-inflammatory/analgesic activity widely studied and known around the world; this bio compound was extracted from plants belonging to the genus *Salix spp* and *Papulus spp*[30].

In Ecuador, approximately 13% of the species described as medicinal in the "Encyclopedia of Useful Plants of Ecuador" possess the anti-inflammatory potential, of which mostly we have the families Asteraceae, Solanaceae, and Malvaceae. There are records of treating external inflammatory processes (in the body) and internal (visceral problems such as kidneys, liver, and other organs)[11, 31].

In vivo studies in mice were carried out with different species of plants used in Ecuador where extracts of *Bonafousia sp*, *Croton methodorum* were administered and where the anti-inflammatory effect declared by the population was tested[32].

Table 1 Some examples of plants species with chemical compounds that have anti-inflammatory activity

Plant species	Common name	Drug	Reference
Salix sp.	Willow	Salicylic acid	(30)
Garcinia mangostana	Mangosteen	Isogarcinol	(33)
Glycyrrhiza uralensis	C. liquorice	Glycyrol	(34)
Malus domestica	Apple	Ursolic acid	(35)
Zanthoxylum piperitum	Japanese pepper	ZPDC glycoprotein	(36)
Olea europaea	Olive	Maslinic acid	(37)
Aesculus hippocastanum	Horsechestnut	Aescin	(38)

2.4 Extraction methods

Extraction methods are used mainly to separate active compounds (secondary metabolites) with medicinal properties (Table 2) housed in plant tissues using different solvents depending on their polarity. During this process, solvents that have been chosen diffuse into the solid material of the plant tissue, diluting and extracting the components with the biological activity of interest[39].

Table 2 Most common secondary metabolites in plants with an essential and relevant biological activity[40].

Secondary metabolite	Biological activity
Phenols	Antimicrobial and Anti-inflammatory
Tannins	Antidiarrheals, urinary antiseptic, and antidote in poisoning
Coumarins	Anti-inflammatory, anticoagulant, and anticancer
Flavonoids	Anti-inflammatory, antiallergic, antithrombotic, and vasoprotective
Chromones and xanthones	Antifungal activity

Alkaloids	Analgesia effect, local anesthesia, vasoconstriction, and muscle relaxation
Saponins	Antitumor activity, expectorant, antitussive agent, and anti-inflammatory
Terpenes	Antibacterial, antifungal, and anti-inflammatory.

One of the most used and easy extraction methods is Maceration, a solid-liquid extraction process where soluble compounds of interest are extracted from plant tissues. For this, the plant material must be finely ground either with dry or fresh material, and a solvent is selected depending on its polarity (Table 3), which can be with lower polarity hexane, petroleum ether, ethyl acetate, or chloroform and for higher polarity methanol, ethanol or water for 5 days with constant stirring[41, 42].

Table 3 Solvents used for separation of different secondary metabolites[39].

Solvents	Secondary metabolite
Water	Tannins, Saponins, Terpenoids
Ethanol	Tannins, Phenols, Terpenoids, Flavonoids, Alkaloids
Methanol	Terpenoids, Saponins, Tannins, Flavonoids, Phenols.
Chloroform	Terpenoids, Flavonoids
Ether	Alkaloids, Terpenoids, Coumarins
Acetone	Phenols, Flavonoids

3. Problem statement

The pharmaceutical field has shown great interest in the anti-inflammatory activity, especially in developing new active compounds that can curb diseases with underlying inflammatory processes. As a result, there has been a wave of new scientific studies to understand the different mechanisms and molecules related to inflammatory processes in recent years. Understanding how the inflammatory process works in the body is key to developing new effective methods to inhibit or attenuate the effects of the inflammatory response, which is why studies have focused on evaluating the anti-inflammatory

response of new compounds, whether natural or synthetic, using *in vitro* and *in vivo* models[43, 44].

4. Objectives

4.1 General objective

Standardize *in vitro* experimental protocol of the anti-inflammatory potential of different natural products from Ecuador.

4.2 Specific objectives

- Employ *in vitro* techniques based on stabilizing the erythrocyte membrane to determine the anti-inflammatory activity of extracts.
- Compare the anti-inflammatory potential between current synthetic drugs such as Aspirin and extracts of natural origin.

5. Methodology

5.1 Extraction process

8 grams of leaves from D – 001 and D - 002 are used, which are stored separately in a 500 mL beaker; 200 mL of methanol are added and left to rest at room temperature for 14 days, then filtered to separate the methanol extracts from the solid residues, the resulting extract it is rotary evaporated, finally obtaining 680 mg of solid final extract.

5.2 Preparation of the extracts

To prepare D-001 extract samples, a stock dilution was made with dimethyl sulfoxide, and later dilutions with water were made to obtain the required concentrations of 0.1mg/mL and 0.25 mg/mL (Annex 2).

To prepare the samples of the D-002 extract, a stock dilution was made with distilled water, and later the dilutions were obtained with the required concentrations of 0.1 mg/mL, 0.25 mg/mL, and 0.05 mg/mL (Annex 3).

5.3 Blood extraction.

Blood is collected from a volunteer who has not consumed any anti-inflammatory drug two weeks before the study; a cap, needles, and vacutainer tubes with 5 ml EDTA are

used; 3 dips are made slowly for a homogeneous mixture of the blood and the anticoagulant. The tubes are centrifuged at 3000 rpm for 5 minutes (Annex 1); the supernatant is discarded. The cell suspension was washed with 0.9% saline using the same volume as the discarded supernatant and centrifuged at 3000 rpm for 5 minutes; this procedure was repeated 3 times, leaving a transparent supernatant. The resulting cellular material was reconstituted in a 40% (v / v) solution of PBS; then, it is refrigerated at 4 ° C until the experiment with a maximum of 24 h[45].

5.4 Anti-inflammatory activity by heat

In 15 mL falcon tubes, 3 mL of each of the extract solutions are added in the concentrations previously obtained in the extraction process, 100 µL of the 40% erythrocyte suspension is added, positive controls are also prepared with Aspirin at the same concentrations of the extracts to be tested, the negative control is added distilled water instead of the active compound, each one of the samples was reproduced in triplicate except the negative control. All samples were incubated at 54 ° C for 20 minutes; they were centrifuged at 3000 rpm for 10 minutes (Annex 4). Finally, the absorbance of the supernatant was measured in a spectrophotometer at 560 nm (Annex 5)[46].

With the data obtained, the percentage of hemolysis inhibition is calculated with the following formula:

$$\% \, Inhibition \; of \; haemolysis = 100 \times \left[1 - \frac{OD_I - OD_{WI}}{OD_{NC} - OD_{WI}}\right]$$

$Where$:

$OD_I = Optical \; density \; of \; sample \; with \; incubation.$

$OD_{NC} = Optical \; density \; of \; the \; negative \; control$

$OD_{WI} = Optical \; density \; of \; the \; sample \; without \; incubation.$

5.5 Anti-inflammatory activity by hypotonicity.

The protocol consists of adding 1 mL of the extracts to be analyzed, 2 mL of hypotonic saline solution, 1 mL of PBS, and 500 µL of the red blood cell solution. Depending on the extracts to be studied, a negative control was added dimethyl sulfoxide in the case of D-001 extracts and distilled water in the case of D-002 instead extracts. In addition, a positive control is prepared with Aspirin instead of extract in the same concentration; all tubes were prepared in duplicates except for the negative control.

All tubes were incubated at 37 ° C for 30 minutes and subsequently centrifuged at 3000 rpm for 20 minutes (Annex 4); the supernatant of the tubes was analyzed using a UV-VIS spectrophotometer at a wavelength of 560 nm (Annex 5)[45]. The data obtained are used to calculate the percentage of inhibition of hemolysis using the following formula:

$$\% \, Inhibition \; of \; haemolysis = 100 \times \left[OD_{wh} - \frac{OD_h}{OD_{wh}} \right]$$

$Where$:

$OD_{wh} = Optical \; density \; without \; sample \; in \; hypotonic \; media.$

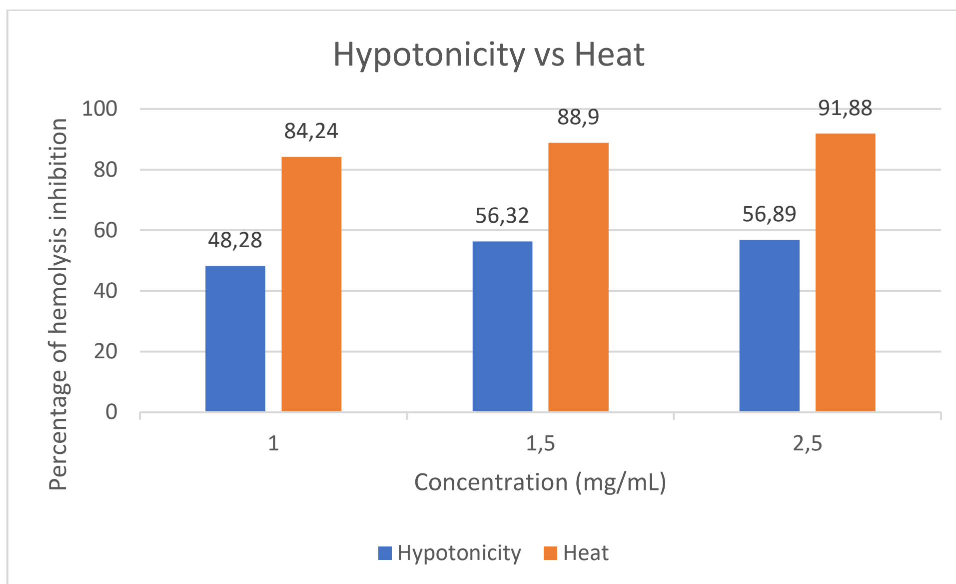
$OD_h = Optical \; density \; with \; the \; sample \; in \; hypotonic \; media.$

6. Results and discussion.

6.1 Comparison between heat and hypotonicity.

Considering the existence of both protocols to test the anti-inflammatory activity of various plant extracts, they were compared with positive control groups (Aspirin) to determine the viability during the experimental process.

In graph 1, the protocol that employs heat as a hemolytic factor generates better results than hypotonic media. However, the resources necessary to be able to carry out this protocol repeatedly generate a problem due to the availability of extract; according to the protocol exposed by Anosike (2012), it is necessary to use large amounts of these (3 mL per tube) this makes the realization of this protocol more costly and complex if the amount of sample is scarce. On the other hand, the protocol proposed by Parvin requires one-third of the sample amount compared to the heat protocol.



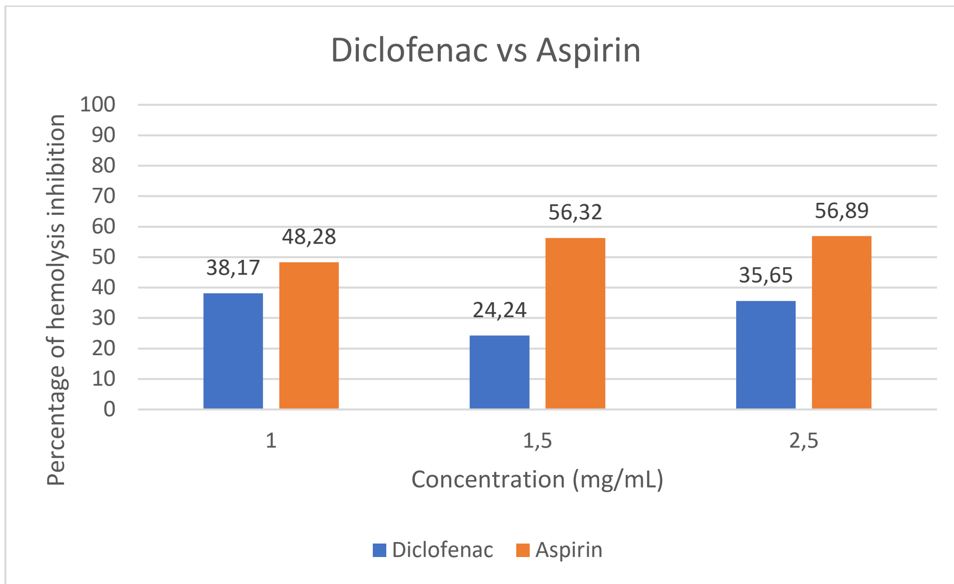
Graph 1. Comparison between both membrane stabilization protocols to determine the anti-inflammatory activity of new compounds.

These results can also be seen in comparative studies such as the one elaborated by Yesmin (2020) or Ranasinghe (2012), who, by using both analytical techniques, obtained a difference in the percentage of hemolysis inhibition by heat and hypotonicity of the erythrocyte membrane, where greater sensitivity is reported in the methodology where heat is used as a trigger of erythrocyte rupture[47, 48].

Both protocols allow us to test the anti-inflammatory activity of various compounds in a fast and simple way, which is why both are widely used by different researchers around the world obtaining positive and encouraging results to discover new molecules with this characteristic. We have several examples of plant extracts that have been tested with this methodology such as *Schinus polygama, Acmelia uliginosa, Acalypha indica*[49,50,51].

6.2 Comparison between aspirin and diclofenac (hypotonicity protocol)

To standardize the protocol and determine the best positive control between both drugs available in the laboratory, the capacity to inhibit hemolysis using a hypotonic medium was compared, and it was found that Aspirin has a greater capacity to stabilize the erythrocyte membrane in the 3 concentrations tested of 1mg/mL, 1.5mg/mL and 2.5 mg/mL (Figure 2). Aspirin is a widely used drug due to its anti-inflammatory and analgesic capacity and tolerance to side effects[25].

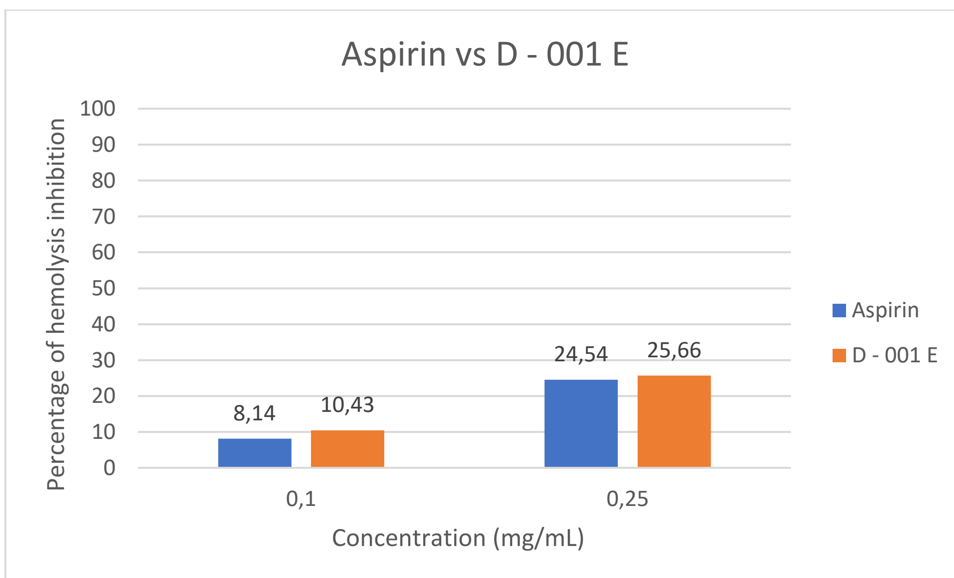


Graph 2. Comparison of the anti-inflammatory activity of two NSAIDs (Aspirin and Diclofenac) using membrane stabilization by hypertonicity.

6.3 Comparison between plant extract and positive controls.

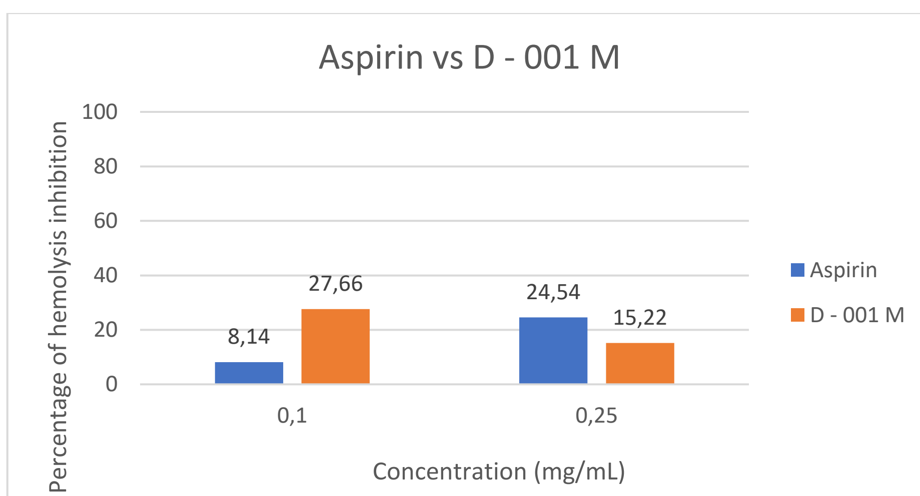
For the analysis of the extracts D - 001 and D - 002, the protocol of inhibition of hemolysis by hypotonicity was chosen.

In graph 3, the anti-inflammatory response of the extract D - 001 in an ethanolic solvent has similar values to the positive control group (Aspirin), having a percentage of hemolysis inhibition at a concentration of 0.1 mg/mL of 10.43% and in concentrations of 0.25 mg/mL of 25.46%.



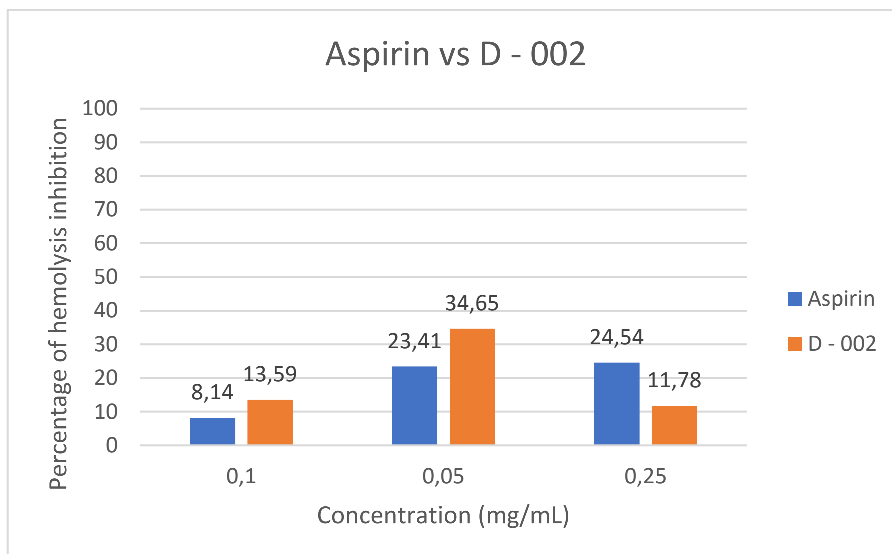
Graph 3. Result of anti-inflammatory activity of the ethanolic extract D - 002 comparing its effectiveness with Aspirin for commercial use.

In the analysis of the methanolic extract of D - 001 shown in graph 4, it can be observed by just a simple comparison that at a concentration of 0.1 mg/mL a higher percentage of inhibition of hemolysis of the extract against the positive control, having a 27.66% of D - 001 M while Aspirin at the same concentration has a value of 8.14%. On the other hand, at a slightly higher concentration of 0.25 mg/mL we have a value of 15.22% in our extract, while Aspirin has a value of 24.54% at the same concentration.



Graph 4. Result of membrane stabilization by hypotonic medium of methanolic extract D -001 against Aspirin.

Graph 5 shows the results obtained with the ethanolic extract D - 002 as an anti-inflammatory compound. A better anti-inflammatory activity than Aspirin is registered at concentrations of 0.1 mg/mL with a value of 13.59% of inhibition against 8.14% registered by Aspirin at the same concentration, at a concentration of 0.05 mg/mL the extract also registers higher values than Aspirin having 34.65% in contrast to the 23.41% achieved by Aspirin. At a slightly higher concentration of 0.25 mg/mL, Aspirin recorded a higher value of 24.54%, while the extract had 11.78%.



Graph 5. Results of the anti-inflammatory analysis of the ethanol extract D - 002 by means of the protocol of membrane stabilization by hypotonicity making a comparison with Aspirin.

The stabilization of the erythrocyte membrane as a methodology to confirm the anti-inflammatory activity of new compounds is ideal. Because the lysosomal membrane is similar, by being able to have more excellent stability in this membrane, the content of the lysosome is not poured into the extracellular space, thus avoiding the progress of the inflammation process, based on this getting higher values in the percentage of hemolysis inhibition the membrane stability rises also having a more significant anti-inflammatory effect[52, 53, 54, 55].

7. Conclusions and Recommendations

7.1 Conclusions

- The protocol was staged and found to be ideal for testing erythrocyte membrane stabilization to test the anti-inflammatory activity of naturally occurring extracts.
- Aspirin shows a more remarkable ability to stabilize the erythrocyte membrane than diclofenac, resulting in an anti-inflammatory response.
- It was determined that the extracts D - 001 and D - 002 have anti-inflammatory properties due to stabilizing the erythrocyte membrane.

7.2 Recommendations.

- To screen the secondary metabolites of the extracts D - 001 and D - 002.
- Quantify the number of secondary metabolites present in the samples.
- To verify the secondary metabolite responsible for the anti-inflammatory activity of the extracts.
- To elaborate encapsulations of the secondary metabolites responsible for the anti-inflammatory activity to facilitate their manipulation.

References

1. Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., ... & Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. Oncotarget, 9(6), 7204.
2. Furman, D., Campisi, J., Verdin, E., Carrera-Bastos, P., Targ, S., Franceschi, C., ... & Slavich, G. M. (2019). Chronic inflammation in the etiology of disease across the life span. Nature medicine, 25(12), 1822-1832.
3. Pahwa, R., Goyal, A., Bansal, P., & Jialal, I. (2018). Chronic inflammation.
4. Patel, A. G., Nariya, M., & Patel, B. (2017). A review on potential families of anti-inflammatory activity. Pharma Science Monitor, 8(1).
5. Rainsford, K. D. (2007). Anti-inflammatory drugs in the 21st century. Inflammation in the pathogenesis of chronic diseases, 3-27.
6. Khan, M. S. A., & Ahmad, I. (2019). Herbal medicine: current trends and future prospects. In New Look to phytomedicine (pp. 3-13). Academic Press.
7. Parveen, A., Parveen, B., Parveen, R., & Ahmad, S. (2015). Challenges and guidelines for clinical trial of herbal drugs. Journal of pharmacy & bioallied sciences, 7(4), 329.
8. Ampofo, A.J., Andoh, A., Tetteh, W., Bello, M., 2012. Microbiological profile of some ghanaian herbal preparations-safety issues and implications for the health professions. Open J. Med. Microbiol. 2 (3), 121-130.
9. Bussmann, R. W., & Sharon, D. (2006). Traditional medicinal plant use in Loja province, Southern Ecuador. Journal of Ethnobiology and Ethnomedicine, 2(1), 1-11.
10. Abad, M. J., Bessa, A. L., Ballarin, B., Aragón, O., Gonzales, E., & Bermejo, P. (2006). Anti-inflammatory activity of four Bolivian Baccharis species (Compositae). Journal of ethnopharmacology, 103(3), 338-344.
11. De la Torre, L., Navarrete, H., Muriel, P., Macía, M. J., & Balslev, H. (2008). Enciclopedia de las Plantas Útiles del Ecuador (con extracto de datos). Herbario QCA de la Escuela de Ciencias Biológicas de la Pontificia Universidad Católica del Ecuador & Herbario AAU del Departamento de Ciencias Biológicas de la Universidad de Aarhus.

12. Ganchozo, B. S. I., Villacreses, J. C. P., Indacochea, B. V. Á., Ganchozo, B. A. Z., & Indacochea, P. A. C. (2018). USO POPULAR DE PLANTAS MEDICINALES DEL CANTON JIPIJAPA, ECUADOR. UNESUM-Ciencias. Revista Científica Multidisciplinaria. ISSN 2602-8166, 2(2), 35-46.
13. Armijos, C. P., Meneses, M. A., Guamán-Balcázar, M. C., Cuenca, M., & Suárez, A. I. (2018). Antioxidant properties of medicinal plants used in the Southern Ecuador. Journal of Pharmacognosy and Phytochemistry, 7(1), 2803-2812.
14. Lima, G. R. D. M., Montenegro, C. D. A., Almeida, C. L. F. D., Athayde-Filho, P. F. D., Barbosa-Filho, J. M., & Batista, L. M. (2011). Database survey of anti-inflammatory plants in South America: A review. International journal of molecular sciences, 12(4), 2692-2749.
15. Alonso, M. R., Anesini, C. A., & Martino, R. F. (2018). Anti-inflammatory activity. In Sesquiterpene Lactones (pp. 325-346). Springer, Cham.
16. Andrade, L. N., Dos Reis Barreto de Oliveira, R., & De Sousa, D. P. (2014). A review on anti-inflammatory activity of phenylpropanoids found in essential oils. Molecules, 19(2), 1459-1480.
17. Netea, M. G., Balkwill, F., Chonchol, M., Cominelli, F., Donath, M. Y., Giamarellos-Bourboulis, E. J., ... & Dinarello, C. A. (2017). A guiding map for inflammation. Nature immunology, 18(8), 826-831.
18. Korbecki, J., Baranowska-Bosiacka, I., Gutowska, I., & Chlubek, D. (2014). Cyclooxygenase pathways. Acta Biochimica Polonica, 61(4).
19. ADNAN, A. Z., ARMIN, F., SUDJI, I. R., NOVIDA, M. D., ROESMA, D. I., ALI, H. A., & FAUZANA, A. (2019). In vitro anti-inflammatory activity test of tinocrisposide and freeze-dried aqueous extract of Tinospora crispa stems on human red blood cells by increasing membrane stability experiment. IN VITRO, 12(5).
20. Osafo, N., Agyare, C., Obiri, D. D., & Antwi, A. O. (2017). Mechanism of action of non-steroidal anti-inflammatory drugs. Nonsteroidal Anti-Inflammatory Drugs, 1-15.
21. García Candela, J. L. E., Pariona Velarde, C. D., & Londoñe Bailon, R. P. (2017). Actividad antiinflamatoria in vitro de los polisacáridos sulfatados de Patallus mollis extraídos mediante digestión enzimática. Rev. peru. med. integr, 759-764.
22. Okoli, C.O., Akah, P.A., Onuoha, N.J. et al. Acanthus montanus: An experimental evaluation of the antimicrobial, anti-inflammatory and immunological properties

of a traditional remedy for furuncles. BMC Complement Altern Med 8, 27 (2008). https://doi.org/10.1186/1472-6882-8-27

23. López, J. Á. S., Lendechy, M. A. V., Hernández, E. G., & Ojeda, G. A. S. (2018). Actividad antiinflamatoria in vitro de los extractos etanólico y hexánico de tallos de Cuscuta jalapensis Schltdl. Investigación y Ciencia: de la Universidad Autónoma de Aguascalientes, (75), 20-27.
24. Sarveswaran, R., Jayasuriya, W. J. A. B., & Suresh, T. S. (2017). In vitro assays to investigate the anti-inflammatory activity of herbal extracts a review.
25. Moreno-Brea, M. R. (2005). Tolerabilidad de aspirina. Revista de la Sociedad Española del Dolor, 12(6), 357-372.
26. Rivera, M. A. G. (2002). La Aspirina. Revista Médica de la Universidad Veracruzana, 2(2), 46-48.
27. Chiclana, C. F., Enrique, A., & Consolini, A. E. (2009). Actividad antiinflamatoria local de Malva sylvestris L.(Malvaceae) en el edema inducido por carragenina en ratas. Latin American Journal of Pharmacy, 28(2), 275-278.
28. Joseph, S. G., Carlos, P. M., Elmer, L. C., & Alberto, S. G. (2019). Efecto antinociceptivo y antiinflamatorio de la metformina en modelos experimentales en ratón. Horizonte Médico (Lima), 19(3), 49-57.
29. Shen, C. Y., Jiang, J. G., Zhu, W., & Ou-Yang, Q. (2017). Anti-inflammatory Effect of Essential Oil from Citrus aurantium L. var. amara Engl. Journal of agricultural and food chemistry, 65(39), 8586-8594.
30. Koparde, A. A., Doijad, R. C., & Magdum, C. S. (2019). Natural products in drug discovery. In Pharmacognosy-medicinal plants. IntechOpen.
31. Martínez, C. E. C. (2006). Plantas medicinales de los Andes ecuatorianos. C. Martinez, Plantas Medicinales de los Andes Ecuatorianos, 285-296.
32. García Bacallao, L., Rojo Domínguez, D. M., García Gómez, L. V., & Hernández Ángel, M. (2002). Plantas con propiedades antiinflamatorias. Revista Cubana de Investigaciones Biomédicas, 21(3), 214-216.
33. Fu, Y., Zhou, H., Wang, M., Cen, J., & Wei, Q. (2014). Immune regulation and anti-inflammatory effects of isogarcinol extracted from Garcinia mangostana L. against collagen-induced arthritis. Journal of agricultural and food chemistry, 62(18), 4127-4134.

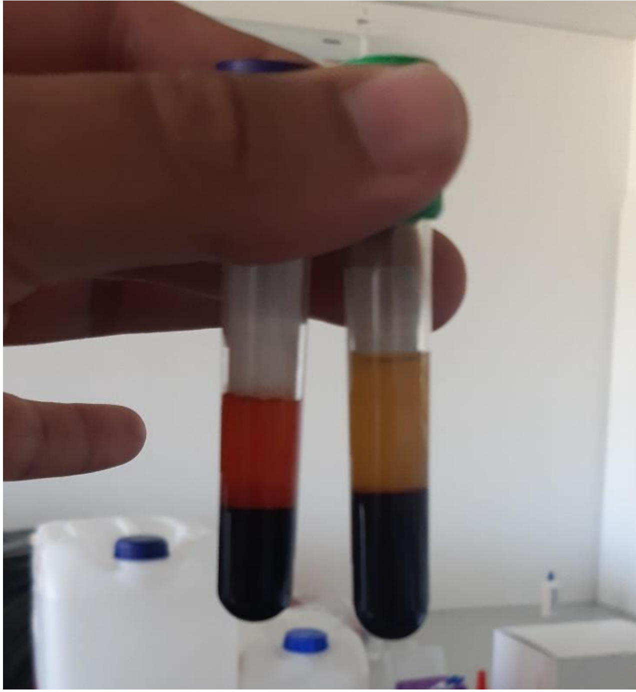
34. Fu, Y., Zhou, H., Wang, S., & Wei, Q. (2014). Glycyrol suppresses collagen-induced arthritis by regulating autoimmune and inflammatory responses. PloS one, 9(7), e98137.
35. Pádua, T. A., de Abreu, B. S., Costa, T. E., Nakamura, M. J., Valente, L. M., das Graças Henriques, M., ... & Rosas, E. C. (2014). Anti-inflammatory effects of methyl ursolate obtained from a chemically derived crude extract of apple peels: potential use in rheumatoid arthritis. Archives of pharmacal research, 37(11), 1487-1495.
36. Lee, J., & Lim, K. T. (2010). Inhibitory effect of phytoglycoprotein (24 kDa) on allergy-related factors in compound 48/80-induced mast cells in vivo and in vitro. International immunopharmacology, 10(5), 591-599.
37. Huang L, Guan T, Qian Y, Huang M, Tang X, Li Y, Sun H. 2011. Anti-inflammatory effects of maslinic acid, a natural triterpene, in cultured cortical astrocytes via suppression of nuclear factor-kappa B. Eur J Pharmacol. 672:169–174.
38. Taylor, L. (2000). Plant based drugs and medicines. Rain tree Nutrition Inc, 1-5.
39. Pandey, A., & Tripathi, S. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry, 2(5).
40. Rehab A. Hussein and Amira A. El-Anssary (November 5th 2018). Plants Secondary Metabolites: The Key Drivers of the Pharmacological Actions of Medicinal Plants, Herbal Medicine, Philip F. Builders, IntechOpen, DOI: 10.5772/intechopen.76139. Available from: https://www.intechopen.com/chapters/61866
41. Verde-Star, M.J., García-González, S., & Rivas-Morales, C. (2016). Metodología científica para el estudio de plantas medicinales. En Rivas-Morales, C., Oranday-Cardenas, M.A., & Verde-Star, M.J. (Eds.). Investigación en plantas de importancia médica. Barcelona, España: OmniaScience. 1-40.
42. Liu, Z. (2008). Preparation of botanical samples for biomedical research. Endocrine, metabolic & immune disorders-drug targets (formerly current drug targets-immune, endocrine & metabolic disorders), 8(2), 112-121.
43. ESTRADA, H. A. G., RUIZ, K. N. G., & Medina, J. D. (2011). Actividad antiinflamatoria de productos naturales. Boletín latinoamericano y del caribe de plantas medicinales y aromáticas, 10(3), 182-217.

44. Libby, P. (2007). Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutrition reviews, 65(suppl_3), S140-S146.
45. Parvin, M. S., Das, N., Jahan, N., Akhter, M. A., Nahar, L., & Islam, M. E. (2015). Evaluation of in vitro anti-inflammatory and antibacterial potential of Crescentia cujete leaves and stem bark. BMC research notes, 8(1), 1-7.
46. Anosike, C. A., Obidoa, O., & Ezeanyika, L. U. (2012). Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (Solanum aethiopicum). DARU Journal of Pharmaceutical Sciences, 20(1), 1-7.
47. Yesmin, S., Paul, A., Naz, T., Rahman, A. A., Akhter, S. F., Wahed, M. I. I., ... & Siddiqui, S. A. (2020). Membrane stabilization as a mechanism of the anti-inflammatory activity of ethanolic root extract of Choi (Piper chaba). Clinical Phytoscience, 6(1), 1-10.
48. Ranasinghe, P., Ranasinghe, P., Abeysekera, W. K. M., Premakumara, G. S., Perera, Y. S., Gurugama, P., & Gunatilake, S. B. (2012). In vitro erythrocyte membrane stabilization properties of Carica papaya L. leaf extracts. Pharmacognosy research, 4(4), 196.
49. El-Nashar, H. A., Mostafa, N. M., Eldahshan, O. A., & Singab, A. N. B. (2020). A new antidiabetic and anti-inflammatory biflavonoid from Schinus polygama (Cav.) Cabrera leaves. Natural Product Research, 1-9.
50. Muzammil, M. S., Manikandan, M., Jafar, A., Sakthivel, P., Geetha, S., & Malarkodi, R. (2015). Anti-inflammatory studies on Acalypha indica L. leaves by membrane stabilization. Indian Journal of Natural Products and Resources (IJNPR)[Formerly Natural Product Radiance (NPR)], 5(2), 195-197.
51. Modak, D., Paul, S., & Bhattacharjee, S. (2017). Anti-inflammatory activity of acmella uliginosa (sw.) cass. flower methanolic extract on membrane stabilization and protein denaturation: an in-vitro evaluation. NBU J Anim Sci, 11, 61-9.
52. Gunathilake, K. D. P. P., Ranaweera, K. K. D. S., & Rupasinghe, H. P. (2018). In vitro anti-inflammatory properties of selected green leafy vegetables. Biomedicines, 6(4), 107.
53. Valdés-Izaguirre, L. E., Arias-Cedeño, Q., Ramírez-Arzuaga, J., & Peña-Fuentes, D. (2018). Actividad antiinflamatoria y antioxidante in vitro de extractos etanólicos de Jatropha aethiopica Müell Arg var inermis. Revista Cubana de Química, 30(3), 440-453.

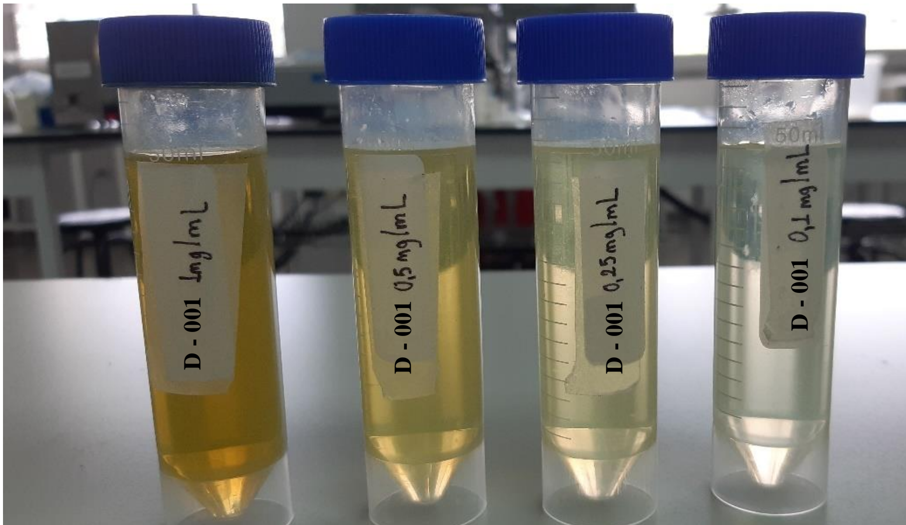
54. Saleem, T. M., Azeem, A. K., Dilip, C., Sankar, C., Prasanth, N. V., & Duraisami, R. (2011). Anti–inflammatory activity of the leaf extacts of Gendarussa vulgaris Nees. Asian Pacific Journal of Tropical Biomedicine, 1(2), 147-149.
55. Sikder, M. A., Rahman, M. A., Islam, M. R., Kaisar, M. A., Rahman, M. S., & Rashid, M. A. (2010). In vitro antioxidant, reducing power, free radical scavenging and membrane stabilizing activities of Spilanthes calva. Bangladesh Pharm J, 13(1), 63-67.

Annex

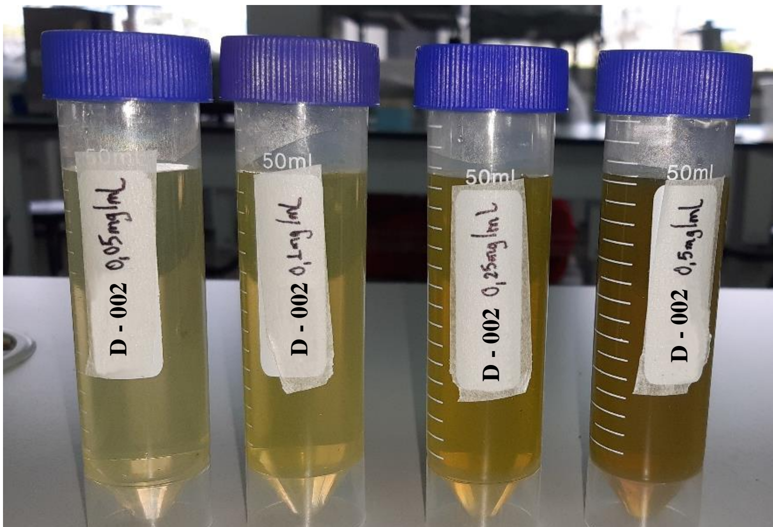
Annex 1. Blood samples after centrifugation.



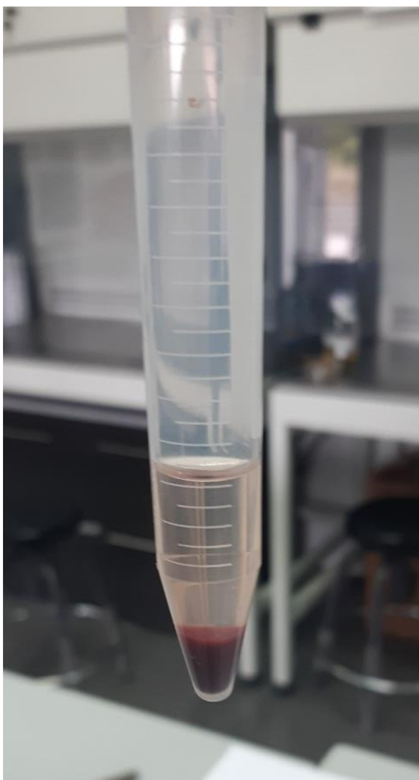
Annex 2. Prepared extract samples D - 001



Annex 3. Prepared extract samples D – 002



Annex 4. Final test tube before absorbance measurements



Annex 5. Absorbance measurements.