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ROSMERY DURAN UBIERA, MD.

ADENOSINE DEPENDENT ANTI-INFLAMMATORY EFFECTS OF *Arrabidaea Chica* VERLOT.

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Thesis Project presented to the Programade Pós-graduação em Imunologia Básica e Aplicada (PPGIBA), Universidade Federal do Amazonas, as a part of the requirements to obtain the title of Doctor of Philosophy (Ph.D.) in Basic and Applied Immunology.

Advisor: Prof. Dr. José Carlos Farías Alves Filho.

Co-Advisor: Prof. Dr. Antônio Luiz Ribero Boechat Lopes.

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"If I have seen further it is by standing on the shoulders of Giants."

Sir Isaac Newton, 1675

DEDICATION

I dedicate this work to GOD almighty, who took me in his hands and brought me here, because I was not strong enough. Because I experimented what says Isaiah 40:31 “But those who hope in the LORD will renew their strength. They will soar on wings like eagles, they will run and not grow weary they will walk and not be faint”

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ABSTRACT

Arrabidaea Chica (Bigonnnace) is commonly used in The Amazon by folks due to its anti-inflammatory properties, properties that have been confirmed in different experimental models of disease, but the molecular processes that govern this effect were unclear. The immune system mediates inflammation through a variety of mechanisms, including the production of pro-inflammatory and anti-inflammatory cytokines, which are directed by different signaling pathways, among them the adenosinergic pathway. The study of new anti-inflammatory molecules provides a long-term solution to the global problem that represents inflammation when uncontrolled and the Amazon is rich in natural products that need to be studied. The objective of this study was to elucidate whether or not the anti-inflammatory effect of *Arrabidaea Chica* VERLOT in bone marrow-derived macrophages (BMDM) is mediated by adenosine. The production of pro-inflammatory (IL-1β and TNFα) and anti-inflammatory (IL-10) cytokines, was assessed by ELISA in the supernatant of BMDM pre-incubated with *Arrabidaea Chica*VERLOT aqueous extract and stimulated with LPS. *Arrabidaea Chica* demonstrated to decrease the production of IL-1β and increase the production of IL-10. The increased production of IL-10 was associated with elevated production of adenosine and this effect was not observed when the different steps of the adenosinergic pathway were pharmacologically inhibited or genetically suppressed. The participation of the metabolism of the cells was also confirmed as the direct source of ATP for the generation of adenosine. We conclude that *Arrabidaea Chica* VERLOT mediates its anti-inflammatory effect via the adenosinergic pathway, increasing the metabolic rate of the cells to generate the substrate for this pathway.

KEYWORDS: *Arrabidaea Chica* VERLOT, Inflammation, Anti-inflammatory, Adenosine, ATP, IL-10, IL-1β.

FIGURES LIST

ABBREVIATIONS LIST

ADO	Adenosine
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
ARES	Adenylate/Uridylate Rich Elements
Arg-1	Arginase 1
ATF1	AMP-dependent transcription factor 1
ATP	Adenosine Triphosphate
BMDM	Bone Marrow derived macrophages
cAMP	Cyclic Adenosine Mono Phosphate
CBP	CREB Binding Protein
CBX	Carbenoxolone disodium salt
CCL	CC chemokine ligands
CREB	Cyclic AMP response element-binding protein
COX	Cycloxygenase
DAMP	Damage Associated Molecular Pattern
DC	Dendritic Cells
ERK	Extracellular signal-regulated kinase
FOXP3	Forkhead box P3
HIF1α	Hypoxia-Inducible factor-alpha
TGFβ	Transforming growth factor β
TLR	Toll-like receptors
TNF	Tumor Necrosis Factor
TPL	Tumor progression locus 2
ICAM	Intercellular Adhesion Molecule 1
iNOS	Inducible nitric oxide synthase
LFA1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
SP1	Specific protein 1

STAT	Signal transducer and activator of transcription
MAPK	Mitogen-activated protein kinase
MCSF	Macrophage Colony-Stimulating Factor.
MGL1	Galactose type C type lectin
MPO	Myeloperoxidize
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFKB	Nuclear Factor-Kb
NSAIDs	Non-steroidal anti-inflammatory drugs
PAMP	Pathogens Associated Molecular Pattern
PFK	Phosphofructo Kinase
PPAR	Peroxisome proliferator-activated receptor.
PRR	Pattern Recognition Receptors
RA	Rheumatoid Arthritis
UTR	Untranslated Regions

SUMMARY

CHAPTER1

1. THEORETICAL FRAMEWORK

1.1.Inflammatory Response and Innate Immunity

The inflammatory response is one of the primary mechanisms that protect homeostasis, involving cells and secreted molecules that aspire to eliminate invading agents that cause initial tissular damage and to eliminate necrotic cells and tissues. Inflammation can also be dysregulated and become detrimental for the internal medium and, if perpetuated for a long time, could be life-threatening. The inflammatory response was described since ancient times, and the first references are from ancient Greece with Hippocrates in the IV century B.C., its clinical manifestations were later compelled by Celso, a contemporary of Jesus of Nazareth, who describe 4 main manifestations including redness, pain, heat, and swelling. Further in the II century A.D. deformity was added by Galeno, a Roman physician. This response can be beneficial when controlled but deleterious when dysregulated, as seen in different conditions like septic shock (DI VITO; DONATO; TOMASSONI, 2017; MEDZHITOV, 2008; VINAY KUMAR, ABUL K. ABBAS, 2013).

Immune cells are the main participants in inflammation; their action margin is unmeasurable, possessing an intriguing biologic and physiologic section that governs and mediates the interaction between the organisms and the environment. Immune and not immune cells can express proteins from a family known as pattern recognition receptors (PRRs), which promote in the initiation of the cascade of events that exhibits the inflammatory response and participate actively in the pathogenesis of a variety of diseases. The PRRs identify Non-self antigens as pathogen-associated molecular patterns (PAMPs), signals as damage-associated molecular patterns (DAMPs) and other detrimental messages (SCRIVO *et al.*, 2011).

When a non-self antigen or a signal resulting from a pathologic interaction reaches the internal medium is exposed to many recognition strategies, the first recognition line is headed by the innate immune cells and molecules. Thus, complement activation, phagocytosis, NETosis, and proteolytic degradation are some of the most memorable innate immunity endeavors to eliminate a recognized non-self antigen. More than 100 years ago, Elie Metchnikoff described phagocytosis as one of the principal mechanisms of a response that he named *natural immunity* against invading pathogens, and this fact is one of the first pieces of

evidence about innate immunity which was further explored and brought us to where we are now(GORDON, 2016).

Innate immune response counts with the participation of molecules and cells, being neutrophils and macrophages the central cells associated, which by different mechanisms have an essential role in the mounting of immune response. One of their weapons in the immune war are their secreted molecules, chemokines, and cytokines, which command the cells migration and response. Among the principal cytokines, IL-10, IL-1β, IL-6 and TNF α are worthy of mention displaying both anti-inflammatory and pro-inflammatory characteristics, and different release pathways govern the secretion of these molecules. The relevance of the innate immunity to health is revealed by the development of diseases when this response is altered(GROH *et al.*, 2018; KIRIPOLSKY; MCCABE; KRAMER, 2017; MESHKANI; VAKILI, 2016).

Innate immune cells are the initiators of the inflammatory response as before mentioned. The inflammation can be acute, lasting hours to days, better exemplified by infection, or chronic, lasting months to years, such as the case of Rheumatoid Arthritis (RA). It is important to point out that inflammation is generated due to the cell's response to a noxious stimulus, typically self-limited. The acute inflammation is initiated by the tissue-resident cells and perpetuated with the migration of other circulating cells to the site of the lesion via plasma extravasation as a consequence of a critical endothelial activation. Important chemoattractant molecules also mediate the recruitment of these cells, and the process is joined by vasoactive amines, eicosanoids, and cytokines, among others (Figure1). Neutrophils are the principal cells involved in the acute inflammatory response, they have a strong role in the front line of homeostasis defense, but their half-life is short, while macrophages, with a longer half-life, participate in the initiation as well as in the resolution of inflammation. The principal stimuli for acute inflammation include infections, necrosis, hypersensitivity reactions, traumas, and foreign bodies (MEDZHITOV, 2008; VINAY KUMAR, ABUL K. ABBAS, 2013).

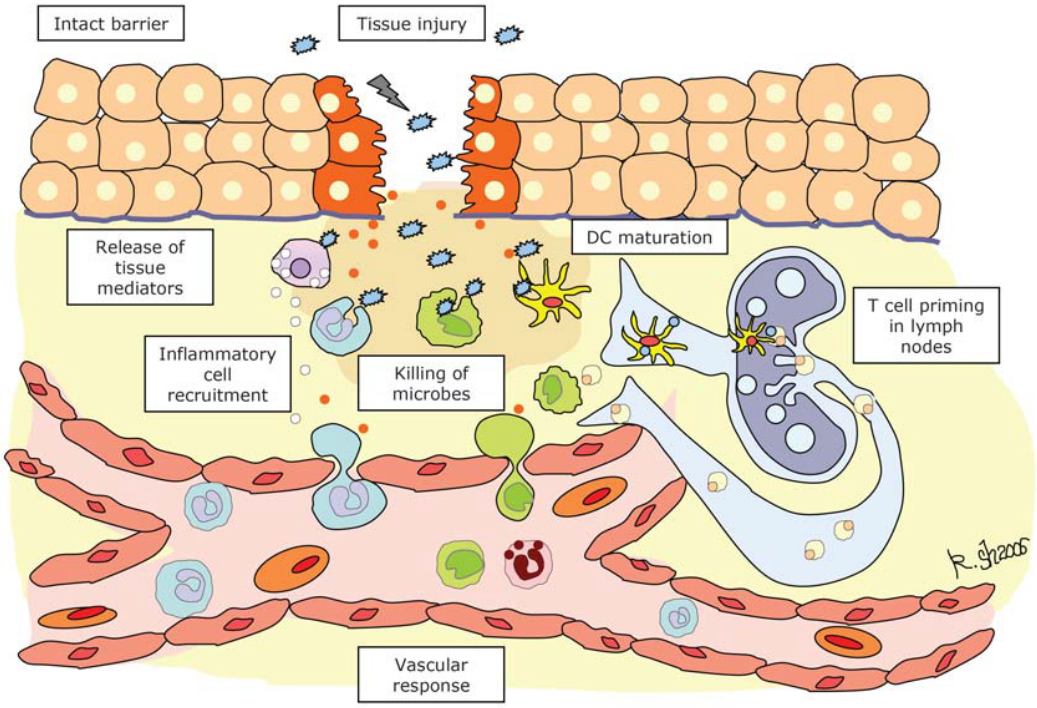

Figure1: Inflammatory response schema, in the context of microbes invasion. When a pathogen cross the anatomical barriers through a wound is recognized by the resident cells which display their different defense mechanisms and recruit other cells from the circulation to combat the pathogen.
Fount:(SHAYKHIEV, 2014)

The recognition of a PAMP or DAMP is a complex process that involves families of intracellular and membrane-bound proteins and the assembly of complex structures such as inflammasomes (important for the activation of IL-1β and IL-18) and intracellular pathways involving proteins like NFκB among others. For the intracellular cascades mediating inflammation, phosphorylation and other post-transcriptional modifications are essential regulators (KANNEGANTI; LAMKANFI; NU, 2007).

The vascular changes during inflammation include the increase in blood flow resulting from the dilation of the vascular beds, induced by substances like histamine that also favors the vascular permeability and subsequent edema. Cyclins also need to be mentioned as they interfere with vascular reactivity and pain during the inflammation. The vascular modifications, as aforesaid, favor the recruitment of circulating cells to the site of the lesion. Leukocyte recruitment depends on the occurrence of different steps that chronologically include the margination and rolling of these cells in the endothelial wall, adherence to endothelium, transmigration through the endothelial cells, and finally, the migration to the injured site following chemoattractant stimuli (Figure 2). For immune cell

migration, the expression of adhesion molecules, both in the leukocytes and in vascular endothelium is essential and is stimulated mainly by specific cytokines in the microenvironment (e.g., IL-1β and TNF-α) and other mediators like leukotriene B4, bacterial products and complement C5. Some of the most relevant molecules are the selectins like P-selectin, E-selectin, and L-Selectin and integrins like ICAM 1, VCAM 1, and LFA 1(VINAY KUMAR, ABUL K. ABBAS, 2013).

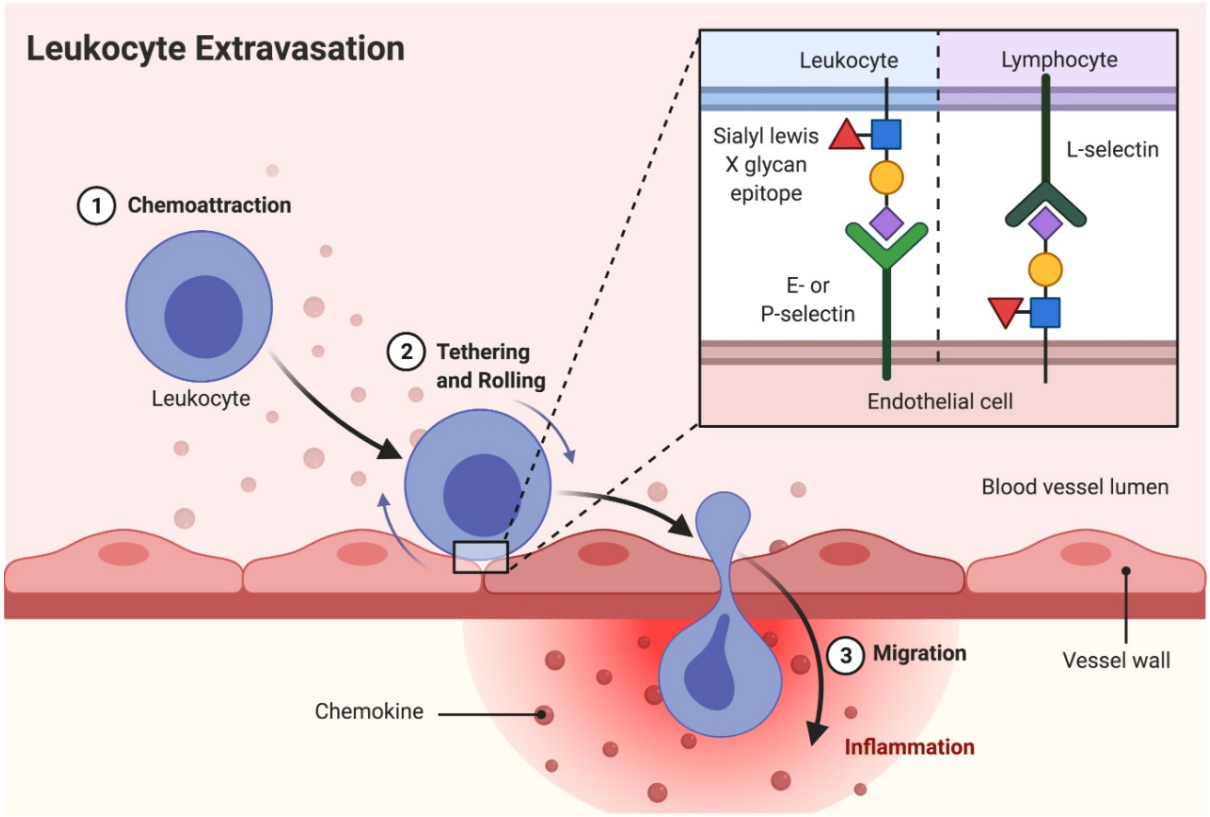

Figure 2: Leukocytes adhesion cascade, how immune cells get to the site of inflammation. Circulating cells are recruited to the inflammation site to help in the fight to maintain the homeostasis, thus contributing to the mounting of the inflammatory response.

Fount: Image designed by the athors.

Once in the lesion site, the immune war continues, and both the resident and recruited cells display their defense mechanisms, using as weapons the phagocytosis, respiratory burst, the transmission of cytolytic signals, and the secretion of cytokines, among others. When the immune war ends, there is a high cost for the body; the lysosomal enzymes and free radicals, along with other molecules, can produce significant tissue damages (SCRIVO *et al.*, 2011).

When inflammation is perpetuated for a broader period than expected, in an uncontrolled manner, it is defined as chronic and occurs as a result of an acute inflammation that progressed due to a poor resolution, continuous or prolonged exposure to the noxious triggers. The same is observed in immune-mediated diseases since the trigger cannot be eliminated because in the majority of the cases is a self-antigen. Chronic inflammatory lesions are lesions where can be found inflammation at different stages coinciding and represent an important contributor to chronic pathologies like obesity, atherosclerosis, cancer, asthma, RA and tuberculosis, the latter being one of the best examples according to the fact that the chronic inflammation can subside along decades. Although in a chronic inflammatory response, the main cells are macrophages and lymphocytes, the latter ones activated in the last stages of the normal inflammatory response. There are other pathological features in chronic inflammation, as the fibrosis of the affected tissues due to the excessive cellular activity and collagen formation (NATHAN; DING, 2010; SCRIVO *et al.*, 2011; VINAY KUMAR, ABUL K. ABBAS, 2013).

In the normal inflammatory response, the phagocytosis of cellular debris and tissue repair are the end steps to restore the tissue's normal function. To achieve this is required the formation of collagen and posterior removal along with the granulation tissue formation and further matrix deposition. In the resolution, macrophages are one of the main cells helping in the tissue's functional regeneration (ALINDA *et al.*, 2018).

1.2.Macrophages

Macrophages are among the most critical cells in immunity; they differentiate from monocytes and are mononuclear phagocytes whose embryologic origin includes the yolk sack and the fetal liver. During inflammation, they are recruited to a specific tissue from bone marrow and bloodstream (SNYDER *et al.*, 2016). Macrophages are important for the progress of many diseases like diabetes, cancer, obesity, and particularly atherosclerosis, where they have one of the prominent roles(EVERAERS, RALFROSA, 2017; CASSETTA; POLLARD, 2018; COCHAIN *et al.*, 2018; EGUCHI; NAGAI, 2017; KLESSENS *et al.*, 2017; MOORE; TABAS, 2011; RUFFELL; COUSSENS, 2015).

There are two different pools of macrophages, the tissue-resident and the circulating ones, but a wide variety of phenotypes. Macrophages respond to microenvironment cytokines, growth factors, pathogens, or other molecules to differentiate and are very plastic cells. The tissue-resident macrophages from different tissues have features to supply the needs of the environment they are located and receive other names like microglia in CNS, histiocytes in the spleen, osteoclast in bone, Kupffer cells in the liver, to mention few examples. Some of their functions include initiation and resolution of inflammation, growth factors production, host defense, phagocytosis, cellular proliferation, and wound healing (ALINDA *et al.*, 2018; MOGHADDAM *et al.*, 2018; SNYDER *et al.*, 2016; ZAKI *et al.*, 2013).

Macrophages polarization (classic or alternative phenotype) and migration depend on specific cytokines or bacterial products. These cells actively phagocyte the cellular debris from the inflammatory environment, which comes mainly from the apoptosis of neutrophils. They also migrate to the secondary lymph organs where they actively present antigens to lymphocytes, participating in the recruitment and differentiation of lymphocytes which in response reinforce the effector functions of macrophages (MOGHADDAM *et al.*, 2018).

Two major cytokines govern the macrophage's differentiation from monocytes, the Macrophage colony-stimulating factor (MCSF) and granulocyte-macrophage colony-stimulating factor (GMCSF). Macrophages differentiated under the presence of each one of these cytokines have different features. When bone marrow-derived macrophages (BMDM) differentiate in the presence of GMCSF, they have both a greater glycolytic rate and a more robust response to lipopolysaccharide (LPS). They also produce more pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) compared to those differentiated in the presence of MCSF, which in contrast have an anti-inflammatory profile (IL-10 production) (NA; JE; SEOK, 2018).

Macrophages can polarize toward a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype and their nomenclature was defined due to their interaction with Th1 or Th2 lymphocytes, but the proposal of only two polarization states became ambiguous and other phenotypes like the intermediate one (M3) have been described (MALYSHEV; MALYSHEV, 2015; NA; JE; SEOK, 2018).

1.2.1.M1 Macrophages

M1 macrophages are pro-inflammatory macrophages whose polarization was driven by the cytokines IFN-γ, TNF-α, and the bacterial product LPS. These cells secrete higher levels of IL-1α and β, IL-6, TNF-α, IL-23, IL-12, but secrete less IL-10. Metabolically M1 macrophages have a high glycolytic rate via pentose phosphate pathway and fatty acids synthesis with a truncated Krebs cycle, which leads to the accumulation of succinate and citrate. Upon stimulation, M1 macrophages increase the synthesis and activity of HIF 1α, which regulates the expression of the ubiquitinated form of phosphofructo kinase 2 (PFK2), increasing the glycolytic flux (SOTO-HEREDERO *et al.*, 2020).

Another important aspect is that the pentose phosphate pathway is enhanced in M1 macrophages. This pathway generates NADPH, an important cofactor for LPS which favors inducible nitric oxide synthase (iNOS) production, that catabolize the production of nitric oxide, an effective pathogen-killing mechanism and a key regulator of M1 macrophages (MOGHADDAM *et al.*, 2018; NA; JE; SEOK, 2018).

1.2.2.M2 Macrophages

Alternative M2 macrophages are anti-inflammatory macrophages induced by fungal cells, immune complexes, parasites, complement proteins, apoptotic cells, and by Th2 cytokines IL-4 and IL-13, which activate STAT6 transcription factor through IL-4 Receptor alpha. Other cytokines like IL-21, IL-10, which interfere with their polarization by activating STAT3 transcription factor via IL-10 Receptor, and IL-33 amplifies the polarization induced by IL-13. M2 macrophages have a low production IL-12 and high production of IL-10 and transforming growth factor β (TGF-β), their phenotype characterization is due to the expression of different molecules both in murine as in human cells like arginase 1 (Arg-1), the mannose receptor CD206, CD 209, galactose type C type lectin (MGL1) and, CCL17 among others. These cells have a high phagocytic capacity and participate mainly in wound healing, angiogenesis, and tissue repair. Still, their anti-inflammatory effects could be detrimental due to their ability to cause allergic inflammation, tumor formation and progression, and the development of some infections (ROSZER, 2015).

Different classifications have been proposed to describe the M2 macrophages subtypes, and one of the most accepted is M2a, M2b, and M2c. The M2a macrophages

respond to the stimulus with IL-4 and IL-13. The M2b macrophages polarize in response to the immune complex and the bacterial LPS, while M2c macrophages are responsive to glucocorticoids and TGF-β. Another proposed group is the M2d which is activated by IL-6 and adenosine. Metabolically the IL-4/IL-13 stimulation activates STAT6, which induces the expression of PPAR-γ and PPAR-δ and inhibits the Mtor (MALYSHEV; MALYSHEV, 2015; ROSZER, 2015).

1.3. IL-10 Production in Macrophages

IL-10 is the principal immunosuppressive cytokine, one of the main ones in maintaining immune homeostasis, which was discovered as a secreted factor of Th2 lymphocyte in a DNA-based screening. IL-10 is secreted by different immune cells, including various subtypes of T and B lymphocytes, natural killer, neutrophils, eosinophils, dendritic cells (DCs), and macrophages. Macrophages are one of the main cells secreting IL-10, and one of the most important mechanisms for its secretion is directed by antigen recognition via PPRs (pattern recognition receptors), especially toll-like receptors (TLR). IL-10 also has an important role in the cancer microenvironment, and some microorganisms can secrete similar molecules to evade immune defense when infecting other organisms (MANNINO *et al.*, 2015).

The functions of this cytokine could be enumerated in a wide list, but an important one is to prevent the generation of a strong pro-inflammatory response, which results in significant metabolic stress for internal medium and consequent tissue damage. This function is exerted by limiting the activation of immune cells and, as a result, preventing the generation of allergy, autoimmune reactions, and other associated immunopathologies. IL-10 is also an important growth factor for B and T lymphocytes, mast cells, NK cells, granulocytes, endothelial cells, keratinocytes, and dendritic cells. This cytokine participates in the pathogenesis of RA and atherosclerosis and is an important target for new cancer therapies due to its role in cancer (CHEN *et al.*, 2019; GALKINA; LEY, 2009; HAN; BOISVERT, 2015; MENG *et al.*, 2018; PARK *et al.*, 2018).

IL-10 production in macrophages and dendritic cells is regulated both transcriptionally and post-transcriptionally. A vast number of transcription factors activated

downstream of ERK1/2 or MAPK p38 are involved in IL-10 gene transcription in macrophages and DCs, among which are found cyclic AMP response element-binding protein (CREB), cyclic AMP-dependent transcription factor 1 (ATF1), c-Fos, and SP1 (SARAIVA; O'GARRA, 2010).

Several MAPKs are involved in the regulation of IL-10 production, but downstream the MAP3 kinase tumor progression locus 2 (TPL2), the activation of ERK1/2 regulates IL-10 production in macrophages and DCs upon the stimulus via toll-like receptors, and this is confirmed by the reduction of IL-10 production with the silencing of TPL2 or ERK1/2 in mice models. Under the stimulation of toll-like receptor, the production of IL-10 is also modulated via MAPK p38 which also have been shown to work jointly with ERK1/2 activating the mitogen and stress-activated protein kinase 1 (MSK1) and MSK2 to stimulate IL-10 production through CREB and ATF1. NFκB has also been reported to participate in IL-10 production after PRR activation (SARAIVA; O'GARRA, 2010).

1.4. Adenosine

Adenosine is a nucleoside belonging to the purinergic family, which is composed of adenine nucleotides, due to its properties works as a signaling molecule mediating different biological processes. The purinergic system, to which this molecule belongs, was proposed by Geoffrey Burnstockin in the 70s, who classified the purinergic receptors in 2 groups, the P1 for adenosine and P2 for ATP/ADP. This molecule is widely distributed among the different tissues, participating in homeostasis maintenance, and also has a role in the pathology of many diseases. One of the pathways through which adenosine is homeostatically synthesized intracellularly is from S-adenosyl homocysteine under the action of the enzyme S-adenosylhomocysteine hydrolase and can be transported to the extracellular space by an equilibrating purine or a concentrative purine as nucleoside transporters. Its average extracellular concentrations are very low (less than 1 uM) but increase during metabolic stress, reaching 4-10 uM in blood samples of septic patients (KAZEMZADEH-NARBAT *et al.*, 2015; KUMAR; SHARMA, 2009; YEGUTKIN, 2020).

Besides its intracellular synthesis, adenosine can also be synthesized extracellularly from the dephosphorylation of ATP due to the action of endo and ectonucleotidases, the ATP leaves the cells mainly through the pannexin channels but there is also vesicular release of

ATP. Once in the extracellular space, ATP is converted to AMP by the action of the ectonucleoside triphosphate dephosphorylate 1 (ENTPD1) (CD39) in the extracellular membrane, which is further converted to adenosine by the ecto-5′-nucleotidase (CD79). Adenosine can suffer the effects of the enzyme adenosine deaminase (ADA), which converts adenosine to inosine, which further becomes uric acid and is eliminated by the kidneys (KUMAR; SHARMA, 2009).

Adenosine can also interact with the cells via four extracellular purinergic1 (P1) receptors coupled to a G protein, giving different results depending on the G protein nature upon its stimulation (classified as A_1, A_{2A}, A_{2B}, and A_3). It is worth mentioning the fact that the affinity to adenosine is not the same for each of its receptors. A_1 Receptor is predominant in the CNS and neutrophils of the circulating pool. This receptor is coupled to a G_i protein, and its stimulation inhibits the adenylyl cyclase, allowing the increased adhesion of these cells to the endothelium and increased chemotaxis (COPPI *et al.*, 2020; KUMAR; SHARMA, 2009).

Adenosine A_{2a}receptors are distributed mainly in immune cells like macrophages, granulocytes, and lymphocytes, they are coupled to a G_s protein and stimulate the Adenylyl Cyclase signaling pathway. In counterpart the A_{2b} receptors can be coupled to a G_s or G_q protein but among all adenosine receptors are the ones with the lowest affinity to adenosine. A_3 receptor is coupled to a Gi protein but has also been associated with a G_q protein, and is predominantly present in immune cells where they increase the anticancer functions of natural killer cells among other functions (BOISON; YEGUTKIN, 2019; COPPI *et al.*, 2020; KUMAR; SHARMA, 2009).

Adenosine is important for the development of different metabolic functions, which affects a vast number of biological systems, including the cardiovascular (exogenously used to treat the supraventricular tachycardia) neurologic, the renal, and the immunologic system. During inflammation, adenosine and its precursors are highly produced by immune cells, which further recognizes and responds to this molecule. This response is different among the cells since not all the immune cells express all the receptors or have the same functions, whereby the effects mediated by this molecule are diverse. In mature dendritic cells, the stimulation of A_{2a} adenosine receptors results in a decrease in the production of IL-12, while in immature dendritic cells, the stimulation of A_1 and A_3 receptors improves their chemotaxis and maturity, other is the case of mast cells where there is an increase in VEGF and IL-18

production and histamine release after the stimulation of the A_3, evidencing the complexity of this response (COPPI *et al.*, 2020; KAZEMZADEH-NARBAT *et al.*, 2015; KUMAR; SHARMA, 2009).

Macrophages are essential cells for the inflammatory response and too much is known about them. These cells express the four types of adenosine receptors and their stimulation have been linked to various effects. Adenosine interferes with the maturation of monocytes into macrophages, sequestering these cells in the G1 phase of the cell cycle; the stimulation of A_1 receptors promotes the formation of multinucleated giant cells while the stimulation of A_2 receptors prevents it. In association with MCSF, Adenosine interferes with the proliferation, maturation, and phagocytic capacity of murine BMDM. In regards to the inflammatory response, adenosine stimulates the production of VEGF in macrophages to facilitate angiogenesis. Also, decrease the production of IL-12 and TNF alpha and NO-induced by the stimulation of TLR4 and post-transcriptionally increase the production of IL-10, regulating the inflammatory response by favoring the resolution and repair of the tissue damage (KUMAR; SHARMA, 2009).

1.4.1. Adenosine and IL-10 Production

Adenosine production constitutes one of the mechanisms that regulate IL-10 production in macrophages following inflammation, participating actively in the control of the inflammatory response, so that the effects of adenosine in IL-10 production have been largely studied, revealing that there are transcriptional and post-transcriptional mechanisms involved. At levels of messenger RNA, there are untranslated regions (UTRs) that contain adenylate/uridylate Rich Elements (AREs). The presence of AREs functions as targets for degradation, preventing the accumulation of messenger RNA for specific proteins and by this way regulating the protein synthesis rate. In the case of the IL-10 gene, upon the activation of adenosine receptor in macrophages, that repressive effect on the AREs is relieved, increasing the bioavailability of the messenger RNA and thus the IL-10 protein Synthesis rate. The aforementioned also prevents the effect of tristetraprolin, which binds to the IL-10 mRNA in the AREs and destabilizes it such as the case of micro RNA mir106a which also targets this mRNA for degradation (SARAIVA; O'GARRA, 2010; TU *et al.*, 2019).

At transcriptional levels, the production of IL-10 in macrophages stimulated with LPS, among other mechanisms, involve the activation of different transcription factors which translocate to the nucleus and stimulate the transcription of IL-10 by binding to its promoter region, among those transcription factors, can be highlighted the specific protein 1 (SP1), specific protein 3 (SP3), CCAATT enhacer binding Protein β (C/EBPβ), STAT3 and c-Maf (BENKHART *et al.*, 2000; BRENNER *et al.*, 2003; BRIGHTBILL *et al.*, 2000; CAO *et al.*, 2005; TONE *et al.*, 2000).In the case of adenosine, A_2 receptors specifically have been proposed to stimulate an axis involving the transcription factor C/EBPβ after the activation of A_{2a} receptors, but less is known about this (CSÓKA *et al.*, 2007; KUMAR; SHARMA, 2009). The production of IL-10 in LPS stimulated macrophages via activation of adenosine receptors is mainly mediated by A_2 receptors, when ATP is released by the cells via its channels (mainly the panexin 1), is dephosphorilated by the ectonucleotidases CD39 and CD73 to generate adenosine, as aforementioned and this set of steps is essential for the IL-10 production stimulated in this pathway (figure 3).

Different transcriptional and post-transcriptional mechanisms have been proposed along the time, but one of the most elucidated and simple ways of IL-10 production under the activation of adenosine receptors implicates the phosphorylation of CREB. When A_{2a} adenosine receptor is activated by the recognition of adenosine, since it is a receptor coupled to a Gs protein, it stimulates adenylyl cyclase, which further converts the ATP molecule into cAMP, the elevated production of this second messenger activates protein kinase A that leads to the activation of cAMP response element-binding protein (CREB) at Ser-133. CREB binds to p300 and the cofactor CBP (CREB Binding Protein) at the promoter region of IL-10, increasing its transcription rate. CREB has also been associated with direct inhibition of NFκB transcription activity, therefore halting the production of pro-inflammatory cytokines like Il-6 and TNF-α for further relief of the inflammatory response.(AL., 2012; BSHESH *et al.*, 2002; CSÓKA *et al.*, 2007).

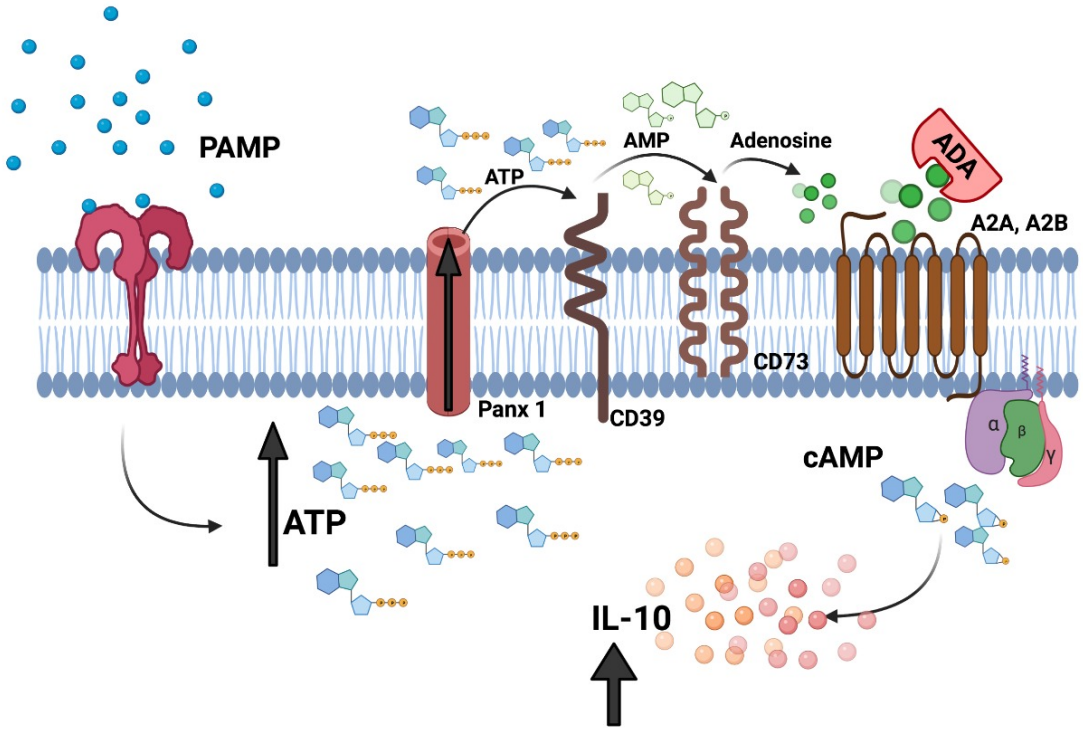

Figure 3: Adenosinergic pathway and IL-10 secretion. The recognition of a PAMP or DAMP can stimulate the cells to increase its ATP production, this ATP is further released by the cells through the panexin 1 channel. Out of the cell the ATP is dephosphorilated by the ectonucleotidases CD39 and CD73 to generate Adenosine, which can suffere the effects of the enzyme adenosine deaminase and become inosine or interact with its receptors, anchored to the cells membranes and stimulate the production of IL-10 by stimulating the generation of cAMP.

Fount: Image designed by the athors.

1.5. Natural products and Immune System

Natural products are widely used, and their impact on society comes even before the pharmacological industry's instauration, there is evidence from both, ancient civilizations with proven medical knowledge and ancient tribes excluded from society that implemented these natural products in their everyday. According to the World Health Organization (WHO) reports, the impact of natural medicine is not only a thing of the ancient times; nowadays, 80% of the world's population access natural sources to supply daily needs and find the cure and prevention of common diseases. There are vast reports about the range of applications that natural products could have; these sources are decked out with applications that include antibiotic, antioxidant, and anti-inflammatory effects. Natural products, especially those

derived from plants, interact with different body systems to mediate their effects, and the immune system is one of the main. Some of these products modulate immune cell response in a wide range of manners (ARULSELVAN *et al.*, 2016).

Due to the continuous damage that the uncontrolled generation of free radicals represents for our health owing to their potential to generate tissue damage and diseases so aggressive like cancer, the health industry develops synthetic substances that promise to ameliorate this problem, but the literature shows that nature offers a lower cost and healthier solution with its free antioxidants, found in roots and shrubs as in other sources. The generation of free radicals is a process that constantly occurs in our body, and its imbalance is detrimental, as before mentioned. Free radicals are atoms with one or more unpaired electrons that need to steal electrons to be stable, compromising other molecule's stability and then generating new free radicals in the act. The better-known free radicals are lipid alkoxyl, superoxide free radical anion, hydroxyl free radicals, and lipid peroxyl. Reactive Oxygen Species (ROS) are radical derivatives important for some of the main functions of immune cells, produced by mitochondria in oxygen-dependent conditions and that under hypoxic conditions produce nitric oxide (NO). Some of the antioxidants found in nature include ascorbic acid, carotenoids, and tocopherols (ARULSELVAN *et al.*, 2016; KRISHNAIAH; SARBATLY; NITHYANANDAM, 2011; POYTON; BALL; CASTELLO, 2009).

Reactive oxygen species are often confused with free radicals, as the first were initially only considered due to their harmful capacity, but nowadays is known that ROS are important for the internal medium homeostasis. Oxidative stress refers to an increase in ROS production from its basal levels concomitant with specific physiological effects. Oxidative stress can be graduated according to its intensity and has become life-threatening due to the challenges that our body has to face counteracting its effects and the risk that synthetic drugs with their adverse effects represent. Studies show that the dietary supplementation of natural sources of antioxidants can improve the incidence of human diseases, another evidence that supports the importance of natural products (HERTOG *et al.*, 1993; RUSSO *et al.*, 2012; SPAGNUOLO; MOCCIA; RUSSO, 2018).

Inflammation, as before described, is an important component of a vast number of diseases, if not of the majority of them, this means that the use of anti-inflammatory drugs worldwide is increasing every day. Many anti-inflammatory drugs are formulated with a synthetic base to target different components of the inflammatory cascade. These drugs come

accompanied by their prominent adverse effects, which include damages to the gastric mucosa and nephron impairment. The most commonly used anti-inflammatory drugs are the nonsteroidal anti-inflammatory drugs (NSAIDs) which inhibit the cyclooxygenase 1-2 (COX 1 and 2), important enzymes for the production of prostaglandins, molecules that participate in the promotion of pain, inflammation, and also fever. Other drugs have other targets in the cascade but always with the counterpart of their already emphasized adverse effects. Nature provides its anti-inflammatory molecules, and there is good scientific literature to sustain the use of these products. Similar to synthetic drugs, naturally obtained products target different points of the inflammatory response and interfere with its progression, but in a less risky mode, with fewer adverse effects. Regions like Africa, the Amazon, India, and the Caribbean are rich in natural products and the folks very commonly use them to treat and prevent inflammation. The African ginger is an example of the above mentioned, whose extract is used to treat inflammation, cough, malaria, and some other conditions like asthma and allergy. Phytochemical studies reveal its potential as an anti-inflammatory drug and due to its extensive demand from the African folks is included in the Red List of South African Plants (ADEBAYO *et al.*, 2021). Another example is Pristimerin, derived from the Chinese medical herb *Celastraceae* and *Hippocrateaceae,* which is used as an anti-inflammatory and is suggested to interfere with NLRP3 inflammasome mediated response (ZHAO *et al.*, 2020). The literature describes many other plant extracts, natural oils, etc., used to treat inflammation (KISHORE *et al.*, 2019; LATRUFFE, 2017).

Medicinal plants are defined as those with therapeutic benefits when administered. The presence of polyphenols, alkaloids, and flavonoids, among other secondary metabolites, are responsible for its effects, including its anti-inflammatory effects (JALILI *et al.*, 2019; TABRIZI *et al.*, 2020). The Amazon, a region filled with flora, offers the natives and folks a big pharmacy to treat their illnesses. Every year new medicinal plants based products are formulated to supply the demand. In the Amazon, the popular knowledge about natural medicine is almost religiously transmitted from one generation to the other. One of the commonly used amazon plants is *Arrabidaea Chica* VERLOT, belonging to the *Bigonacea*family, use to treat inflammatory infectious and non-infectious diseases (MAFIOLETI *et al.*, 2013). In Brazil, due to the immense biodiversity, especially in The Amazon, the use of medicinal plants and other natural products is regulated by Agência Nacional de Vigilância Sanitária (ANVISA), which is the principal organ of surveillance

followed the by Brazillian Public Health system which since 2007 works offering plants derived phytotherapy to the population via the Health Ministry (CARVALHO *et al.*, 2008)

1.6. Flavonoids

Flavonoids are found in plants and fruits as secondary metabolites, responsible for protecting against microorganisms and ultraviolet (UV) radiation, and they are considered classic antioxidants and regulators of the immune response. Flavonoid's chemical structure comprises 15 carbons, and they can be classified according to the oxidation level of their C ring into flavones, flavonols, flavanols, and anthocyanins. According to the C ring's carbon to which B ring is linked are classified into isoflavones, and a third classification exists due to a similar synthesis process that includes the chalcones and dihydrochalcones (MARTÍNEZ; MIJARES; DE SANCTIS, 2019; SPAGNUOLO; MOCCIA; RUSSO, 2018).

There is a vast biological difference between all the flavonoids that depends on their conjugation, glycosylation, and methylation patterns mainly, while other processes are responsible for their structural differences. Flavonoids have been used over the years to counter inflammation, and they can influence the expression of inflammatory markers and mediators. Evidence links flavonoids like Fisetin to the blockage of transcription of specific cytokines, demonstrating how wide the margin of reach of these molecules (MARTÍNEZ; MIJARES; DE SANCTIS, 2019).

In the immune system, the effects mediated by these molecules can vary according to the target cell and the moment of the response in which they are administered. Their impact on the immune system is unmeasurable but goes from modulation of B cell proliferation to inhibition of histamine production during allergy or Myeloperoxidase (MPO) in neutrophiles and NFκB in macrophages (SPAGNUOLO; MOCCIA; RUSSO, 2018). As examples of the aforementioned can be highlighted the apigenin which is a flavone found in many plants and has a demonstrated anti-inflammatory capacity by impeding the effects mediated by the pro-inflammatory molecules IL-6 and TNF-α and the anthocyanins contained in *Vacciniummyrtillus* L. bilberry and Korean black soybean (KHAN *et al.*, 2016; PRIOR; WU, 2006; REZAI-ZADEH *et al.*, 2008).

Anthocyanins are polyphenols known as potent anti-oxidants, have anti-cancer and anti-inflammatory properties, studies demonstrated that decrease the acute phase reactant C

reactive protein and also improve cardiovascular health. They are abundant in fruits, flowers, and leaves, are the pigments responsible for many of the blue-violet and red-orange colors in nature, thus are used in the industry to manufacture food colorings. There is not toxicity associated with the consumption of anthocyanins and they are essential for the Europeans and Asian diet, associated with improved health. Is scientifically known that this flavonoid alters the mammalian's metabolism, demonstrated in adult rats with the administration of cyaniding-3 glucoside and in many other randomized trials that evidence their impact on cholesterol metabolism. One of the important characteristics of anthocyanins is their rapid absorption, the majority of them have already reached circulation 1 hour after the ingestion, which means that the body takes rapid advantage of their benefits, and this characteristic jointly with their benefits for health has become the target of many types of research (FALLAH *et al.*, 2020; FANG, 2014; TANG ET AL., 2008; VANZO *et al.*, 2013; WALLACE, 2013).

1.7. *Arrabidaea Chica* VERLOT

Arrabidaea Chica VERLOT belongs to the division *Magnoliophyta*, class *Magnoliopsida,* subclass *Asteridae*, order *Scrophulariales*, family *Bigonacea,* gender *Arrabidaea*, and specie *Arrabidaea Chica*. This woody plant was taxonomically classified in 1981 by CROQUIST and is widely distributed in South America and Africa, although it is native to The Amazon. This plant is used by amazonian folks, who prepare teas, baths, and also compresses from its leaves to help with wounds healing, treat inflammation, gastrointestinal diseases, leishmania, enterocolitis, anemia, and gynecological conditions (CORTEZ DE SÁ *et al.*, 2016; JORGE *et al.*, 2008; VASCONCELOS *et al.*, 2019).

This plant receives different names given by the folks of various regions, like Crajiru, capiranga, cajuru, crejeru, carajunu, chica, cipó-paucarajiru, carajuru, cipó-cruz, pariri, paripari and crejer. *Arrabidaea Chica* is not only used for medicinal purposes since the natives also use this plant in their rituals, mainly as a dye for skin and trees. In 1927, referring to one of the first investigations with this plant, the main flavonoid was identified in methanol extracts of fresh *Arrabidaea* leaves, the 6,7-dihydroxy-5,4′-dimethoxy-fla-vylium (a3-desoxyanthocyanidin, termed carajurin), to which was attributed its reddish color. The chemical structure of the principal flavonoids present in this plan was elucidated in 2001, but even before, many kinds of research have been performed which recognized the presence of

other flavonoids, steroids, and organic acids, among other molecules in this plant extract (MAFIOLETI *et al.*, 2013).

Figure 4: *Arrabidaea Chica* VERLOT leaves and aqueous extract.

Photos by Adriane Dâmares De Sousa Jorge Batalha

As aforementioned, *Arrabidaea Chica* leaves are rich in anthocyanins, which are flavonoids present in colored fruits, flowers, roots, and leaves as water-soluble pigments with a potent anti-inflammatory and antioxidant capacity. Anthocyanins also have anti-cancerogenic activity and are associated with a reduction in cardiovascular risk evidenced in epidemiological studies (LEE *et al.*, 2017; MARTÍNEZ; MIJARES; DE SANCTIS, 2019; PAULA *et al.*, 2013).

Different extracts and fractions can be obtained from this plant, varying only on the solvent used and the preparation method, but even though the raw material is the same, each of the extracts is more specifically applied to a particular scenario. Due to the knowledge construction about this plant in the last decades, is known that ethanolic extract is more specific for wound healing, aqueous extract as an anti-inflammatory, dichloromethanic

extract as anti-microbial, hydroethanolic extract for hepatic protection, and chloroform fraction as an antioxidant. The flavonoids are unstable molecules that lose stability with heat and pH variations but anthocyanins are the most stables among them and constituted the main flavonoids in the *Arrabidaea Chica* VERLOT; this aspect is responsible for the stability of this molecule (CORTEZ DE SÁ *et al.*, 2016; PAULA *et al.*, 2013; ZORN *et al.*, 2001).

CHAPTER II

1. INTRODUCTION

Medicinal plants are used all over the world to treat and improve the progression of multiple conditions, they are widely distributed, and regions like The Amazon have a great variety of those products, used by natives and the general population without a clear knowledge of how do they perform their target effects. *Arrabidaea chica*, a woody plant belonging to the Bignoniaceae family, contains flavonoids and is used as an anti-inflammatory, leishmanicidal, antibiotic and healing agent (CORTEZ DE SÁ *et al.*, 2016; JORGE *et al.*, 2008; VASCONCELOS *et al.*, 2019).

The immune system constitutes the target for many natural products, comprises very plastic cells and others, not that plastic, as well as molecules. Macrophages are essential cells for the functioning of the immune system, interfering in innate and adaptive responses, whose profile can be regulated by the microenvironment to which they are exposed. When stimulated with LPS, macrophages can produce pro-inflammatory cytokines such as IL-1β and TNF alpha in an early phase and anti-inflammatory cytokines as IL-10 in a late phase. IL-10 is one of the most important anti-inflammatory cytokines, which is secreted by Treg, and macrophages mainly. Its synthesis in macrophages is regulated by different mechanisms, and one of them, activated in metabolic stress, is the purinergic system, represented by the adenosine (SARAIVA; O'GARRA, 2010).

Adenosine is a nucleoside and signaling molecule synthesized in the intracellular space and transported to the extracellular under stress or extracellularly generated from the hydrolysis of ATP mediated by the ectonucleotidases CD39 and CD73 in the cells surface. Adenosine posses its receptors on the cell surface (A_1, A_{2a}, A_{2b}, and A_3), each of them is classically coupled to a G protein, which could be S, I, or q, depending on the G protein nature. The A_2 receptors are coupled to a Gs protein, and their activation stimulates the adenylyl cyclase, leading to an increase in the cAMP production and IL-10 synthesis (NÉMETH, 2012; SARAIVA; O'GARRA, 2010).

The active principle of many drugs is a substance commonly found in nature, a root or a leaf, used indiscriminately by the folks of a specific region. Gathering the scientific information that has been exposed by previous researches about *Arrabidaea Chica* VERLOT in experimental models of diseases where macrophages actively participate and given the

preliminary results of our research group, the objective of this study was to demonstrate that the anti-inflammatory effect of *Arrabidaea Chica* VERLOT in bone marrow-derived macrophages is mediated by adenosine.

2. OBJECTIVES

2.1. General Objective

- To investigate whether the anti-inflammatory effect of *Arrabidaea Chica* VERLOT in bone marrow-derived macrophages (BMDM) is mediated by adenosine.

2.2. Specific Objectives

- To determine the production of pro-and anti-inflammatory cytokines in bone marrow-derived macrophages (BMDM) treated with *Arrabiaea Chica* VERLOT aqueous extract and activated with LPS.

- To evaluate the production of IL-10 in BMDM treated with different concentrations of both, *Arrabidaea Chica* VERLOT aqueous extract and LPS.

- To identify if the effects of *Arrabidaea Chica* VERLOT aqueous extract increase adenosine production in BMDM activated with LPS.

- To demonstrate the participation of the adenosinergic pathway in the production of IL-10 in BMDM treated with *Arrabidaea Chica* VERLOT and activated with LPS.

- To detect the metabolic alterations induced by *Arrabidaea Chica* VERLOT aqueous extract in BMBDM activated with LPS that could be interfering in the activation of the adenosinergic pathway.

- To define if the effects of *Arrabidaea Chica* VERLOT aqueous extract in the adenosinergic pathway could result in the activation of NLRP3 inflammasome.

- To validate in vitro the anti-inflammatory potential of the *Arrabidaea Chica* VERLOT aqueous extract to control the inflammation in gout disease.

3. MATERIALS AND METHODS

3.1. Animals

Specific pathogen-free male c57BL/6, A2a $^{-/-}$ (background BALB/c), and CD39$^{-/-}$ were (background C57BL/6) obtained from the genetic department isogenic mice bioterium and the immunology department of the Ribeirao Preto Medical school (FRP/USP). Mice were maintained in the pharmacology Department Bioterium FMRP, at room temperature of 21±1 ℃, with light and dark cycles of 12 hours and food and water access ad libitum. All the experiments were conducted with the approval of the animal research ethics committee of the Ribeirao Preto Medical School, Univerity of Sao Paulo (Protocol number 117/2020).

3.2. Vegetal Material Origin

Arrabidaea chica leaves were collected in November 2019 with the help of Francisco Célio M. Chaves Ph.D. in the Medicinal Plants section of Embrapa, Western Amazon, located in the AM 010 highway, km 29 Manaus road, Itacoatiara - AM. *Arrabidaea Chica* botanical samples were deposited as exsiccates in the EAFM Herbarium of the Instituto Federal do Amazonas-East zone, with the access number EAFM 6791.

3.3. *ArrabidaeaChica* VERLOT Aqueous extract preparation

For *Arrabidaea Chica* aqueous extract preparation, the leaves were collected, dried under room temperature, and further crushed with a knife mill to obtain a red powder. In 1L of distilled water, previously boiled in a Beaker, 150 g of the obtained red powder were diluted and covered for 30 minutes. After the infusion, the extract was filtered and dried at 90 ℃ through nebulization using the mini spray dryer (LABMAQ DO BRASIL LTDA), without any drying adjuvant and obtaining 9.028 % of the dried extract. The aqueous extract of *Arrabidaea Chica*was processed in the cell culture laboratory of the pharmaceutical sciences school of the Universidade Federal do Amazonas (UFAM).

3.4.Chemical Prospecting of *Arrabidaea Chica* VERLOT aqueous extract

A stock solution (1mg.mL-1) of the *Arrabidaea Chica* VERLOT aqueous extract in methanol was prepared. An aliquot (5µL) of the stock solution was subsequently diluted to 5µg.mL-, and the resulting solution was analyzed via direct infusion in the mass spectrometry. All the mass spectrums were registered in the continuous monitoring mode.

The spectrums were acquired from the media of at least ten spectrums using the LCQ Fleet equipment with an ESI fount, operated in the positive acquisition mode. The samples were infused into the ESI through the syringe bomb of the equipment (10µL.min-1). The conditions of the analysis were the following: spray voltage (5kV), sheath gas (10arb), auxiliary gas (5 arb), sweep gas, (0 arb), capillary temperature (200 ºC), capillary voltage (40 V), tube lens (115 V), mass range m/z 200 to 400 collision gas, He. The ESI-MSn spectrum was obtained by applying 20 to 30% of energy.

3.5. MSU Crystals preparation

According to the protocol described previously (NISHIMURA *et al.*, 1997) 800mg of sodium mono-urate were diluted in 155 mL of boiling water, already containing 5 mL of 1 N NaOH. The pH was adjusted to 7.2 and the temperature of the solution was gradually reduced to the room temperature to further collect the crystals by centrifugation and subsequently heat them at 180 ̊C for 2 hours to purify and sterilize them, after which the crystals were stored until the experiments.

3.6. BMDM Isolation and Differentiation

Mice were euthanized under the effect of anesthesia (xylazine hydrochloride (10mg/kg) and ketamine hydrochloride (80mg/kg) followed by cervical dislocation, and femurs were collected and placed in a 5 mL falcon tube containing 2 mL of sterile saline solution. After removing the surrounding muscles and sterilization of the femurs, the diaphysis was cut with a scalp exposing the medullar canal and the bone marrow was flushed out with a 26 gauged needle and a 3 mL syringe filled with complete RPMI medium. The suspension was homogenized and centrifuged at 400 g, at 4 ̊C for 10 minutes, after this time, the supernatant was discarded, and the cellular pellet was conserved and resuspended in

complete RPMI medium (4 mL/mice) enriched with 10% of fetal bovine serum and 20% of L929 conditioned medium. The resuspended cells were placed in low adherence petri dishes (Corning 430591) (1 ml/dish), already containing 9 mL of the aforementioned culture medium and incubated in the CO_2 stove for 7 days at 37 ℃ with a refill of the macrophages differentiation medium (10 mL) on the day 3 and frequent monitorization.

After 7 days of differentiation, the macrophages were removed from the petri dishes with the help of cold saline phosphate buffer (PBS) and a cell scraper (Corning NY), the obtained solution was centrifuged at 400 g, at 4 ℃, for 10 minutes and the supernatant was discarded and the cells were resuspended in complete PRMI medium. The differentiation was confirmed with flow Cytometry analysis due to the expression of F480.

3.7.Cytokine production

The cell culture supernatants were harvested and the concentrations of murine IL-10, IL-6, IL-1β, and TNF-α were determined by ELISA using the immunoassay kit*DuoSet*® ELISA *Development System* (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

3.8. *Arrabidaea Chica* VERLOT Assays.

After bone marrow-derived macrophages isolation and differentiation, the obtained cells were used to assess different parameters related to this investigation. For the different experiments, macrophages were plated in a 96 wells costar plate (Sigma) at $2x10^5$ cells/well or in a 12 wells costar plaque (Sigma) at $1x10^6$ cells/well and incubated for 1 hour in the CO_2 incubator at 37 ℃ for adherence. After adhesion to the plate, macrophages were treated with *Arrabidaea Chica* VERLOT aqueous extract (1, 3, 10, and 30 ug/mL) or medium and incubated for 4 hours in the CO_2 incubator. In specific experiments, the cells were treated with different inhibitors, agonists, and antagonists drugs as 8-(3-Chlorostyryl) caffeine CSC (100μM, Sigma), adenosine deaminase (ADA) (1U/ml, Sigma), MRS 1754 (1μM, Tocris), ARL 67156 trisodium salt ARL (200μM, Tocris) adenosine 5'-(α,β-methylene) diphosphate APCP (100μM, Sigma) and carbenoxolone disodium salt CBX (50μM, Sigma). After 4 hours of incubation, the cells were treated with LPS (100ng/ml, Sigma) and incubated for 24 hours,

after which we collected the supernatant of the cell culture to perform ELISA or the cells to flow cytometry analysis.

3.9. Flow Cytometry Analysis

BMDM were or not incubated with *Arrabidaea Chica* VERLOT aqueous extract (10ug/mL) for 4 hours and stimulated or not with LPS (100ng/mL) for 24 hours and then collected in a proportion of $1x10^6$ cells/tube. Once collected, after the incubation time, the cells were stained as follows with F480 FITC, CD73 PE, CD39 PEcy7, CD206 APC 1uL (1ug) and incubated for 30 minutes at 4 ℃. After incubation, the cells were washed twice with PBS containing 2% of fetal bovine serum and centrifuged at 400 g for 10 minutes, after which the cells were resuspended in 100 uL of PBS containing 1% of formaldehyde. After the aforementioned, the samples were acquired in a FACSVerse (BD Immunocytometry System, Franklin Lakes, NJ) and analyzed with the software FlowJo™ v10 (BD Biosciences).

To analyze mitochondrial parameters the cells were treated and stained as mentioned above but not fixed. The cells were stained with Mito Tracker Red (PE), Mito Sox (PE), or Mito Tracker Green (FITC) and analyzed as aforesaid.

3.10. Lactate Production.

The lactate production was assessed in the supernatant of the cell with the help of a commercial colorimetric testing kit, according to the manufacturer's instructions (Quibasa-Bioclin, Belo Horizonte, MG, Brasil),

3.11. Adenosine Production

To access the adenosine production, BMDM were treated for 2 hours with *Arrabidaea Chica* VERLOT Aqueous extract (10ug/mL) and stimulated with LPS for 3 hours, thereafter the supernatant was collected and analyzed by mass spectrometry. The collected supernatant was treated with acetonitrile 66% to stabilize and extract the adenosine to further dilute it in 33% of water. The liquid chromatographic separation was performed with the system Xevo TQ-S Waters Acquity UPLC HSS, using a C18 Acuity UPLC HSS column with 1,0x150mm and using adenosine (Sigma) diluted in methanol in the different concentrations to calibrate

the machine. The column temperature was 40 ℃ and the solvents system was composed of 0,1% of acetic acid in methanol and 0.1% of acetic acid in water. The flow rate was defined to 500µl/min and the injection volume used during the whole execution time was 5µl, to 4.5 minutes execution. The positive ions fragment 268 and 136 m/z were observed. The adenosine concentration and the area under the peak were calculated with the help of the software MassLynx V4.1 SCN 843.

3.12. MSU

To mimic in vitro, the process that occurs in the joints during the pathogenesis of gout, the BMDM were isolated and plated in 96 wells plaque in a proportion of $1x10^6$ cells/wells as mentioned, then the cells were placed in the CO_2 incubator for 1 hour to adhere, after which macrophages were primed with LPS (100ng/mL) and incubated for 3 hours. Subsequently, cells were incubated for 1 hour with *Arrabidaea Chica* aqueous extract (3,30, and 300ug/mL) or complete RPMI medium and then stimulated with MSU (450 ug/mL) crystals and incubated for 1 more hour to complete the last experimental time. After the end of the experiment, the supernatants were harvested for ELISA of IL-1β, TNFα, and IL-10.

3.13. Statistic Analysis

Statistical significance between two groups was estimated using multiple two-tailed *t*-tests for parametric data and a Mann–Whitney *U*-test for non-parametric data. The data from experiments with three or more groups were analyzed using one-way ANOVA with the Tukey test for parametric data and the Kruskal–Wallis test for non-parametric data. The data are expressed as the mean ± standard error of the mean. Values of $P < 0.05$ were considered significant. The data were analyzed with PRISM 6.0 software (GraphPad Software, Inc., San Diego CA, USA).

4. RESULTS

4.1. Biochemical Characterization of *Arrabidaea Chica* VERLOT

The biochemical profiling of the *Arrabidaea Chica* VERLOT aqueous extract was performed via mass spectrometry, revealing the ions that are present in the extract. These ions represent different substances or contamination. The *A. Chica* VERLOT aqueous extract is complex but revealed the two primary pigments in the molecule, the ion 299 and 285, that correspond to the carajurina and carajurona, respectively (Figure 5 A). The carajurina is the main component of the *Arrabidaea Chica* species and, jointly with the carajurona is responsible for giving the reddish color to the leaves (Figure 5 B-C).

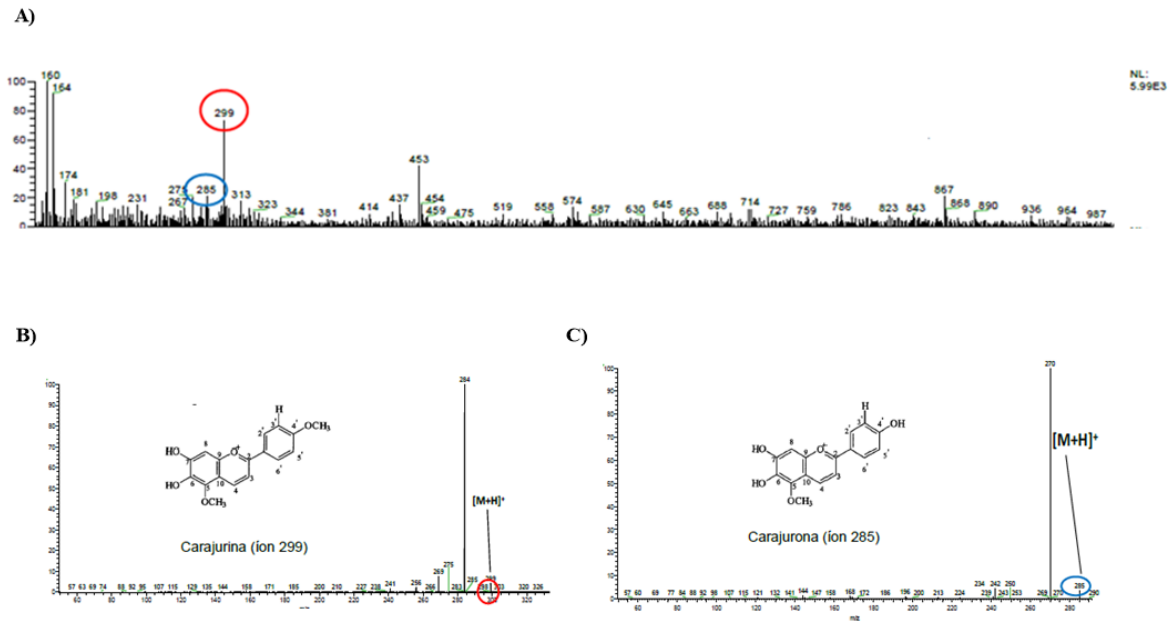

Figure 5: Biochemical characterization of *Arrabidaea Chica* VERLOT Aqueous extract. A) *ArrabidaeaChica* VERLOT extract was diluted in methanol and directly infused in the mass spectrometry for analysis. B and C) The fragmentation of the ions 299 and 285, significantly expressed in the *A. Chica* Aqueous extract, was performed via mass spectrometry, identifying in spectrum B, the carajurina, and in the spectrum C, the carajurona.

4.2 *Arrabidaea Chica* VERLOT aqueous extract decreases the production of IL-6 while increasing the production of the anti-inflammatory cytokine IL10.

The anti-inflammatory capacity of *A. Chica* VERLOT aqueous extract was evidenced as noticed in vivo by other authors and reported in the popular believes of Amazonian folks. BMDM isolated from wild type mice, were incubated with *A. chica* VERLOT aqueous extract (1, 3, 10, 30 ug/ml) for 4 hours and stimulated with LPS (100ng/mL) for 6 or 24 hours, after which the supernatant was harvested and ELISA detected the production of the pro-inflammatory cytokines IL-6 and TNFα and the anti-inflammatory cytokine IL-10. Here we demonstrated that macrophages pre-incubated with *A.Chica* VERLOT aqueous extract and stimulated with LPS during both 6 and 24 hours have a decreased production of IL-6 and similar production of TNFα when compared with macrophages treated exclusively with LPS (Figure 6)**.**

The production of the IL-10 was also determined in the supernatant of the cells, noticing that when the macrophages were pre-incubated with *A. Chica* VERLOT aqueous extract in the concentrations of 3 and 10 ug/mL, the production of IL-10 was greater than the stimulated by LPS itself (Figure 7). After demonstrating that the extract increased the production of this important anti-inflammatory cytokine, further steps were oriented to elucidate this phenomenon.

After confirming the effect of the extract in the production of IL-10, we proceed to explore other concentrations of *A. Chica* VERLOT (10, 30, 100, 300 ug/mL) and also stimulated the cells with different concentrations of LPS (10 and 100ng/mL) obtaining similar results about the production of this cytokine. The production of IL-10 was greater when the cells were pre-incubated with *A. Chica* VERLOT aqueous extract (10ug/mL) and stimulated with LPS rather than just using LPS. Given that the concentration of 10 ug/mL was more effective in stimulating the production of IL-10, this concentration was chosen for the following experiments (Figure 6 and 7).

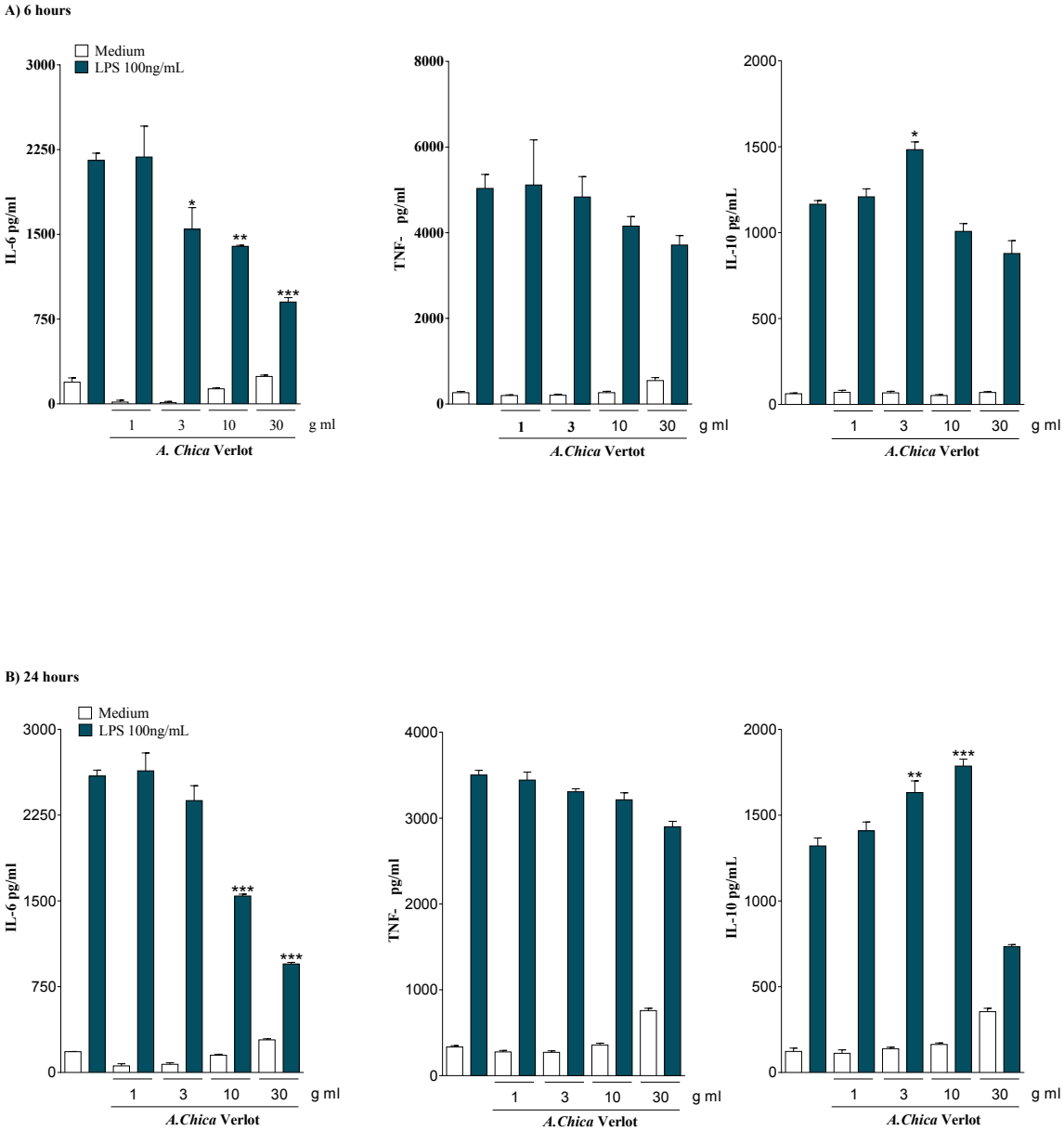

Figure 6: *A. Chica* VERLOT decrease the production of Pro-inflammatory cytokines and increase the production of the anti-inflammatory cytokine IL-10 in BMDM activated with LPS. A and B) BMDM were incubated for 4 hours in the presence of *A. Chica* VERLOT Aqueous Extract (1, 3,10, and 30 ug/mL) and activated with LPS for 6 hours (A) or 24 hours (B), the supernatants were collected to dose the cytokines IL-6, TNF-α, and IL-10 by ELISA. The graphs represent the media ± SEM, *p<0,05 indicates the values significantly different from the LPS group.

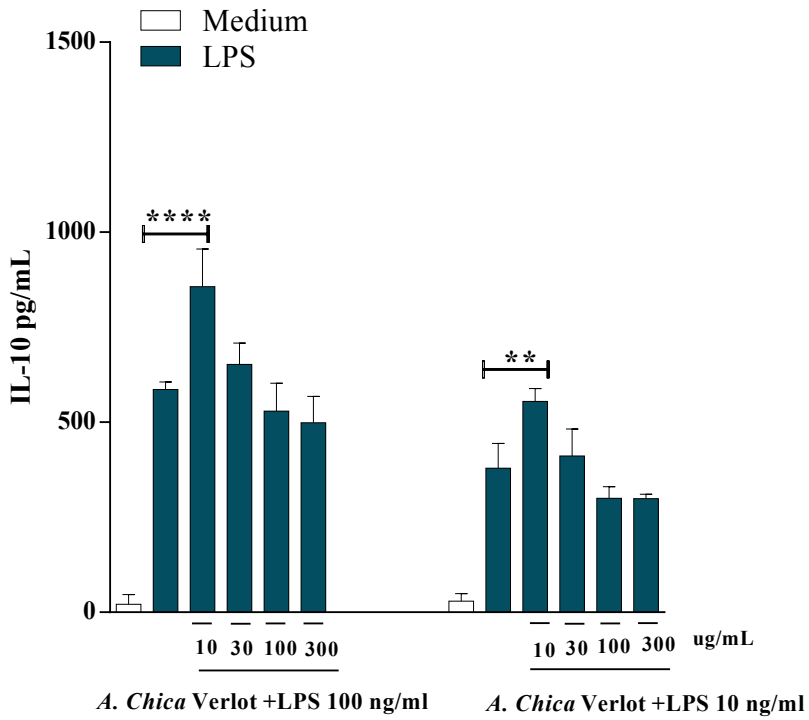

Figure 7: *A. Chica* VERLOT increases the production of the anti-inflammatory cytokine IL-10 in BMDM activated with different concentrations of LPS. A) BMDM were incubated for 4 hours in the presence of *A. Chica* VERLOT aqueous extract (10, 30, 100, 300 ug/mL) and activated with LPS 100 ng/mL or 10 ng/mL during 24 hours. The graphs represent the media ± SEM, *p<0,05 indicates the values significantly different from the LPS group.

4.3. *Arrabidaea Chica* VERLOT aqueous extract increases the production of adenosine in BMDM activated with LPS

The mechanism by which *A. chica* generates an anti-inflammatory response has not been elucidated, and the majority of the studies only demonstrate that the extract of this leaf has anti-inflammatory properties, but the molecular aspects involved are not well known. After showing that *A. Chica* VERLOT aqueous extract decreases the production of pro-inflammatory cytokines while increasing the production of the anti-inflammatory cytokine IL-10, we proceed to explore the molecules involved in the different signaling pathways that lead to the production of IL-10.

One of the molecules that we considered and explored was adenosine. To do this, we pre-incubated the BMDM with *A. Chica* VERLOT aqueous extract, using the concentration of 10 ng/mL, therefore is the best increasing the production of IL-10, and stimulated with LPS 100 ng/mL as already described. We determine the production of adenosine in the supernatant of the cell's culture via mass spectrometry. After analyzing the obtained data, we observed that the production of adenosine was greater in the cells pre-incubated with *A. Chica* VERLOT (10ug/mL) and stimulated with LPS than in the cells only stimulated with LPS (Figure 8). The aforementioned opened the doors to explore the adenosinergic pathway as a possible mechanism involved in the anti-inflammatory response stimulated by *A. Chica* VERLOT extract.

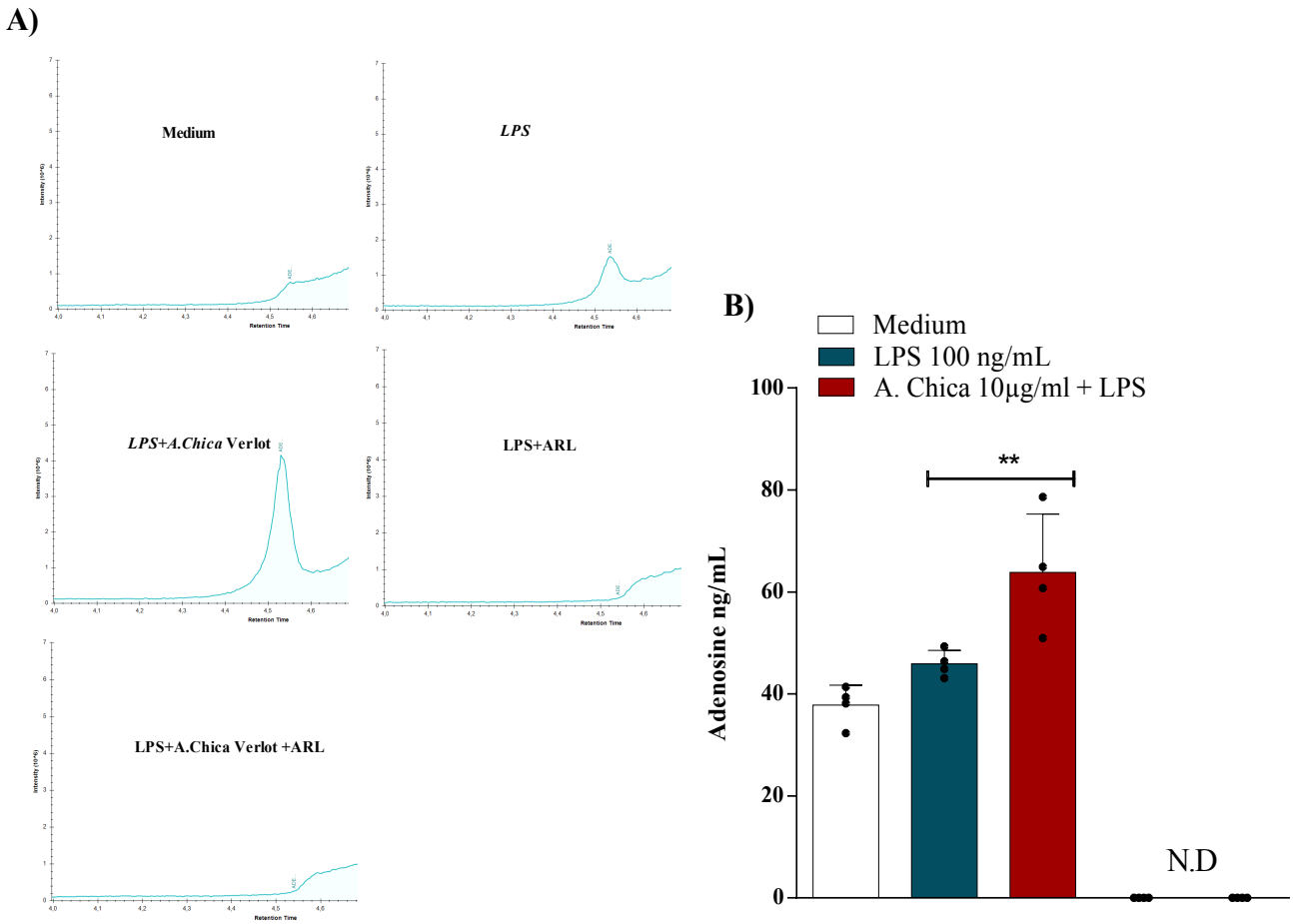

Figure 8: *A. Chica* VERLOT aqueous extract increase the production of adenosine in BMDM activated with LPS. BMDM were incubated for 2 hours in the presence of *Arrabidaea Chica* VERLOT aqueous extract (10 ug/mL) and activated with LPS (100ng/mL) for 3 hours, and the adenosine production was determined in the supernatant of the cells. A) Quantification of adenosine in the cell's supernatant using the system UPCL-MS, the representative chromatogram of adenosine in the cells culture. B) Adenosine concentration detected via UPCL-MS in the supernatant of BMDM in the presence or not of ARL (inhibitor of CD 39). The graphs represent the media ± SEM, *p<0,05 indicates the values significantly different from the LPS group.

4.4. The adenosinergic pathway contributes to the increase in the production of IL-10 in BMDM incubated in the presence of *A. Chica* VERLOT aqueous extract and further stimulated with LPS.

After correlating the adenosine levels with the increase in the production of Il-10, we proceed to explore the adenosinergic pathway role in the production of this cytokine in macrophages incubated with *A. Chica* VERLOT aqueous extract (10ug/ML)and stimulated with LPS. When adenosine is produced by the cells can be degraded to inosine, an immune-active substance that is further eliminated by the kidneys. This degradation of adenosine is given by the enzyme Adenosine deaminase (ADA). Hence, we treated or not the BMDM with adenosine deaminase simultaneously with the *A. Chica* VERLOT (10 ug/mL) and proceeded to stimulate with LPS (100ng/mL) as described. We observed that the production of IL-10 decreased when BMDM were treated with adenosine deaminase together with *A. Chica* VERLOT aqueous extract and stimulated with LPS compared to the cells only treated with the extract and stimulated with LPS(Figure 9), revealing that the degradation of adenosine interferes with the effects of *A. Chica* VERLOT in the production of IL-10.

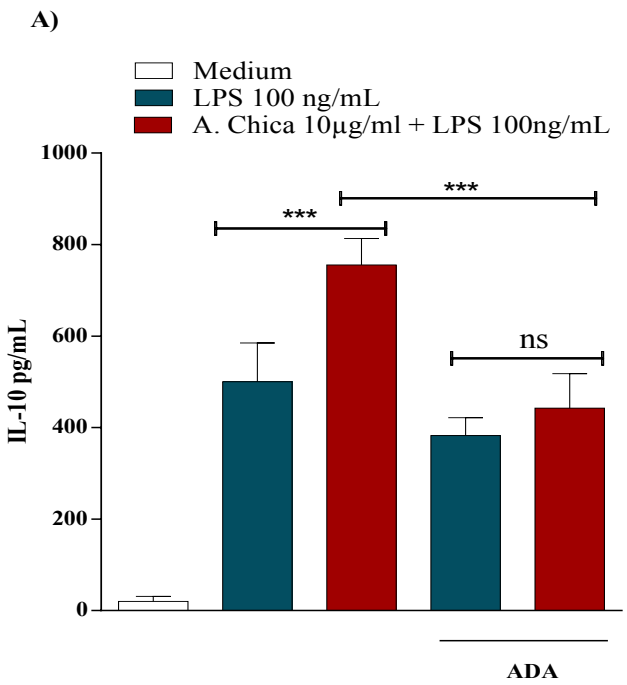

Figure 9: The degradation of adenosine by adenosine deaminase interferes with the effect of *A. Chica* VERLOT in the production of adenosine in BMDM stimulated with LPS. A) BMDM were pre-incubated or not with *A. Chica* VERLOT (10ug/mL) for 4 hours in the presence of Adenosine deaminase or not and activated with LPS (100ng/mL) during 24 hours. The graph represents the media ± SEM, *p<0,05 indicates the values significantly different between the groups.

Adenosine is generated in the extracellular space, from the ATP that release the cell by the pannexin 1 channel. This is the first step in the adenosinergic pathway, thus after observing the effects of the enzyme adenosine deaminase in the production IL-10 stimulated by the *A. chica* VERLOT aqueous extract (10ug/mL) the further step was to inhibit pharmacologically the release of the ATP from the cells, for this purpose we pre-incubated the BMDM with *A. Chica* VERLOT aqueous extract (10 ug/mL) in the presence or not of Carbenoxolone disodium salt CBX (50µM) and stimulate then with LPS (100 ng/mL). We observe that when the release of ATP from the macrophages was impaired by the inhibition of the pannexin 1 channel, the effect of *A. Chica* on IL-10 production was also impaired, the difference between the group stimulated with LPS and the group pre-treated with *A. Chica* VERLOT, was not statistically significant in the presence of CBX, revealing that the ATP is involved in the mechanism of action of this extract (Figure 10).

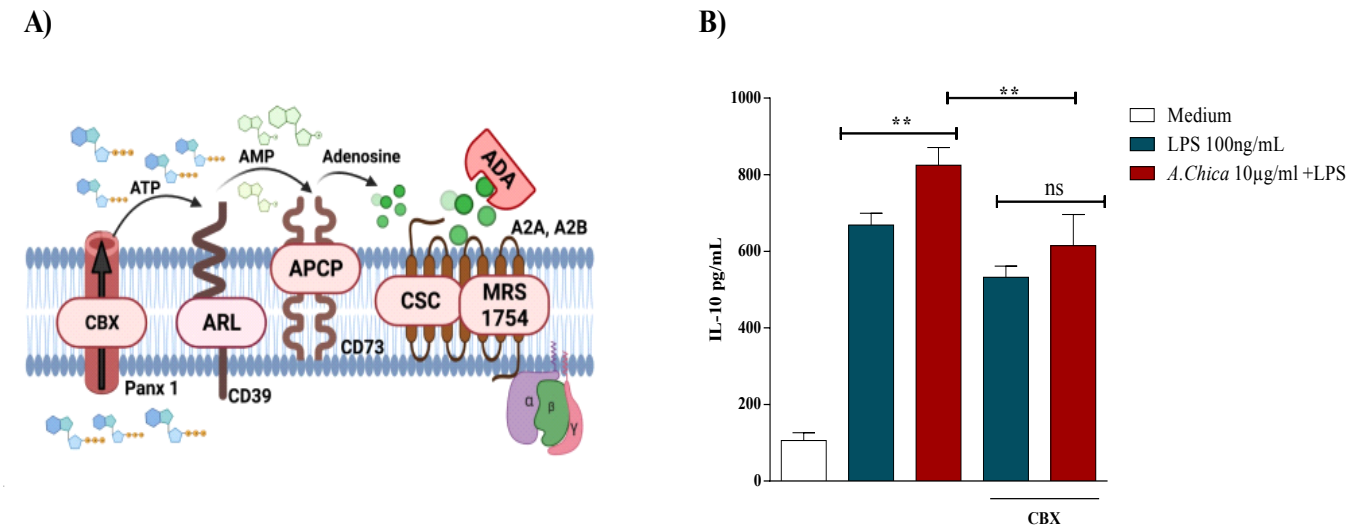

Figure 10: The inhibition of pannexin 1 channels from the BMDM impairs the effects of *A. chica* VERLOT in the production of IL-10. A) Schematic representation of the adenosinergic pathway and its inhibitors. B) BMDM pre-incubated with *A. Chica* aqueous extract for 4 hours and stimulated with LPS (100ng/mL), in the presence or not of CBX (50uM). The graph represents the media ± SEM, *p<0,05 indicates the values significantly different between the groups.

When the ATP is released by the cells, is exposed to the extracellular space, where multiple events can occur, but the macrophages can process this ATP and convert it to adenosine, this effect is mediated by the ectonucleotidases CD73 and CD39, anchored to the extracellular membrane of macrophages, our further step was pharmacologically inhibit this ectonucleotidases, as before, we pre-incubated BMDM with *A. Chica* VERLOT aqueous extract (10 ug/mL) in the presence or not of ARL (inhibitor of CD39) or APCP (inhibitor of

CD73) for 4 hours and stimulate then with LPS (100ng/mL) for 24 hours, the production of IL-10 measured by ELISA in the supernatant of the cells revealed that when the cells were incubated with *A. Chica* VERLOT in the presence of any of this inhibitors (ARL and APCP) the production of IL-10 decrease or was similar, APCP and ARL respectively, to the cells stimulated with LPS(Figure 11 A-B)., thus, the inhibition of this ectonucleotidases interferes with the anti-inflammatory effects of *A. Chica* VERLOT.

To confirm the results obtained with the pharmacological inhibition of CD39 and CD73, BMDM isolated from CD39 KO mice were incubated with LPS in the presence or not of APCP for 4 hours and stimulated with LPS (100ng/mL). The results obtained with the cells belonging to the CD39 KO mice were compared with others obtained from WT mice, both experiments occurred at the same time and with the same experimental conditions, but the effect of *A. chica* VERLOT on the production of IL-10 was abolished in the CD93 KO mice confirming the importance of this ectonucleotidases to the mechanism of action of the extract (Figure 11 C).

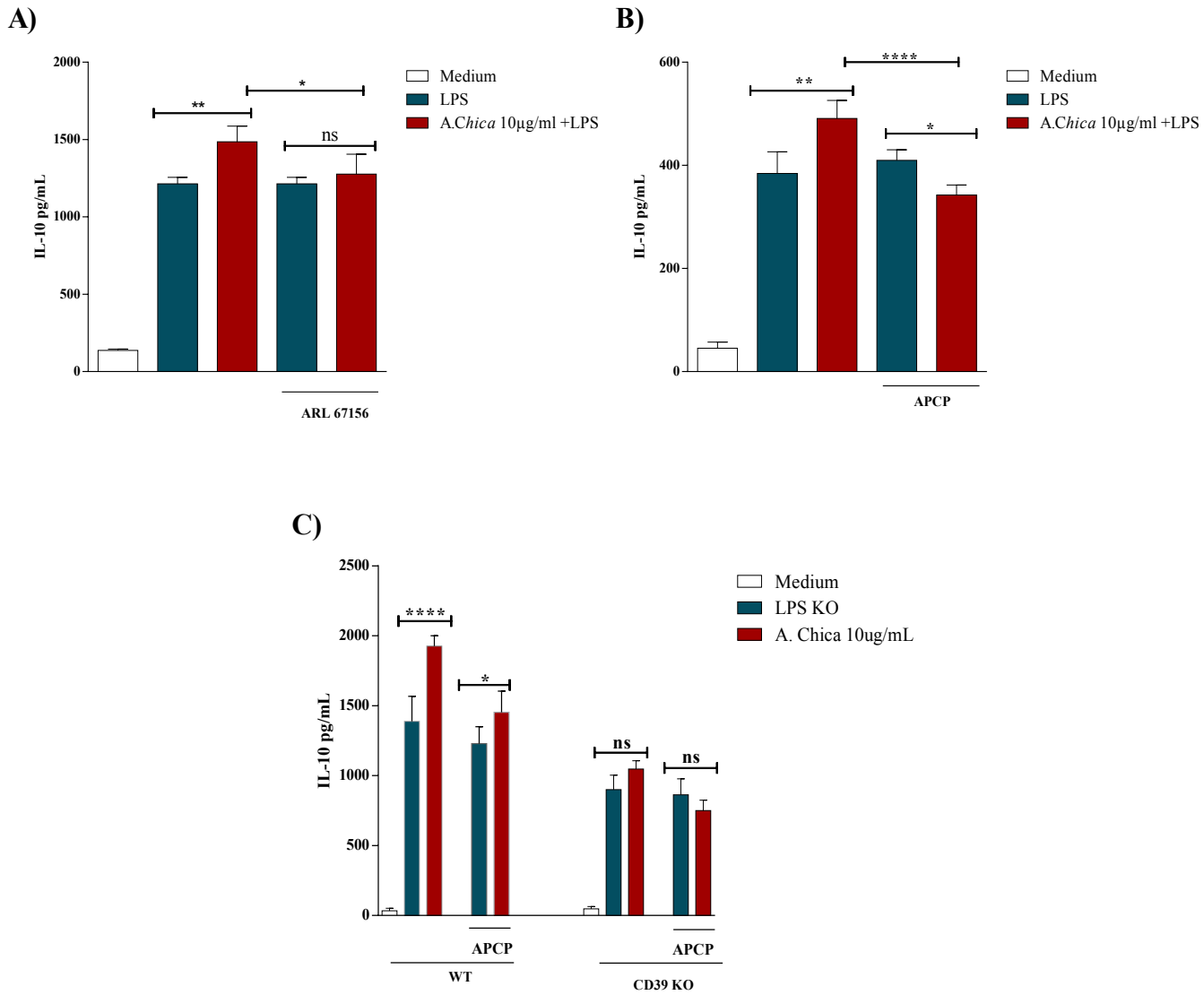

Figure 11: The mechanism by which *A.Chica* VERLOT increases the production of IL-10 in BMDM involves the participation of CD39 and CD73. BMDM isolated from WT or CD39 KO mice and pre-incubated with *A.Chica* VERLOT in the presence or not of the drugs ARL 67156 or APCP and stimulated with LPS. Quantification of A) IL-10 in the presence of ARL 67156 (inhibitor of CD39). B) IL-10 in the presence of APCP (inhibitor of CD73). C) IL-10 production in BMDM isolated or not from CD39 KO mice. The graph represents the media ± SEM, *p<0,05 indicates the values significantly different between the groups.

The expression of CD39 and CD73 was also explored by flow cytometry in BMDM incubated with *A.Chica* VERLOT aqueous extract (10 ug/mL) and stimulated with LPS (100 ng/mL), not revealing any difference in the expression of these proteins between the macrophages pre-incubated with *A. Chica* VERLOT and stimulated with LPS, and the ones exclusively stimulated with LPS (Figure 12 A-C).

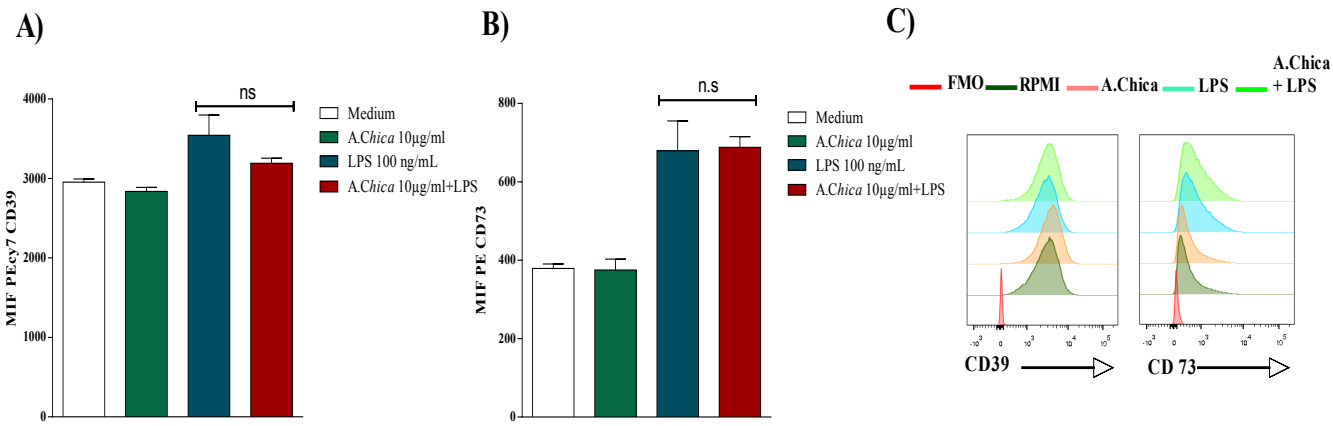

Figure 12: *A.Chica* VERLOT does not interfere with the expression of the ectonucleotidases CD39 and CD73. BMDM were incubated with *A. Chica* aqueous extract and further stimulated with LPS A and B) Quantification of the Median intensity of fluorescence of the fluorochromes PEcy7 (CD39) and PE (CD73) C) expression of CD39 (right) and CD73 (left). The graph represents the media ± SEM, *p<0,05 indicates the values significantly different between the groups.

Our next step was to explore the participation of adenosine receptors in the production of IL-10 induced by *A. Chica* aqueous extract because these receptors are one of the essential points of the pathway giving the fact that without them, adenosine can not be recognized by the cell, to do this BMDM were as before, incubated with *A. chica* aqueous extract (10 ug/mL) in the presence of CSC (inhibitor of $A2_A$) or MRS 1754 (inhibitor of A_{2B}) and 4 hours later, stimulated with LPS (100ng/mL). The production of IL-10 in BMDM stimulated by *A. Chica* aqueous extract was reduced to the level of the medium with any difference with the cells treated exclusively with LPS, evidencing that A_{2A} receptor is crucial for the anti-inflammatory effects of *A. Chica* VERLOT (Figure 13 A). When explored the participation of A_{2B} receptor in the production of IL-10 mediated by *A. Chica* VERLOT, the latter also exhibit an important effect in this phenomenon showed by the impaired effects of the extract on the cytokine after the inhibition of this receptor (Figure 13 B).

The information revealed by the pharmacological inhibition of Adenosine receptors was further confirmed. BMDM isolated from WT and A_{2A} KO mice were incubated with *A. chica* aqueous extract for 4 hours and stimulated with LPS (100ng/mL); when collected and analyzed, the supernatant of the cells revealed that the effects of *A. Chica* aqueous extract in the production of IL-10 were maintained in the cells from WT mice but abolished in the cells

from the A_{2A} KO mice, reaffirming how essential is this receptor for *A. Chica*mediated effects on IL-10 production(Figure 13 C).

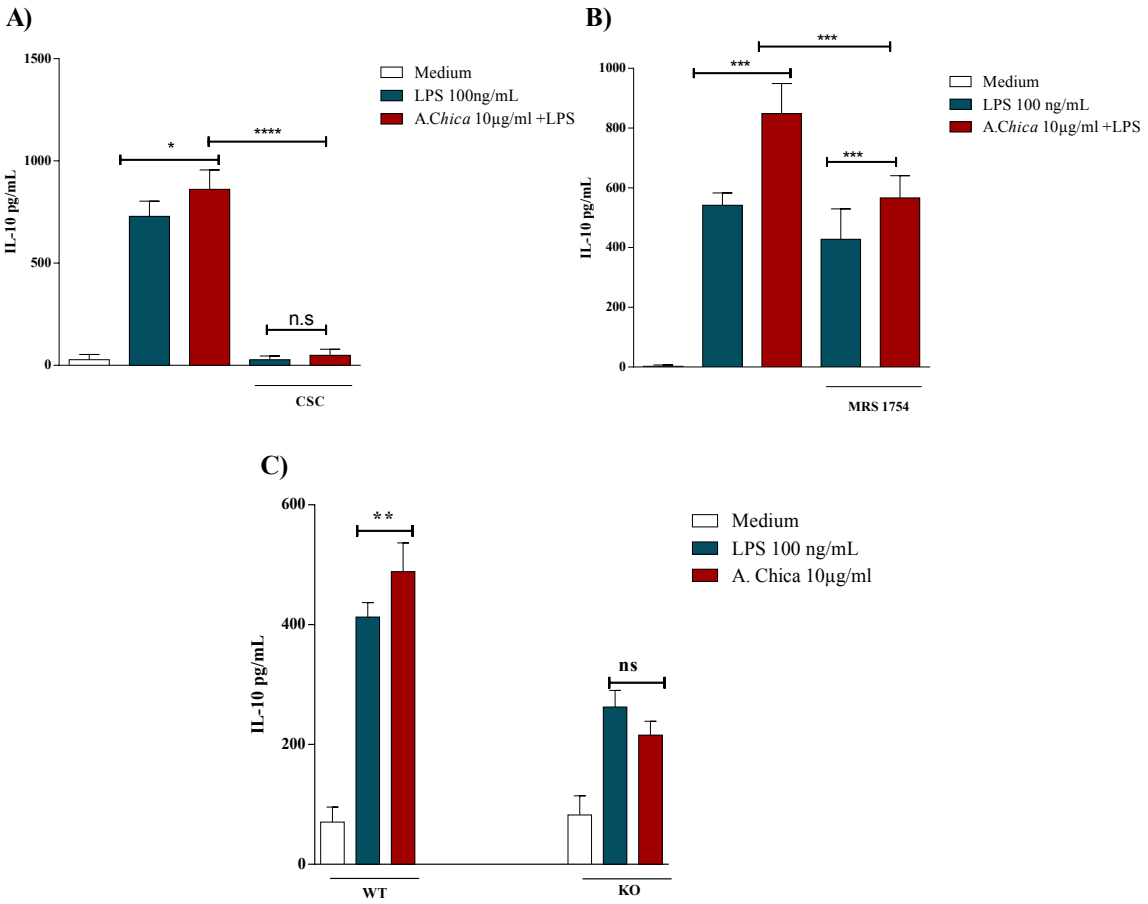

Figure 13: A_{2a} and A_{2b} receptors have one of the prominent roles in the anti-inflammatory response stimulated by *A.Chica* VERLOT. BMDM were isolated from WT or CD39 KO mice and pre-incubated with *A.Chica* VERLOT (10ug/mL) in the presence or not of CSC (CD39 Inhibitor) or MRS1754 (CD73 Inhibitor). Quantification of A) IL-10 in the presence of CSCS. B) IL-10 in the presence of MRS 1754. C) IL-10 in BMDM from CD39 KO mice. The graph represents the media ± SEM, *p<0,05 indicates the values significantly different between the groups.

4.5. *Arrabidaea Chica* VERLOT alters the metabolism of BMDM to generate the ATP that further is directed to the adenosinergic pathway.

The cells have different ways to generate ATP, as is their energy molecule, essential for many functions and also crucial for the immune system. After showing how the adenosinergic pathway interferes with the IL-10 production in the presence of *A. chica*

VERLOT aqueous extract, BMDM were isolated and incubated with *A. Chica* VERLOT aqueous extract for 4 hours and activated with LPS. The cells from the cell culture were harvest and stained for flow Cytometry analysis, The analysis revealed that in the presence of *A. Chica* VERLOT, the mitochondrial mass (mito-Tracker green) and the mitochondrial membrane potential (Mito-Tracker red) increase in BMDM while mitochondrial ROS do not suffer any alteration (Figure 14). This result confirms that the ATP production of the cells is increased in the presence of the extract and that the increase in the mitochondrial mass could be the source of this ATP.

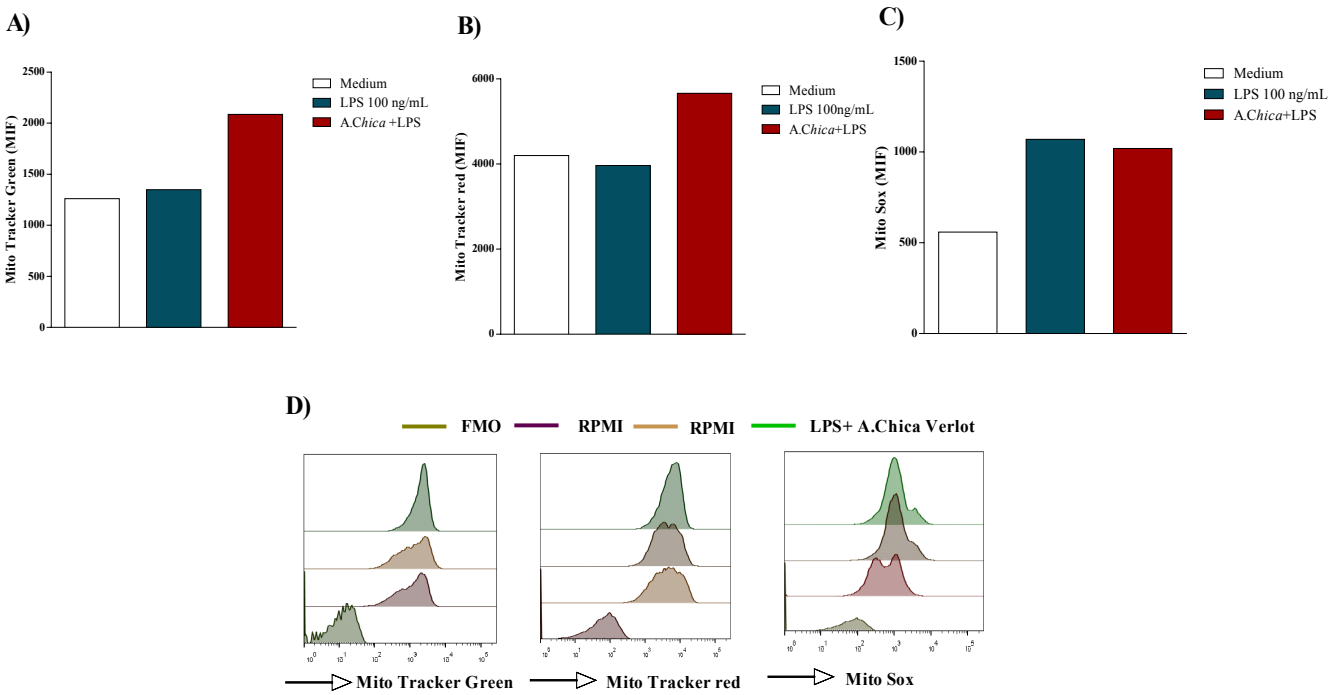

Figure 14: *A.Chica* VERLOT alters mitochondrial parameters important for the generation of ATP. BMDM were pre-incubated with *A. Chica* VERLOT for 4 hours and activated with LPS. 24 hours later, the cells were collected and stain for flow cytometry analysis. A) Median intensity of fluorescence of the mito-tracker green that corresponds to the mitochondrial mass. B) Median intensity of fluorescence of the mito-tracker red that corresponds to the mitochondrial membrane potential C) Median intensity of fluorescence of the mito-sox that corresponds to the mitochondrial ROS. D) Histograms representing the parameters of mass, electric membrane potential, and ROS.

When the cell demand of ATP increase, the lactate production also increase, being the latter a reflex of activation of the basal metabolism of the cell, thus with the help of a colorimetric kit, we proceed to dose lactate in the supernatant of BMDM incubated with *A. Chica* VERLOT (10ug/mL) and activated with LPS (100ng/mL) for 24 hours and observed that when the cells were pre-incubated with *A. Chica* before being stimulated with LPS, the lactate production was greater than the pre-incubated with medium and stimulated

exclusively with LPS. Those above revealed that the *A.Chica* aqueous extract increases the lactate production in BMDM stimulated with LPS(Figure 15 A).

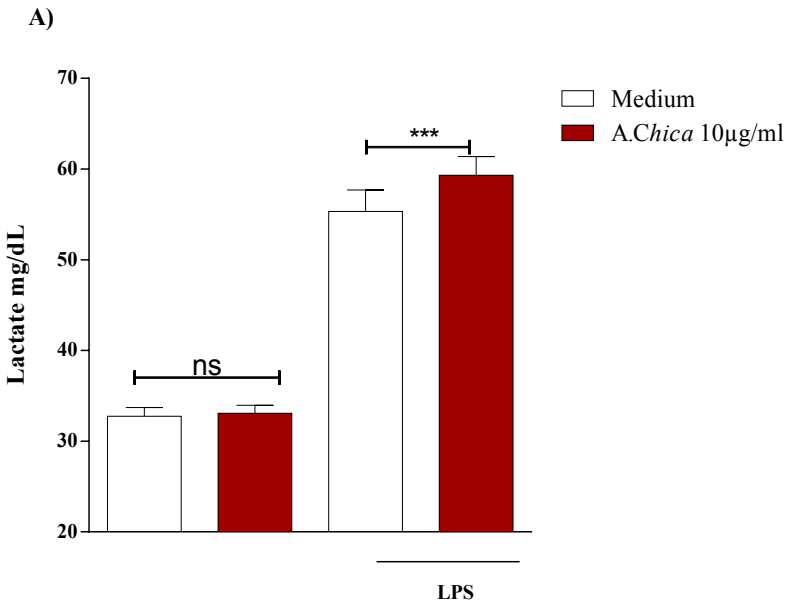

Figure 15: *A. Chica* VERLOT increases the lactate production in BMDM stimulated with LPS. BMDM were incubated with *A. Chica* for 4 hours and stimulated with LPS for 24 hours. A) Quantification of lactate production via colorimetric assay. The graph represents the media ± SEM, *p<0,05 indicates the values significantly different from the LPS group.

4.6. *A.Chica* VERLOT aqueous extract decreases the production of pro-inflammatory cytokines and increases the production of IL-10 in BMDM primed with LPS and stimulated with Monosodium Urate Crystals.

After observing the participation of the adenosinergic pathway in the anti-inflammatory response mediated by *A. Chica* aqueous extract, we proceed to explore the production of the pro-inflammatory cytokines IL-1β and TNFα and the anti-inflammatory cytokine IL-10 in the context of the stimulation with the MSU crystals. The stimulation of macrophages with MSU crystals mimics the process that occurs in the joints of individuals with gout disease and gives us a better idea of how efficient could be *A.Chica* in a model of this disease. BMDM were primed with LPS for 3 hours, then incubated for 1 hour with *A.Chica* aqueous extract (10,30,100,300 ug/mL) and further stimulated with MSU crystals for

5 hours. A. Chica VERLOT increases the production of IL-10 in BMDM incubated with this extract (300 ug/Ml) and decreases the production of IL-1β reaffirming its anti-inflammatory potential and evidencing that it can counter the effects of MSU crystals (Figure 16 A-C).

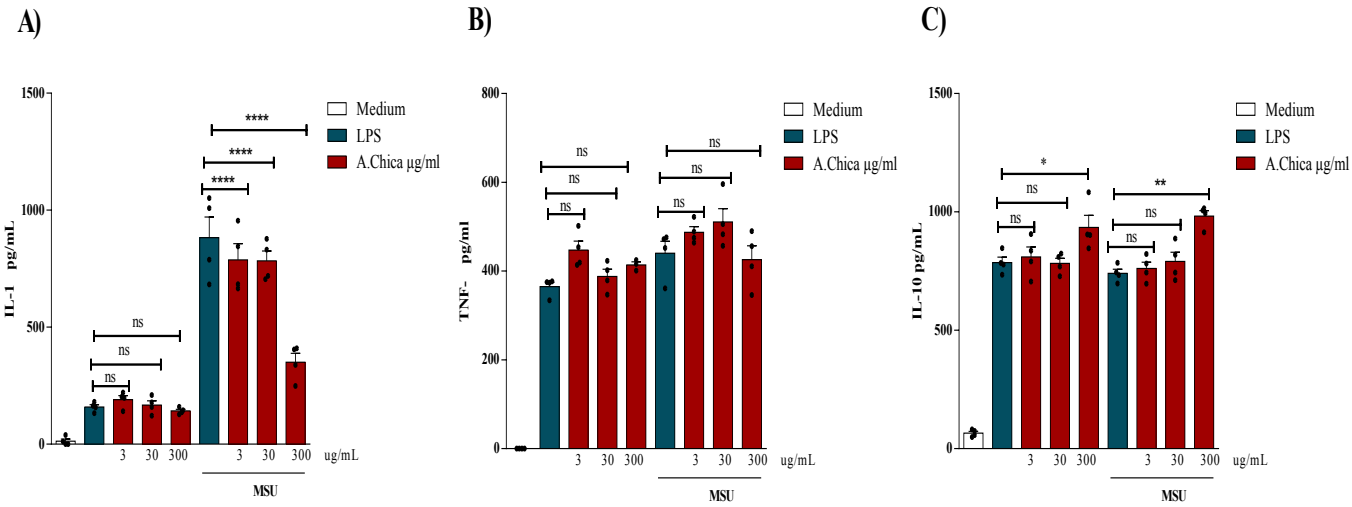

Figure 16: *A. Chica* aqueous extract decreases the production of IL-1β while increases the production of IL-10 and does not alter the production of TNFα in BMDM primed with LPS and stimulated with MSU crystals. BMDM were primed with LPS for 3 hours, incubated for 1 hour with *A.Chica* aqueous extract (3, 30,300 ug/mL), and stimulated with MSU crystals for 5 hours. Quantification of A) IL-1β production in BMDM stimulated with MSU crystals. B) TNFα production in BMDM stimulated with MSU crystals. C) IL-10 production in BMDM stimulated with MSU crystals. The graph represents the media ± SEM, *p<0,05 indicates the values significantly different between the groups.

5. DISCUSSION

Inflammation is one of the most accurate and efficient mechanisms that the body has to defend itself, but understand and control this response has always been a challenge. Back in 1844, Doctor Jhon Hughes Bennett said, “it has been the object of British pathologists to give precision to the old term inflammation, rather than change it for another, perhaps more unsatisfactory”(TRAVERS; BENNETT, 1845), nowadays not too much has changed, give precision to the term is still the object. In understanding the inflammatory response, its control is an important aspect, and now we know that inflammation can occur in an uncontrolled manner and how high is the price that the body pays.

Inflammation is an important component of many diseases like obesity, atherosclerosis, and RA, in the course of these conditions, the mere control of the inflammatory response can impair incredible the evolution of the individuals (WOLF; LEY, 2019; YING *et al.*, 2020). The times we live expose us to pro-inflammatory substances every day, in the fast food that we eat or the air that we breathe. We suffer from inflammation and pain every day; hence, we need to explore new anti-inflammatory drugs. New synthetic drugs appear every day; these drugs are designed to target specific components of the inflammatory cascade, and science has grown exponentially, but it is necessary to go back to the beginning, to those days when the observation of the normal practices of the folks opened the gates for new researches and gave the first step of the controversial and simple scientific method that laid the foundation of modern medicine.

The inflammatory response includes different potential targets, many pro-inflammatory molecules have been described, and some researchers are focus on understanding them to control their activation. On the other hand, the anti-inflammatory molecules represent another attractive target, with the principle of potentiating the mechanisms that the body has itself to control inflammation, which is also a challenge.

Natural products are potential sources of anti-inflammatory drugs; thereby, the fruits, the roots, and leaves that the folks use to prepare herbal teas are subjects of study. *A. Chica* VERLOT is an example of the aforementioned and have been the focus of many kinds of

researches, the characterization of the molecule in 2001 (ZORN *et al.*, 2001) confirm the presence of the anthocyanin 6,7-dihydroxy-5,40-dimethoxy-flavylium or carajurin (responsible for the red color of the extract). Our group characterized the aqueous extract of the *A. chica* VERLOT leaves and identify two important molecules via HPLC, the carajurin and carajurone, these molecules were also identified by other groups before (PAULA *et al.*, 2013). Carajurin and carajurone are anthocyanins and exist evidence showing that this group of flavonoids interferes with metabolic processes (VANZO *et al.*, 2013); therefore, these molecules could be the responsible of many of the effects associated with this plant.

There is scientific evidence that confirms the anti-inflammatory properties of the extract applied to many disease models like osteoarthritis (VASCONCELOS *et al.*, 2019), oral mucositis (QUEIROZ *et al.*, 2018), and wound healing (ARO *et al.*, 2013). Other properties like leishmanicidal have also been attributed to this plant (CORTEZ DE SÁ *et al.*, 2016); among all the investigations that demostrate the properties of this plant, no one has gone deep enough to understand the molecular principles that govern these properties. The plant's safety has been extensively proved (DOS SANTOS, V. C. *et al.*, 2013; GEMELLI *et al.*, 2015), and our investigation group confirms that there is no cytotoxicity associated with *A. Chica* VERLOT.

It is a proven fact that *A. Chica* has anti-inflammatory activity, but what is not that clear is how these effects are driven molecularly. The immune cells have different mechanisms to combat inflammation, and one of them is the secretion of soluble molecules like cytokines. Cytokines are the double-edged swords of the cells, and their secretion needs to be regulated. Exist natural products with the capacity of regulating immune response by regulating the message that the cytokines represent, such as the case of *A. Chica*. Here we demonstrate that the aqueous extract of this plant increases the production of the anti-inflammatory cytokine IL-10 in BMDM stimulated with LPS. IL-10 has immunomodulatory and anti-inflammatory properties that could explain some of the uses that the folks give to this plant. Other natural products share this property, such is the case of *Allium Savitum* which increase IL-10 production in human monocytes while decreasing the production of Th1 cytokines (HODGE; HODGE; HAN, 2002); other is the case of the aqueous fraction of the ethanol extract of *Cissampelos sympodialis Eichl.* (Menispermaceae) leaves which also increase the production of this cytokine (PIUVEZAM *et al.*, 1999).

A. Chica also decreases the production of the pro-inflammatory cytokine IL-1β and tends to decrease the production of TNF-α (not statistically significant) in BMDM, evidencing the anti-inflammatory effects of this extract. IL-1β plays an essential role in inflammation; this cytokine promotes the leukocytes infiltration by upregulating the adhesion molecules in the endothelium, also promotes the type 1 immune response and favors the generation of Th17 cells from Treg lymphocytes, this switch of class results from alternative splicing of FOXP 3 (KULDO *et al.*, 2005; MAILER *et al.*, 2015). The promotion of the inflammatory response favors the clearance of the infections, important to maintain homeostasis. As before mentioned, when the process is perpetuated over time can be detrimental and the overproduction of IL-1β is an important contributor to chronic inflammation, this response is upregulated in diseases like type 2 diabetes and favors the pancreas β islets destruction (BÖNI-SCHNETZLER; DONATH, 2013; MAEDLER *et al.*, 2002). IL-1β also participates in tumorigenesis (COSTES *et al.*, 1998), a clear example of chronic inflammation. Thus inflammation cannot be perpetuated along the time because when this occurs, the damage is unmeasurable and diseases establish. *A. Chica* aqueous extract is widely used by folks to control inflammation and jointly with the increase of IL-10 production, the decrease in IL-1β production observed in vitro is a key component of the mechanism of action of this plant. The aforementioned give us the direction to deeply understand how *A. Chica* mediate its effects. In nature, there are other examples of products that decrease the production of IL-1β, such as the case of *Pinus maritime* (CHO *et al.*, 2001).

One of the mechanisms by which Il-10 production is regulated is the adenosinergic pathway, but the literature describes that flavonoids normally antagonize this pathway, thus we analyze the production of adenosine, the key component of the pathway, in BMDM stimulated with LPS and pre-incubated with *A. Chica.* To our surprise, the extract of this plant which as demonstrated contains anthocyanins in its structure, increase the production of adenosine, assessed by HPLC, and it is known that adenosine has anti-inflammatory properties. The aforementioned does not mean that the effects of *A. Chica* in adenosine production are mediated by the flavonoids contained in the extract, but it is too early to affirm the opposite, and our research group is working on a fraction of the extract.

After discovering that *A. Chica* increases adenosine production in BMDM, we proceed to study the whole pathway. After the extracellular generation of adenosine, this

nucleoside can interact with its receptors and mediate important effects in the cell, which vary from one to the other. Adenosine also suffers the effects of the enzyme adenosine deaminase, which converts adenosine into inosine, which has immunosuppressive actions as well and can be eliminated through the kidneys. Knowing the effects of this enzyme under adenosine production, we incubated BMDM with *A. Chica* but this time in the presence of adenosine deaminase and observed that the effect of the extract in the production of IL-10 significantly decreased, evidencing that adenosine was involved indeed in the production of IL-10 in the presence of *A. Chica*. There are also Flavonoids like hibifolin, which can inhibit the catalytic activity of this enzyme (ARUN *et al.*, 2016)

Adenosine is generated from the ATP that leaves the cell, the release of ATP can result from the damage of the cell membranes, via exocytosis in cells like neurons or through specific channels, which include the pannexin 1, which represents one of the most important (TARUNO, 2018). Nowadays, exist valuable tools to explore this channel's role, which is a direct reflex of the role of ATP, one of them is its pharmacological inhibition with CBX(MICHALSKI; KAWATE, 2016). Here we demonstrate that when this channel is inhibited, the production of IL-10 observed in the presence of *A. Chica* in BMDM decreases significantly, which makes sense because adenosine is generated from ATP, evidencing that the ATP that leaves the cell is an essential contributor to the effect of the extract.

The ATP needs to be cleaved to generated adenosine, and this process is mediated via two important ectonucleotidases in the cell membranes, CD39 and CD73. These molecules are essential for the progression of the pathway; thus our next step was pharmacologic inhibition of them, demonstrating that their activation is essential for the effects mediated by *A. Chica* in the production of IL-10, the role of this ectonucleotidases was confirmed with the CD39 KO. This ectonucleotidases are constitutively expressed in the surface of BMDM, and some substances increase their expressions, such as the case of 8-Bromoadenosine 3',5'-cyclic monophosphate (CD39) and IL-2 (CD39 and CD73) and Uncaria Tomentosa extract (CD39) (SANTOS, K. F. *et al.*, 2016).Here we demonstrate that *A.Chica* aqueous extract, do not interferes with the expression of CD39/CD73, thus the increased production of Adenosine is a direct reflex of increased activity of these ectonucleotidases in the presence of the extract, different than what is observed with quercetin, which inhibits the activity of CD73 (BRAGANHOL *et al.*, 2007).

After the dephosphorylation of ATP and adenosine generation, the latter must interact with the cells to induce its mediated effects. There are four purinergic receptors for adenosine (A_1, A_{2a}, $A_{2b,}$ and A_3) and it is known that flavonoids and phytochemicals can interact with them (JACOBSON *et al.*, 2002; JI; MELMAN; JACOBSON, 1996). This interaction tends to antagonize the effects of adenosine in its receptors, cirsimarin, a flavonoid found in extracts of MicroteaDebilis antagonizes adenosine at the $A_1$and A_2 receptors (HASRAT *et al.*, 1997). Conversely *A. Chica*needs the participation of adenosine receptors to increase IL-10 production in BMDM, a mechanism that mainly relies on the participation of the A_{2a} receptor, demonstrated with the complete reduction of the effect of this extract over the cytokine IL-10 when we inhibit the A_{2A} receptor pharmacologically. The role of A_{2b} receptor was also evidenced but is known that the molecule of adenosine has less affinity to A_{2b} receptor which minimally compensates the lack of A_{2a} receptor when we treat with the extract BMDM isolated from A_{2A} KO mice, thereby confirming the essential role of this receptors. Adenosine interacts with the A_{2a} and A_{2b} receptors, which are coupled to a G stimulatory protein, altering the production of cyclic AMP, which results in inhibition of the NF-kB pathway and increase synthesis of IL-10 and growth factors (NÉMETH *et al.*, 2003; SARAIVA; O'GARRA, 2010).

A. Chica mediates its anti-inflammatory effects by increasing the production of the cytokine IL-10 in BMDM, which is mediated by the adenosinergic pathway, but is also clear that adenosine is generated from ATP. After noticing this pathway's participation and the direct impact of the pharmacologic inhibition of the Pannexin 1 channel in IL-10 production in the presence of *A. Chica*, we hypothesized that the extract could be altering the BMDM metabolism to increase ATP production (BONORA *et al.*, 2012). The ATP is generated mainly from cellular respiration and glycolysis, thereby one of these processes could be altered, leading to increase production of ATP. We observed that both the mitochondrial mass and electric membrane potential increase in the presence of *A. Chica,* but the ROS production remains unaltered, revealing that the increase production of adenosine in BMDM in the presence of *Arrabidaea Chica* VERLOT aqueous extract is a late result of an increase mithocondrial biogenesis.

Another proof of the impact of *A. Chica* on cellular metabolism is the increase in the production of lactate, which is a final metabolite of the glycolytic pathway and is not

generated only under low oxygen conditions, as previously thought. It is known that when the glycolytic rate of the cells increases, this glycolytic product is a direct indicator (SOTO-HEREDERO *et al.*, 2020). The increased mitochondrial mass and lactate production indicates that *A.Chica* aqueous extract alters the metabolism of BMDM to stimulate ATP production.

ATP activate NLRP3 inflammasome, thus we investigate whether the NLRP3 is activated or not in the presence of *Arrabidaea Chica* VERLOT. Aqueous extrac in BMDM primed with LPS and stimulated with MSU crystals demonstrating that the extract counters the stimulus for NLRP3 activation reducing the production of IL-1β. The aqueous extract also increases the production of IL-10 and does not alter the production of TNF-α in this model of assessment. The aforementioned is *in vitro* evidence of the anti-inflammatory potential of the extract because the MSU stimulation of BMDM primed with LPS mimics the process that occurs in the inflamed joints of gout disease patients (RUIZ-MIYAZAWA *et al.*, 2018).

6. CONCLUSIONS

As a conclusion of the set of results presented in this study, we can infer that the aqueous extract of *Arrabidaea Chica* VERLOT has anti-inflammatory properties, demonstrated by its ability to reduce the production of the pro-inflammatory cytokine IL-1β, And increase the production of the anti-inflammatory cytokine IL-10 in BMDM.

The increased production of IL-10 in BMDM in the presence of *A.Chica* is dependent on the production of adenosine, evidenced by the active participation of the adenosinergic pathway. Adenosine results from the dephosphorylation of ATP, and the macrophages themselves are the source of this ATP; therefore, *A.Chica* alters the metabolism of the cells to produce more ATP and subsequently more adenosine.

The aqueous extract of *A. Chica* also has the potential to treat anti-inflammatory diseases such as Gout disease in a dose and time-dependent manner.

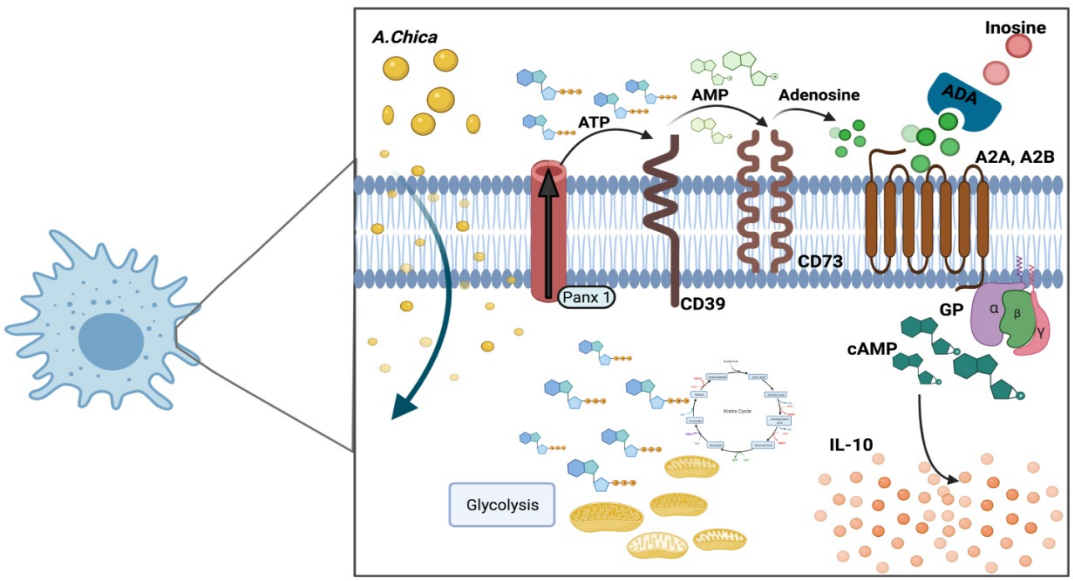

Figure 17: Schematic representation of the Influence of Arrabidaea Chica VERLOT aqueous extract.
Arrabidaea Chica VERLOT aqueous extract interacts with the cells, increasing the rate at which extracellular ATP is converted to adenosine to further pearse the cytoplasmic membrane and increase the production of the anti-inflammatory cytokine IL-10.
Fount: Image designed by the athors.

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APPENDAGE

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A U T O R I Z A Ç Ã O

A CEUA-FMRP autoriza a execução do projeto intitulado: ***"Efeito anti-inflamatório da Arrabidaea chica VERLOT dependente da adenosina."***, registrado com o número do protocolo **117/2020**, sob a responsabilidade do **Prof. Dr. José Carlos Farias Alves Filho,** envolvendo a produção, manutenção ou utilização de animais pertencentes ao *filo Chordata, subfilo Vertebrata* (exceto humanos) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009 e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA). O Protocolo foi **APROVADO** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, em reunião de 31 de agosto de 2020.

Colaboradores: Rosmery Duran Ubiera
Antonio Luiz Boechat
Paula Ramos Viacava
Adriane Dâmares de Sousa Jorge Batalha

Lembramos da obrigatoriedade do Relatório Final, em modelo da CEUA, para emissão do Certificado, como disposto nas Resoluções Normativas do CONCEA.

Finalidade		() Ensino (X) Pesquisa Científica		
Vigência da autorização		31/08/2020 a 12/10/2022		
Espécie/Linhagem	Nº de Animais	Peso/Idade	Sexo	Origem
Camundongo / C57Bl 6	105	22g / 42 dias	Macho	Serviço de Biotério
Camundongo / Balb c	06	22g / 42 dias	Macho	Serviço de Biotério
Camundongo / A2A KO	06	22g / 42 dias	Macho	Centro de Criação de Camundongos Especiais
Camundongo / CD39 KO	06	22g / 42 dias	Macho	Centro de Criação de Camundongos Especiais

Ribeirão Preto, 31 de agosto de 2020

Profa. Dra. Katiuchia Uzzun Sales
Coordenadora da CEUA–FMRP–USP

Comissão de Ética no Uso de Animais
CEUA-FMRP / CEUA-PUSP
Avenida Bandeirantes, 3900 - Ribeirão Preto-SP
CEP 14049-900
Telefones: (16)3602-3301 e 3602-4469

CERTIFICADO DE CAPACITAÇÃO TEÓRICA PARA USO ÉTICO DE ANIMAIS

A Comissão de Ética no Uso de Animais da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (CEUA-FMRP) e da Prefeitura do Campus de Ribeirão de Ribeirão Preto da Universidade de São Paulo (CEUA-PSUP) em parceria com a Comissão Interna de Prevenção de Acidentes (CIPA-FMRP) certifica que o aluno rosmery Duran ubiera, apresentou rendimento considerado suficiente em prova teórica básica referente ao curso Ética no Uso de Animais e Segurança no Ambiente de Trabalho. Ele está autorizado a progredir para o próximo módulo de capacitação prática que deverá ser realizado no próprio biotério e/ou laboratório de experimentação/ensino, sob responsabilidade do orientador e/ou do(s) responsável(is) pelo(s) biotério(s). Ao final desta nova etapa, ele deverá apresentar a esta CEUA um checklist preenchido certificando a aquisição de habilidades necessárias para execução do seu projeto, devidamente assinada pelo orientador e pelos responsáveis pelo biotério.

Ribeirão Preto, 18 junho 2018.

Presidente da Comissão de Ética no Uso de Animais da FMRP/PUSP

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