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ERICLICIA DORALICE AMANCIO BISPO DEIRÓ

BIOLOGICAL AND PHYTOCHEMICAL STUDY OF LEAVES OF *Verbesina macrophylla* (CASS.) BLAKE (ASTERACEAE)

Alagoinhas - BA
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Dissertation presented to the Postgraduate Program in Biosystems Modeling and Simulation at the State University of Bahia as a partial requirement for obtaining the title of Master in Biosystems Modeling and Simulation , M.Sc.

Area of knowledge: Interdisciplinary
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Advisor: Prof. Dr. Edson de Jesus Marques.

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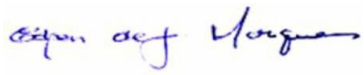

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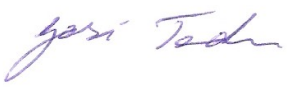

Prof. Dr. José Tadeu Raynal Rocha Filho
Federal University of Bahia - UFBA

To my daughter Júlia and my parents
Edvaldo and Cristina, with all my love.

I dedicate!

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I thank God for giving me life and health to be able to fulfill another dream.

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It doesn't matter where you stopped... at what point in life you got tired...
What matters is that it is always possible and necessary to start over.
Starting over is giving yourself a new chance...
It's renewing hope in life and, most importantly...
Believe in you again. If we strongly desire the best and...
Mainly, we fight for the best...
The best will come into our lives.
Because I am the size of what I see.
And not as tall as I am.”

Carlos Drummond de Andrade

ABSTRACT

Plants are considered promising resources in the treatment of many diseases that plague humanity, especially in the case of bacterial and fungal infections that have been a matter of worldwide concern, due to the increase in microbial resistance to antibiotics available on the market. This perspective is particularly interesting for Brazil, due to the richness of its biodiversity and the traditional use of medicinal plants by the population. Among these medicinal species is *Verbesina macrophylla* (Cass.) Blake (Asteraceae) used in folk medicine for the treatment of bacterial and fungal infections of the urinary tract, respiratory tract, bronchitis, kidney problems, inflammation and fever. In this study, the species was submitted to biological activity assays and phytochemical studies. The antimicrobial activity of crude extracts in hexane, ethyl acetate and ethanol from leaves of *V. macrophylla*, through the microdilution method, revealed activity against Gram positive bacteria (*Bacillus cereus, Staphylococcus aureus, Bacillus subtilis and Micrococcus luteus*), with inhibitory concentration minimum (MIC) between 500 µg/mL and 7.81 µg/mL. The same extracts were inactive against fungi at concentrations up to 500 µg/mL. The fractionation of the extract in ethyl acetate using Column Chromatography (CC) resulted in 51 fractions, analyzed by Analytical Thin Column Chromatography (ADCC), which antimicrobial action can be observed in low polarity against Gram positive bacteria with concentration of 1000 µg/mL and against filamentous (Aspergilus niger) and yeast (Candida albicans) fungal strains. The phytochemical study revealed two compounds (6-O-b-E-p-coumaroyl-4a-hydroxyeudesmane and 6-O-b-Z-p-coumaroyl-4a-hydroxyeudesmane). These substances were active against Gram-positive bacteria with MIC between 2.5 and 3.9 µg/mL and bactericidal action against *S. aureus* and *B. cereus*. The cytotoxicity assay in red blood cells of the crude extracts in hexane and ethyl acetate indicated slight hemolysis only for the hexane extract, demonstrating the toxicological safety of the extracts. The study of microbial growth kinetics of the VM-F19-20 fraction using the time-kill method revealed a concentration-dependent bacteriostatic effect for the *Bacillus subtilis* strain.

Key Words: *Verbesina macrophylla;* biological activity; medicinal plants; sesquiterpenos.

RESUMO

As plantas são consideradas recursos promissores no tratamento de muitas doenças que assolam a humanidade, principalmente no caso de infecções bacterianas e fúngicas que tem sido motivo de preocupação mundial, devido ao aumento da resistência microbiana aos antibióticos disponíveis no mercado. Essa perspectiva é particularmente interessante para o Brasil, em razão da riqueza de sua biodiversidade e ao uso tradicional de plantas medicinais pela população. Dentre essas espécies medicinais está inserida a *Verbesina macrophylla* (Cass.) Blake (Asteraceae) utilizada na medicina popular para o tratamento de infecções bacterianas, fúngicas, do trato urinário, respiratório, bronquite, problemas renais, inflamações e febre. Neste trabalho a espécie foi submetida a ensaios de atividade biológica e estudos fitoquímicos. A atividade antimicrobiana dos extratos bruto em hexano, acetato de etila e etanol das folhas de *V. macrophylla*, através do método de microdiluição, revelou atividade contra bactérias Gram positivas (*Bacillus cereus*, *Staphylococcus aureus*, *Bacillus subtilis* e *Micrococcus luteus*), com Concentração Inibitória Mínima (CIM) entre 500 µg/mL e 7,81 µg/mL. Os mesmos extratos apresentaram-se inativos contra fungos em concentrações até 500 µg/mL. O fracionamento do extrato em acetato de etila utilizando Cromatografia em Coluna (CC) resultou em 51 frações, analisadas através de Cromatografia em Coluna Delgada Analítica (CCDA), as quais a ação antimicrobiana pode ser observada em baixa polaridade contra bactérias Gram positivas com concentração de 1000 µg/mL e frente as cepas fúngicas filamentosa (*Aspergilus niger*) e leveduriforme (*Candida albicans*). O estudo fitoquímico revelou dois compostos (6-O-b-E-p-coumaroyl-4a-hydroxyeudesmane e 6-O-b-Z-p-coumaroyl-4a-hydroxyeudesmane). Essas substâncias foram ativas contra bactérias Gram-positivas com CIM entre 2,5 e 3,9 µg/mL e ação bactericida para *S. aureus* e *B. cereus*. O ensaio de citotoxidade em hemácias dos extratos bruto em hexano e acetato de etila apontou ligeira hemólise apenas para o extrato hexânico, demonstrando a segurança toxicológica dos extratos. O estudo de cinética do crescimento microbiano da fração VMF19-20 utilizando o método time-kill revelou efeito bacteriostático dependente da concentração, para a cepa *Bacillus subtilis.*

Palavras-chave: *Verbesina macrophylla;* atividade biológica; planta medicinal; sesquiterpenos.

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LIST OF ABBREVIATIONS AND ACRONYMS

AcOEt	Ethyl Acetate
ATCC	American Type Culture Collection
BaSO4	Barium sulphate.
CIM	Minimum Inhibitory Concentration
CC	Column chromatography
CCDA	Analytical Thin Layer Chromatography
CTC	Tropical Culture Collection
d	Doublet
dd	Double doublet
t	Triplet
DMSO	Deuterated dimethyl sulfoxide
EHexFV	Hexane Extract from the leaves of *V. macrophylla*)
EAcOEtFVM	Ethyl Acetate Extract from the leaves of *V. macrophylla*)
EEtnFVM	Ethanolic Extract of *V. macrophylla leaves*)
F	Fraction
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
LABEXP	Experimental Biology Laboratory
m	Multiplet
MHz	Megahertz
MTT	Tetrazoline 3-(4,5–dimethylthiazol-2yl) -2,5-diphenyl bromide)
ml:	Milliliters.
NCCLS	National Committee for Clinical Laboratory Standards
WHO	World Health Organization
ppm	Parts per million
RPM	Rotations per minute
^{13} NMR	Carbon 13 Nuclear Magnetic Resonance
NMR H ^{1}	Hydrogen Nuclear Magnetic Resonance
RF :	Retention Factor.
SDC	Defibrillated sheep blood

s	Simple
sl	Simplex _
δ	Chemical shift in ppm
UFC	Colony Forming Units.
UNEB	University of the State of Bahia
UFBA	federal university of Bahia
µg	Micrograms.
µl	Microliters.

SUMMARY

1. INTRODUCTION

The use of plants for medicinal purposes is one of the oldest practices of humanity, many species were used to help cure diseases, with this knowledge being passed on from generation to generation. Thus, they represent the first pharmacological resources to be used in society. The healing properties of plant constituents come from the plant defense system, which generates compounds with varied molecular structures, far superior to those derived from synthetic products (PRADDEPA et al., 2014). Therefore, the plant kingdom is an inspiration for the development of new drugs (NEWMAN; CRAGG, 2012), being the focus of modern research, due to its great chemical diversity, which can provide substances with varied pharmacological effects (WANG et al., 2011; YANG et al., 2017).

A large part of the population uses medicinal plants because it is considered a less aggressive treatment for the body, and as a result of the high cost of synthetic medicines, which makes them less accessible (NÓBREGA et al., 2017). In more isolated communities and poorer regions, medicinal plants stand out as the only therapeutic resource, even though their chemical constituents are not fully known (MACIEL, 2002; BERTINI, 2005). Knowing this, popular use constitutes an important strategy for discovering new drugs (BARNES et al *.,* 2012).

According to the World Health Organization (WHO), approximately 80% of the population uses traditional medicine or herbal medicine to treat their illnesses (KHAN; AHMAD, 2019). In Brazil, in 2006, the Ministry of Health launched the National Policy on Integrative and Complementary Practices (PNPIC), which offered users of the Unified Health System (SUS) services that included Phytotherapy, mainly within the scope of Primary Health Care. (APS) (BRASIL, 2006; MATTOS et al., 2018). Currently, in the public network, the Ministry of Health (MS) promotes the use of 13 herbal medicines (BRASIL, 2020).

The main advantages of herbal medicines are low cost, accessibility and generally fewer side effects. Research carried out with medicinal plants is very important to confirm their safety and effectiveness (BHATTACHARYA, 2017). The evaluation of the biological activity of a plant must cover aspects associated with the pharmacological and toxicological activity of the isolated substances, obtained fractions or total extracts of the plant, this information is fundamental for the transformation of the medicinal plant into a phytotherapeutic product (TOLEDO et al. ,

2003). In countries with diverse flora, there are already several studies on the antimicrobial activities of extracts and essential oils from native plants (HOLETZ et al., 2002; ARAÚJO, 2010).

The diversity of plant species present in Brazil constitutes one of its greatest riches, the country is home to approximately 20% of the total number of species on the planet (CALIXTO, 2003). This makes it possible to study many of these plants to prove their therapeutic action and subsequently develop herbal medicines that help prevent or cure diseases (GIULIETTI et al., 2005; KLEIN et al., 2009). However, even though the Brazilian territory has this rich flora with widespread use of medicinal plants by the population, there remains a need to stimulate research related to the therapeutic potential of plant metabolites (FAGUNDES et al., 2017). Although it is considered to have the greatest plant diversity in the world, Brazil has gaps to be filled in relation to research with an emphasis on natural products, since only 8% of the total species in the country have been studied (GONÇALVES, 2015).

With the emergence of highly resistant bacteria, studies of plant extracts that exert some antimicrobial action provide the opportunity for the development of new agents against difficult-to-treat infections (ELOFF, 1998). Infectious diseases continue to be the leading cause of morbidity and mortality worldwide. One explanation for this fact is that microorganisms have a great capacity to acquire resistance to antimicrobials, a fact that is mainly due to the indiscriminate use, without medical or pharmaceutical guidance, of antibiotics by the population (MADDILA; HEMALATHA, 2017).

Knowing this, it is important to report that substances obtained from species native to Brazil, which present antimicrobial activity and low toxicity, can become a viable alternative for obtaining natural antibiotics, at low cost and accessible to the needy population in the locations where these species are found. (COSTA et al *.,* 2005). Antibiotics originating from natural products generally have complex chemical structures that are important for specific interactions and recognition by macromolecular targets in pathogenic bacteria (WALSH, 2003)

Among the wide variety of plants with medicinal properties are species belonging to the Asteraceae family, which have great value for the pharmaceutical industry, and as a result, have been the subject of much research, in order to serve as a subsidy for new drugs with antimicrobial, anti-inflammatory, antiprotozoal and analgesic action (LORENZI; MATOS, 2021; MARTINEZ et al., 2020).

The *Verbesina* genus, one of the largest in the Asteraceae family with around 300 species (KARIS; RYDING, 1994), has been widely used in traditional medicine for the treatment of various illnesses (MORA et al., 2013). The species *Verbesina macrophylla,* for example, is popularly used in the treatment of infections, kidney problems and fever. Terpenes, monoterpenes and sesquiterpenes have already been isolated from this species (BOHLMANN et al., 1980, MAIA et al., 2011, BEZERRA et al., 2018, DE VERAS et al., 2021). According to Lorenzi and Matos (2021), terpenes are mainly responsible for the antiseptic, anti-inflammatory and antipyretic effects.

Given the above, it can be stated that the investigation of alternative therapeutic compounds from medicinal plants is a necessity. It is of great importance to deepen information about species with potential for the development of new drugs. Therefore, the present study is very relevant, as it contributes to the chemical and biological knowledge of the Asteraceae family.

1.1 Objectives

1.1.1 General objective

Contribute to the knowledge of the chemotaxonomic profile of the Asteraceae family through the biological and phytochemical study of the species *Verbesina macrophylla* (Cass .) B lake (Asteraceae).

1.1.2 Specific objectives

- Determine the Minimum Inhibitory Concentration (MIC) of crude extracts in hexane, ethyl acetate and ethanol from *Verbesina macrophylla leaves* against Gram-positive and Gram-negative bacterial strains, and filamentous and yeast-like fungi;

- Perform chromatographic fractionation of the extract with the best biological activity;

- Evaluate the antibacterial and antifungal activity of the fractions;

- Determine the Minimum Inhibitory Concentration (MIC) of the fractions with the best activity;

- Determine the classification of the antimicrobial action of the fractions with the best activity;

- Evaluate the cytotoxicity in red blood cells of crude extracts;

- Evaluate the microbial death kinetics of the fraction with the best activity against Gram-positive bacteria *Bacillus subtilis* ;

- Carry out the phytochemical study of active fractions;

- Characterize the active components of the purest sample;

2 THEORETICAL FOUNDATION

2.1 Antibiotics and microbial resistance

Antibiotics can be defined as natural or synthetic compounds capable of inhibiting the growth or causing the death of bacteria. Depending on the mechanism of action, they can be classified as bactericidal, when they cause cell death, or bacteriostatic, when they cause the inhibition of microbial growth (WALSH, 2003).

The first antimicrobials were produced synthetically. In 1910, the German bacteriologist biologist Paul Ehrlich made a huge contribution to the treatment of syphilis through the discovery of arsphenamine , a compound that has arsenic in its structure, being marketed as Salvesan (PATRICK, 2005).

However, the milestone in the therapy of bacterial infections occurred with the discovery of Penicillin by the English doctor Alexander Fleming, in 1928 (NICOLAOU, 2008). A few years later, in 1934, proflavin was introduced , an agent widely used in the Second World War, especially against deep wound infections. This compound presented high toxicity for use in systemic bacterial infections, a fact that highlighted the need to search for new agents (PATRICK, 2005).

In 1935, Gerhard Domagk found that the red dye prestasil showed *in vivo* activity against infections caused by Streptococcus species *,* this fact was a milestone in antibacterial chemotherapy. Prontosil gave rise to a new class of antibiotics of synthetic origin , sulfa drugs or sulfonamides . Introduced in the early 1940s, they established themselves as the first class of effective drugs against systemic infections (PATRICK, 2005) .

The therapeutic response of penicillin was superior to that of sulfa drugs and the proof that fungi produced substances capable of inhibiting the proliferation of bacteria motivated a new front of research in the search for new antimicrobial agents: prospecting in cultures of microorganisms, mainly fungi and actinobacteria. . It was only in 1940 that penicillin G, or Benzylpenicillin, was introduced as a therapeutic agent and only then did penicillin begin to be produced on an industrial scale, especially as a result of the Second World War (PROJAN, 2004).

The discovery made by Alexander Fleming began the era of antibiotic therapy, contributing to an increase in the life expectancy of people suffering from infectious

diseases, which were difficult to treat and had high mortality (SCHAECHTER et al., 2002)

The period between 1940 and 1970 is marked by the emergence of new antibiotics, considered the “golden age”. Since then, many others have been developed from different sources, with different targets and mechanisms of action being more efficient and causing fewer side effects (KONG et al., 2010)

According to Walsh (2003), the literature records numerous antibiotics, many of which originated from microorganisms, and although this large portion is produced through these microorganisms, another significant portion resulted from the chemical modification of known antimicrobials, or microbial metabolites. , semisynthetic penicillins and cephalosporins, modified tetracyclines and rifamycins , clindamycin and troleandomycin are just a few examples. The author also reports that there are also antibiotics obtained entirely synthetically, such as chloramphenicol .

Plants also produce a vast number of natural substances with antimicrobial and immunomodulatory potential in an attempt to adapt to environmental aggressions (WILLIAMS, 2001). The search for knowledge about the presence of antimicrobial substances in higher plants gained great momentum after the discovery of penicillin (LIMA, 2001).

Antibiotics play a fundamental role in the treatment of diseases, they are the second most used class of drugs in the world, being responsible for 20 to 50% of hospital expenses, corresponding to a significant portion of prescriptions in outpatient care (SÁEZ-LLORENS, 2000) .

Antimicrobial drugs have different pharmaceutical forms and treatment dosages depending on the pathology. If used independently, consumed in a different dosage than prescribed or interrupted treatment without medical advice, some negative consequences may occur (DEL FIOL et al., 2010). The consequence of this scenario is that the disordered use of antibiotics can lead to serious public health problems, such as increased mortality, due to the emergence of superbugs resistant to antibiotics currently available on the market (MONTEIRO et al., 2020). This resistance has progressively increased since the use of the first antibiotics, posing a threat to an entire century of advances in medicine (LOUREIRO et al., 2016). Above all, due to the indiscriminate use that contributed decisively to the selection of microorganisms and consequently the problem we face: resistance to antimicrobials.

Before the arrival of the 21st century, bacterial resistance predominated in hospital environments. Nowadays, this resistance is associated with different environments and can even affect healthy individuals (WOODFORD, 2005). One possibility to be adopted in an attempt to overcome this problem that increases year after year is the use of associated therapies (FERNANDES, 2006). However, the extensive and often irrational use of antibiotics, precarious hygiene conditions, continuous flow of travelers, the increase in immunocompromised patients and the slow diagnosis of bacterial infections have led to an increase in resistance (VON NUSSBAUM, 2006).

Understanding the biochemical and genetic mechanisms involved in bacterial resistance is extremely important for understanding the process that leads bacteria to develop resistance. Although these mechanisms vary from pathogen to pathogen, resistance is promoted by some fundamental factors: inactivation of the antimicrobial directly in the bioactive molecule by chemical changes, commonly caused by bacterial enzymes (WRIGHT, 2005); modification of the target, which leads to a decrease in sensitivity to the antibiotic (LAMBERT, 2005); changes in the efflux pump and external membrane permeability, which causes a reduction in the antibiotic concentration without its chemical modification (ALLINGTON, 2001); target transfer: which means that certain bacteria become insensitive to some antibiotics due to their ability to transmit the inactivation of a certain enzyme, that is, antibiotics that have mechanisms of action that require enzyme inhibition become inactive because they do not have the target to act on (HAPPI et al., 2005).

It is important to highlight that although the difficulties with discovering antibacterials are already known to the population, the problems with creating antifungal drugs are even more acute (NEWMAN ; CRAGG, 2020) . All new agents are effectively azole-based, while new infectious fungi are being discovered in patients. Data from 2019 from the Center for Disease Control demonstrate that *Candida auris infections* are increasing with resistance to fluconazole, echinocandins (with resistance being established during treatment), this reality is observed even with amphotericin B in around 30% of patients. clinical isolates (NEWMAN ; CRAGG, 2020) . The fact that some antifungals have fungistatic and non-fungicidal action (azoles) may contribute to the emergence of resistant microorganisms (RANG et al., 1997; ZACCHINO et al., 2003).

The Global Action Plan, approved at the World Health Assembly in 2015, establishes strategies that can be adopted to delay the emergence and mitigate the spread of microorganisms resistant to these therapeutic agents, reducing the burden of disease and the spread of infection: prevention of infectious diseases with the use of vaccines; rational use of antibiotics; control and prevention of the spread of resistant microorganisms; discovery and development of new drugs; population awareness; action of hygienic and sanitary measures; decreased use of empirical therapies by health professionals; Furthermore, identifying the genes that cause resistance, as well as their location and diversity, is important for understanding the factors involved in resistance (WHO, 2015).

AMR (antimicrobial resistance) monitoring was carried out using different methods in each country that participated in the 2015 Global Action Plan, and adjustments were made according to local technological and financial conditions. The National Action Plan for the Prevention and Control of Antimicrobial Resistance in the Scope of Single Health (PAN-BR), published by the Ministry of Health in 2018, describes that the monitoring of antimicrobial resistance in Brazil will take place through the implementation of a network national integrated information system for the surveillance and monitoring of AMR in the sphere of human health, through laboratory safety methodologies and models, guiding clinical protocols for treatment and analyzing epidemiological trends (BRASIL, 2018).

Although several initiatives have been developed and are in place to combat Bacterial Resistance, the solutions are still insufficient to measure the severity and impact of infections (ESTRELA, 2018). In this sense, Souza (2008) adds that given the growth of drugstores and pharmacies, supervision becomes more complex in these establishments, demonstrating in research that the Collegiate Board Resolution (RDC) no. 20 of July 25, 2011, which controls the sale of antibiotics, do not prove to be as effective, since prescription control is not equally monitored in all places of sale.

There is no doubt that antibiotics markedly reduced the human mortality rate as a result of infections, as well as preventing the occurrence of many other pathologies, contributing to the health and well-being of the population during the last half of the 20th century (RIPOLL-COZZANO, 2002). However, bacterial resistance resulting from the indiscriminate use of antimicrobials will always be a serious public health issue, but in order to maintain minimal control over this global problem, professionals need to be

increasingly aware of this evil and responsible for their day-to-day duties with patients. Still, it will not be the complete solution (OLIVEIRA, 2020).

Given this scenario, it is possible to state that the importance of researching the application of new substances obtained from the extraction of active principles from different plant species lies in the possibility of finding ways to combat pathogens, which constantly acquire resistance to industrialized antibiotics. (GONÇALVES et al., 2011). In this context , Newman and Cragg (2020) report that natural products still offer the best options for finding new active compounds, or models that can help cure a variety of diseases that affect humanity. In line with this statement, the authors also describe that of the 162 antibacterial agents approved between January 1, 2015 and September 30, 2019, just over 48% of this total fell into the categories of Unaltered Natural Product (N) or Product Derived Natural (ND), while 22.2% of these agents were completely synthetic.

With regards to antifungal agents, there are two natural product-based compounds in phase III trials that may increase natural product statistics in the relatively near future, one of which is enfumafungin, a triterpene that led to the semi-synthetic ibrexafungerp , which is in four current phase III trials. It is necessary to emphasize that phase III studies on anti-infective agents are randomized comparative studies in sick patients compared with the best current treatment (NEWMAN ; CRAGG, 2020).

2.2 Secondary metabolites and bioprospecting potential

The set of chemical reactions that occur in each cell is called metabolism. Chemical compounds that are synthesized, degraded or modified are called metabolites (SIMÕES et al., 2010), which in turn can be divided into primary metabolites and secondary metabolites (WAKSMUNDZKA-HAJNOS et al., 2008).

Primary metabolism promotes a set of reactions that perform essential functions for the survival of plants. Among the compounds that are involved in this process are proteins, carbohydrates, amino acids and nucleic acids. These molecules will play an important role in regulating biological activity, respiration, photosynthesis and solute transport (CUNHA et al., 2016; DE ARAÚJO et al., 2018).

On the other hand, secondary metabolites are substances produced in a smaller proportion, when compared to primary ones, and which are not always involved in the

vital functions of the plant or even present in all of them. Furthermore, they are known to be produced in specific types of cells and at different stages of plant development, which makes their isolation and purification strenuous (UNDERHILL et al., 1980).

Secondary metabolism compounds can be stored in different parts of the plant's organs. These molecules are extremely diverse. Each family, genus, and species produces a characteristic chemical category or a mixture of them, which in turn can be used as taxonomic characters in the classification of plants (WAKSMUNDZKA-HAJNOS et al., 2008). These chemical constituents favor seed dispersal, act as antibiotics, antifungals and antivirals, protecting the species from pathogens. There are also compounds that have great relevance in absorbing ultraviolet light, preventing leaves from being damaged (VIZZOTO et al., 2010; FUMAGALI et al., 2008).

It should also be noted that secondary metabolites act in interactions that occur between plants, in competition for resources (allelopathy), including when this competition occurs between individuals of the same species (autopagy), so that the association of specific metabolites can be toxic to the plant that is affected (POSER; MENTZ, 2010).

Knowing this, it can be said that the production of secondary metabolites is strongly influenced by the environment, and depending on environmental conditions, it is diverted to different routes resulting in products of multiple forms and variations during this influence. The modifications occur as a response of plants to several factors such as: seasonality, circadian rhythm, development; temperature; water availability; altitude and nutrients (GOBBO-NETO; LOPES, 2007). Other possible sources of interference in this production are: ontogenetic factors, which include variation in concentration and composition according to the age and stage of development in which the plant is; genetic factors, since secondary metabolites are biosynthesized from pre-existing primary metabolites, and for these transformations to occur, molecular catabolism reactions are essential, which is carried out through cellular enzymes, and these are expressed by genes, which They are guided, like other cellular processes, by genetic control (LEITE, 2009).

Secondary metabolites are divided into three large chemically distinct groups: phenolic compounds, terpenes and alkaloids (TAIZ; ZEIGER, 2009). They are classified according to their chemical structure: having an aromatic ring, and may or may not have sugar units; composition, that is, whether or not it contains nitrogen; and the route by which they are biosynthesized, which would be the origin of the plant or

their solubility in different solvents (BRESOLIN; CECHINEL FILHO, 2003; WAKSMUNDZKA-HAJNOS et al., 2008).

Terpenes originate from mevalonic acid (in the cytoplasm) or pyruvate and 3-phosphoglycerate (in the chloroplast). They are chemically formed mainly by unsaturated hydrocarbons designated as "natural alkenes", with varying degrees of oxygenation (alcoholic, ketonic, etc.) in the substituent groups linked to the skeleton of the basic carbon chain (FELIPE; BICAS, 2017; HARBORNE, 1999). They are known for their vast industrial applications, including: perfume fixative, solvents, raw material for the production of paints, greases and waxes, in addition to being part of popular medicine, acting in the cure of many illnesses (HARTMANN, 2007; FELIPE; BICAS, 2017).

Phenolic compounds are characterized by having at least one aromatic ring in their structure with one or more hydroxyl substituent groups (CUNHA et al., 2016). They can be synthesized by two metabolic pathways, the shikimic acid pathway and the mevalonic acid pathway. The class of phenolic compounds of secondary metabolites has several biological effects, including: antioxidant, anti-inflammatory, antitumor action, inhibition of collagen damage, reduction of serum cholesterol, stimulation of the immune system, among others (CUNHA et al., 2016 ; FUMAGALI et al., 2008; VIZZOTTO et al., 2010).

Alkaloids are cyclic organic molecules that have at least one nitrogen (N) atom in a negative oxidation state and whose distribution is limited among living organisms. They are pharmacologically active compounds and are mostly found in angiosperms (HENRIQUES et al., 2002). These bioactive substances have a range of physiological effects, including antimicrobial activity and a pronounced effect on the nervous system. Bioactive substances correspond to active principles common in medicinal and toxic plants and can be used as poisons and hallucinogens (FUMAGALI et al., 2008; VIZZOTTO et al., 2010; LOPEZ et al., 2012). These metabolites arouse great interest among researchers, due to the group's chemical heterogeneity, restricted distribution in nature and impressive bioactive potential (ROBBERS et al., 1997)

The substances produced by secondary metabolism stand out due to the diversity of chemical structures already reported as well as their bioactivity and wide applicability (NEWMAN; CRAGG, 2012). From a pharmaceutical perspective, the main interest in the secondary metabolism of plants is the high number of pharmacologically important substances. Many techniques can be used to detect and quantify secondary

metabolites in samples of plant species, and studies involving spectrophotometric methods are the most practical, reproducible and accessible than other methodologies, allowing the investigation of compounds in the ultraviolet or visible region (PEIXOTO SOBRINHO et al., 2011).

The characterization of these metabolites generally includes classical phytochemical methods that involve the purification and subsequent structural determination of the isolated substances. These methods are quite efficient, however they consume a lot of time, but the large amount of data currently available on secondary metabolites isolated from plants in conjunction with spectroscopic and spectrometric techniques makes it possible to analyze a large number of samples with a reasonable characterization of the main classes of secondary metabolites (JANSEN et al., 2010; KIM et al., 2011; OKADA et al., 2010). The study of the metabolic profile of plants is covered by a variety of analysis techniques, however the combination of H ^{1} Nuclear Magnetic Resonance (NMR) and principal component analysis (PCA) has been widely used (FLORES-SANCHEZ et al ., 2009; KIM et al., 2010; LIU et al., 2010).

The therapeutic potential of secondary metabolites contributed to vegetables being used for centuries in folk medicine as a way to prevent, treat and cure diseases (BRAIBANTE et al., 2014). The isolation of these compounds as well as the determination of biological activity may provide greater information, stimulating their possible use as a method of disease control (SCHWAN-ESTRADA et al., 2003). These comprehensive biological effects of metabolites present in plants demonstrate that it is essential to know them more and more, as a better understanding of how these molecules act can lead to countless research possibilities that direct the search for solutions to important global problems currently faced, such as for example, combating microbial resistance to synthetic drugs available on the market (BEZERRA, 2008).

2.3 Botanical considerations

2.3.1 Asteraceae Family

Asteraceae is considered one of the largest families of Angiosperms, covering between 24,000 and 30,000 species allocated between approximately 1600 and 1700 genera. With a cosmopolitan distribution, it is present on all continents, and can be

found mainly in tropical, subtropical and temperate regions, with the exception of Antarctica (FUNK et al., *2009*).

In Brazil, the Asteraceae Family is represented by approximately 2,000 species and 300 genera, however, it is believed that these values are underestimated. These plants can be identified in the form of herbs, subshrubs, bushes, small trees or Eudicot lianas (SOUZA; LORENZI, 2008; DO NASCIMENTO , 2020). Among the Angiosperms, the family is especially recognized by the presence of capitulum-type inflorescences surrounded by bracts, by the fusion of the anthers around the style forming a ring, and by the cypsela-type fruit, often accompanied by a set of apical appendages called papus. (FUNK et al., 2009). It has a greater occurrence of species in grassland formations, being less significant in humid tropical forests and lowlands (JEFFREY, 2007).

In folk medicine, many remedies are extracted from species of *Arnica, Calendula* and *Echinacea* . *Mikania* Species are used as remedies for snake bites. *Calea divaricata* (Benth), is used in Venezuela to treat fever. Species of *Acmella* , from South America, and *Salmea scandens* (L) DC., from Central America, are used to relieve toothache (PRUSK; SANCHO, 2004). *Baccharis ,* whose species are popularly known as carqueja is a genus of great relevance, as it has high socioeconomic value, being potentially indicated as a source of volatile oil for industrial use (VERDI et al., 2005). *Baccharis tridentata* (Vahl), for example, is cited by Correia (1926), as a febrifuge and diuretic). Carqueja, (*B. dracunculifolia*), is used to combat gastric disorders, physical fatigue, lack of appetite, feverish conditions and organic weakness (MORS et al., *2000* ; SILVA JÚNIOR, 1997).

Among the best-known species of the Brazilian cerrado we can mention *Lychonophora ericoides* (Mart) known in common sense, as arnica, used in folk medicine to combat inflammation and *Vernonia ferruginea* (Less) [= *Vernonanthura ferruginea* (Less .) H. Rob.], honey plant whose leaves are considered diuretic and whose flowers can be used in perfumeries (ALMEIDA et al., 1998).

Many scientific works carried out with species from the Asteraceae family, resulted in the isolation of a variety of secondary metabolites, with emphasis on flavonoids, allocated as important chemotaxonomic markers (EMERENCIANO et al., 2001). However, the chemical-biological potential of species in this family can be lost due to deforestation, which is worsened by the fact that some of its species are endemic (TELES et al., 2009).

According to Cronquist , (1988) the evolutionary success of this family can be partially attributed to the development of a chemical defense system that incorporates a combined production of secondary metabolites originating from polyacetylenes, sesquiterpene lactones , carbohydrates such as oligosaccharides, alkaloids, terpenoids , flavonoids, in addition to phenolic acids, benzofurans and coumarins (EMERENCIANO et al ., 2001).

Sesquiterpene lactones are striking compounds of essential oils and Asteraceae resins (SPRING, 2000), they are one of the groups of secondary metabolites responsible for the anti-inflammatory, analgesic, antimicrobial and antitumor activities of a variety of medicinal plants, these actions being investigated and proven through pharmacological trials (LORENZI; MATOS, 2002; SIEDLE et al . , 2003). This phytochemical potential is mainly responsible for the socioeconomic relevance of plants from the Asteraceae family in traditional medicine, as well as justifying the fact that they are widely studied regarding their chemical composition and biological activity, with some of their compounds being used for the production of pharmaceuticals. and insecticides (VERDI et al., 2005). In addition to medicinal use, many species are used in food, cosmetic production or even as ornamental plants (ROQUE, 2008).

2.3.2 Genus *Verbesina*

The genus *Verbesina* is classified in the tribe Heliantheae , (subtribe Verbesininae), has around 300 species distributed throughout the Americas, with the majority occurring in Mexico, Brazil and the Andes. The species belonging to this genus are of great economic importance due to their chemical properties (BEZERRA et al., 2018; PANERO; CROZIER, 2016).

A study comprising 47,000 occurrences of flavonoids, corresponding to 800 different compounds, using a computational system specially developed for chemotaxonomic purposes records that the maximum expression of flavonoids in Asteraceae is found in Heliantheae (EMERENCIANO et al., 2001). In this sense, several studies indicate that flavonoids, in addition to playing an important role in plant survival, may be mainly responsible for the antimicrobial potential (ROZATTO, 2012)

Verbesina can be found , with confirmed occurrences in the Northeast, Center-West, Southeast and South regions (MOREIRA, 2022). They can be characterized by

subshrub to shrub representatives, more rarely arboreal, with alternate, rarely opposite, sessile or petiolate leaves, with entire, pinnatifid or pinatipartite blades, serrated to toothed margin, with capitulescences corymboid to panniculoid (MOREIRA; CAVALCANTI, 2020).

The *Verbesina genus* is differentiated by presenting substances such as eudesmane derivatives and esterified sesquiterpenes , often as cinnamates or coumarates . The first to be isolated were α- and β- verbesinol coumarates extracted from the roots of *Verbesina subcordata .* (DC), collected in Paraguay. Several sesquiterpene compounds were isolated, among them, derivatives of germacradiene , and several cinnamates derived from eudesmane (JAKUPOVIC et al., 1987). Agra in (2007), reported that sesquiterpenes , diterpenes and triterpenes were isolated in species belonging to this genus.

Verbesina species have been widely used in traditional medicine by communities in the preparation of drinks, teas, infusions and extracts, as a form of treatment for diabetes, hypertension, infections and inflammations (MORA et al., 2013), with some biological activities already validated. , such as the antibacterial and anti-inflammatory activities of *V. turbacensis* (GUALTERI et al., 2005; LOBITZ et al., 1998), antiparasitic and antitumor activities of *V. encelioides* (EZZAT et al., 2016; AL-OQAIL et al., 2016), antibacterial activity of *V. negrensis* (MORA et al., 2015) and antihypertensive activity of *V. caracasana* (BOTTA et al., 2003).

The species *Verbesina encelioides* (Cav.) Benth and Hook plant native to North America, known for presenting toxic activity, causing death in cattle and sheep, its toxic principle is guanidine galegin (3-methyl-2-butenyl-guanidine) (AMARO-LUIS et al., 2002). Toursarkissian , (1980) mentions that *V. encelioides* It has anti-hemorrhagic and healing activity. Roig (2001) adds that this species has ophthalmic properties.

2.3.3 *Verbesina macrophylla species* (Cass .) Blake

The first description of the species *Verbesina macrophylla* (Figure 1) was made by Cassine , later revised by Blake (MONDIN, 2015). It is characterized by being a subshrub plant, with winged branches, alternate leaves, varying from 12-23 x 5-8 cm, deltoid , spatulate and divided into horizontal segments; copious, corymbose and panicled chapters; ligule small and lanceolate, but wide at the tip (BAKER, 1882). It is recognized by its pinatipartite leaves, the wide, branched paniculoid inflorescence, the

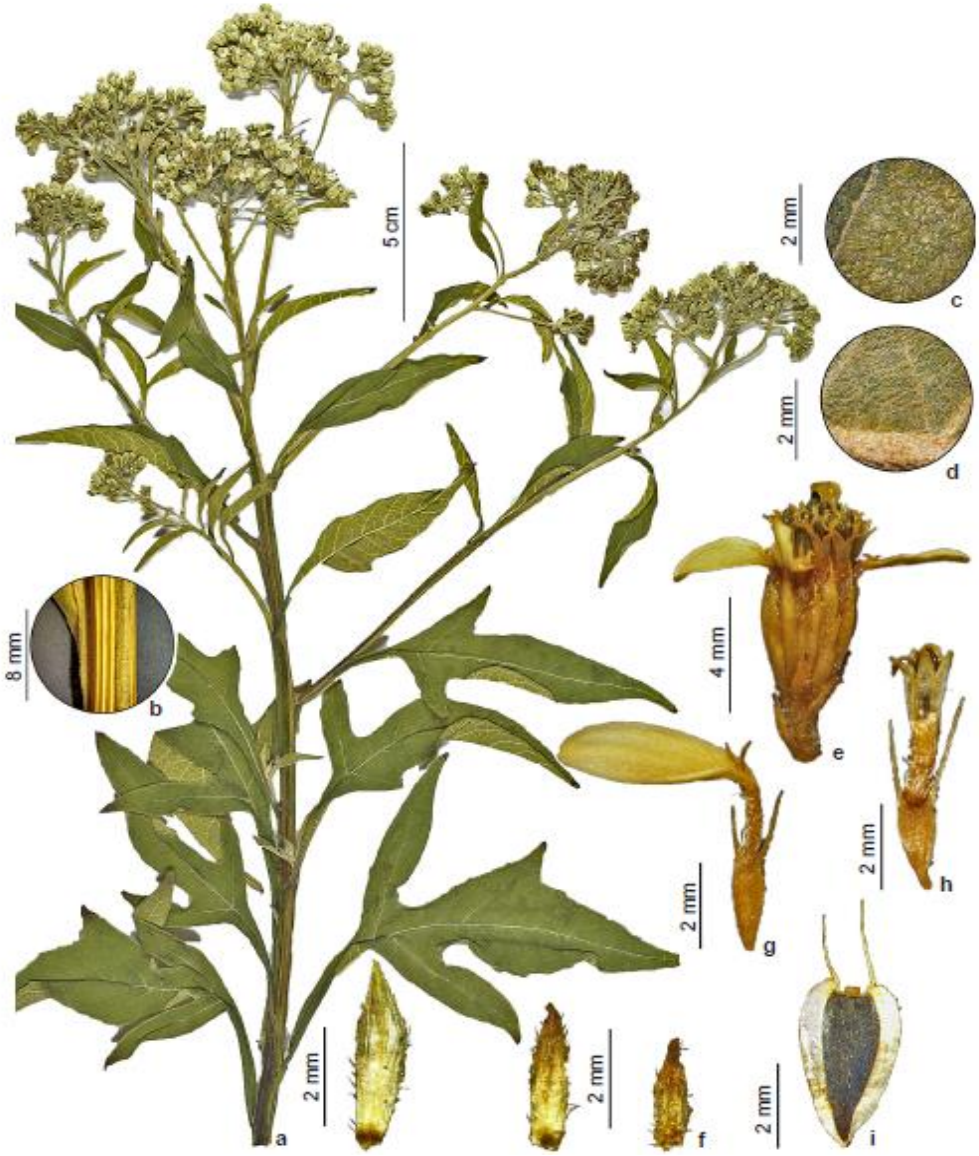
radiated capitulums with white ray flowers and a cylindrical envelope. In nature, it is possible to smell the strong sour odor dispersed by the capitulescences (MOREIRA; CAVALCANTI, 2020).

With wide distribution in South America, it has confirmed occurrences in the Northeast (Alagoas, Bahia, Ceará, Paraíba, Pernambuco, Sergipe), Southeast (Espírito Santo, Minas Gerais, Rio de Janeiro) and South (Paraná) regions of Brazil. It occurs from sea level to approximately 1,500 meters of altitude, in large populations, and can easily be observed on roadsides and in degraded environments. Flowering and fruiting occurs more concentrated from June to September, and October to February (MOREIRA; CAVALCANTI, 2020).

V. macrophylla is popularly known in the Northeast region of Brazil as “assa pesca”, it is a species with wide distribution in areas of caatinga, rupestrian fields and cerrado. It occurs in sandy, clayey and dry soils and is known by traditional communities for its healing powers in the form of teas, infusions and liqueurs, for the treatment of bacterial infections and fungal infections of the urinary and respiratory tract, bronchitis, kidney problems, inflammation and fever (BOHLMANN et al., 1980; BEZERRA et al., 2018).

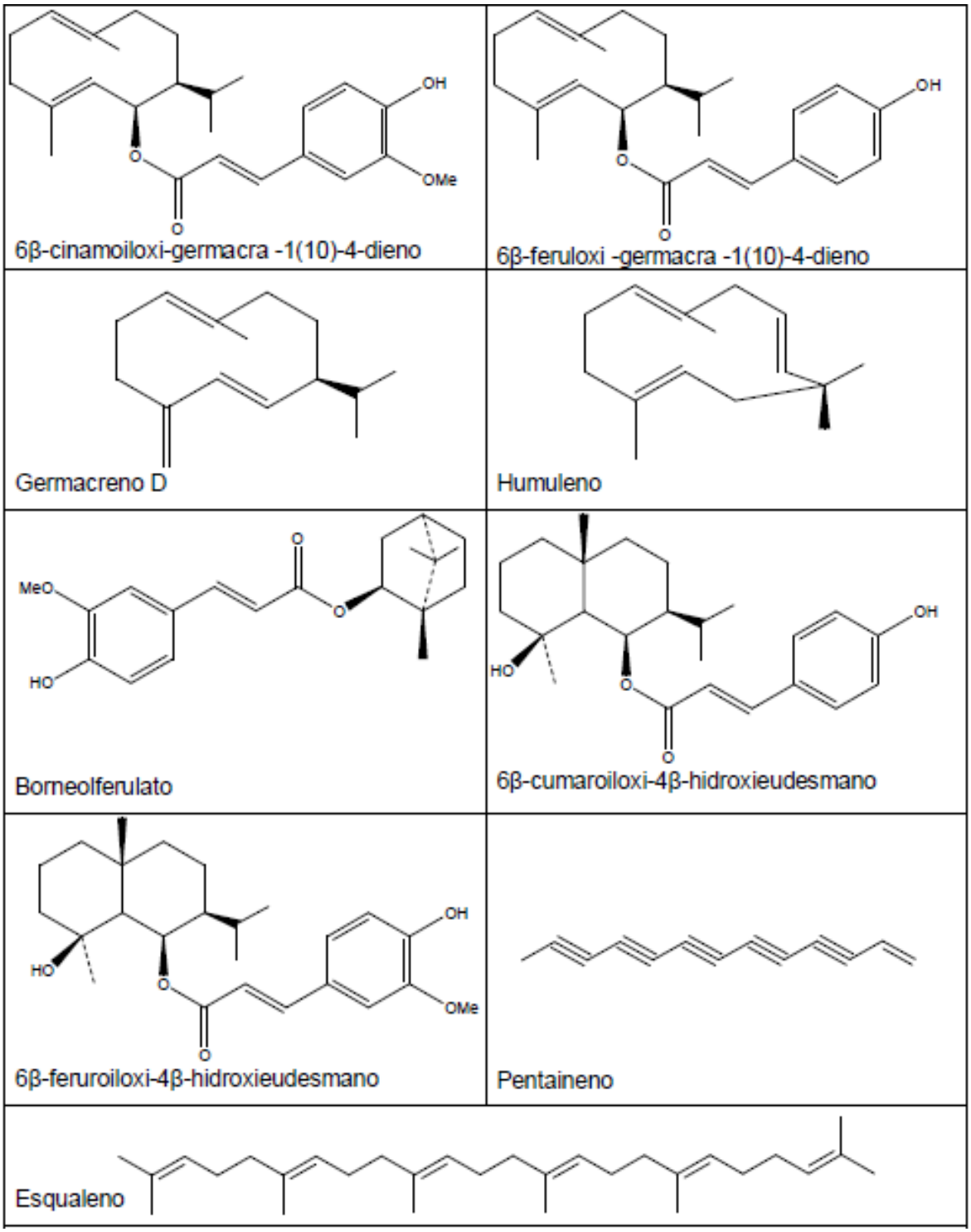
The literature reports phytochemical analyzes that revealed the presence of terpenes: 6 β - coumaroyloxy - germacra-1; 4-diene; 6 β -feruloyloxy-germacra-1; 4-diene; 6 β - coumaroyloxy- 4 β - hydroxyeudesmane ; 6 β -feruloyloxy- 4 β - hydroxyeudesmane ; _ germacrene D; squalene ; epoxy-caryophyllene ; humulene ; pentainene ; bornyl ferulate (BOHLMANN et al . , 1980) (Figure 2). monoterpenes: bornyl -p-trans- coumarate and bornyl -p-cis- coumarate (MAIA et al., 2011) (Figure 3). Studies on *V. macrophylla essential oil* pointed out the presence of sesquiterpenes , with the main components being germacrene D, germacrene D-4-ol, (E) - caryophyllene , δ - cadinene and bicyclogermacrene (BEZERRA et al., 2018, DE VERAS et al., 2021). According to data from the study presented by De Veras et al. (2021), the essential oil of this species presented antimicrobial, anti-inflammatory activity and antipyretic effects found with toxicological safety. These findings suggest that further studies should be carried out to evaluate the biological potential of *V. macrophylla* in originating phytomedicines , since publications related to their biological activity are scarce.

Figure 1: Details of the species *Verbesina macrophylla* – a. fertile branch; B. winged branch; w. adaxial face; d. abaxial face; It is. chapter; f. involucral bracts; g. ray flower; H. disc flower; i. cypsela.



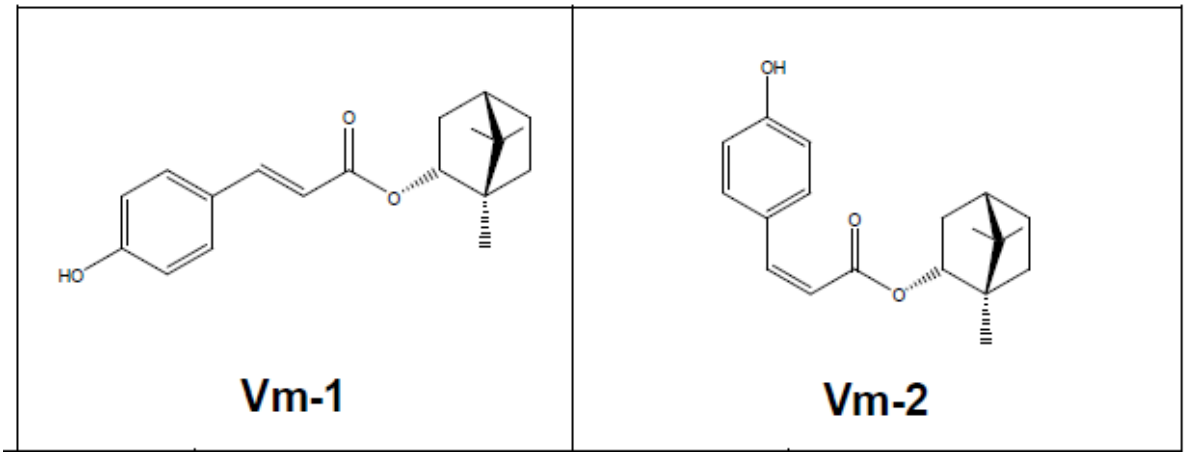
Source: (MOREIRA; CAVALCANTI et al., 2020)

Figure 2: Substances isolated from *Verbesina macrophylla* by Bohlmann and collaborators in 1980.



Source: (BOHLMANN et al., 1980)

Figure 3: Substances isolated from *Verbesina macrophylla* by Maia and collaborators in 2011, monoterpenes: bornyl p- *trans* - coumarate (Vm-1) and bornyl -p- *cis* - coumarate (Vm-2).



Source: (MAIA et al., 2011)

3 RESEARCH METHODOLOGY

3.1 Collection and identification of botanical material

The botanical material (leaves) of *Verbesina macrophylla* (Cass.) Blake was collected in August 2021 in the municipality of Alagoinhas – Bahia, in the area of the State University of Bahia (UNEB) – Campus II. The collection site was georeferenced with the following coordinates (12°10'42"S and 38°24'43"W). The identification was carried out by taxonomist professor Dr. Gracineide Selma Santos de Almeida. An exsiccate of the species collected was prepared and registered in the HUNEB Herbarium of the State University of Bahia under registration number No. 14858.

3.2 Processing of botanical material

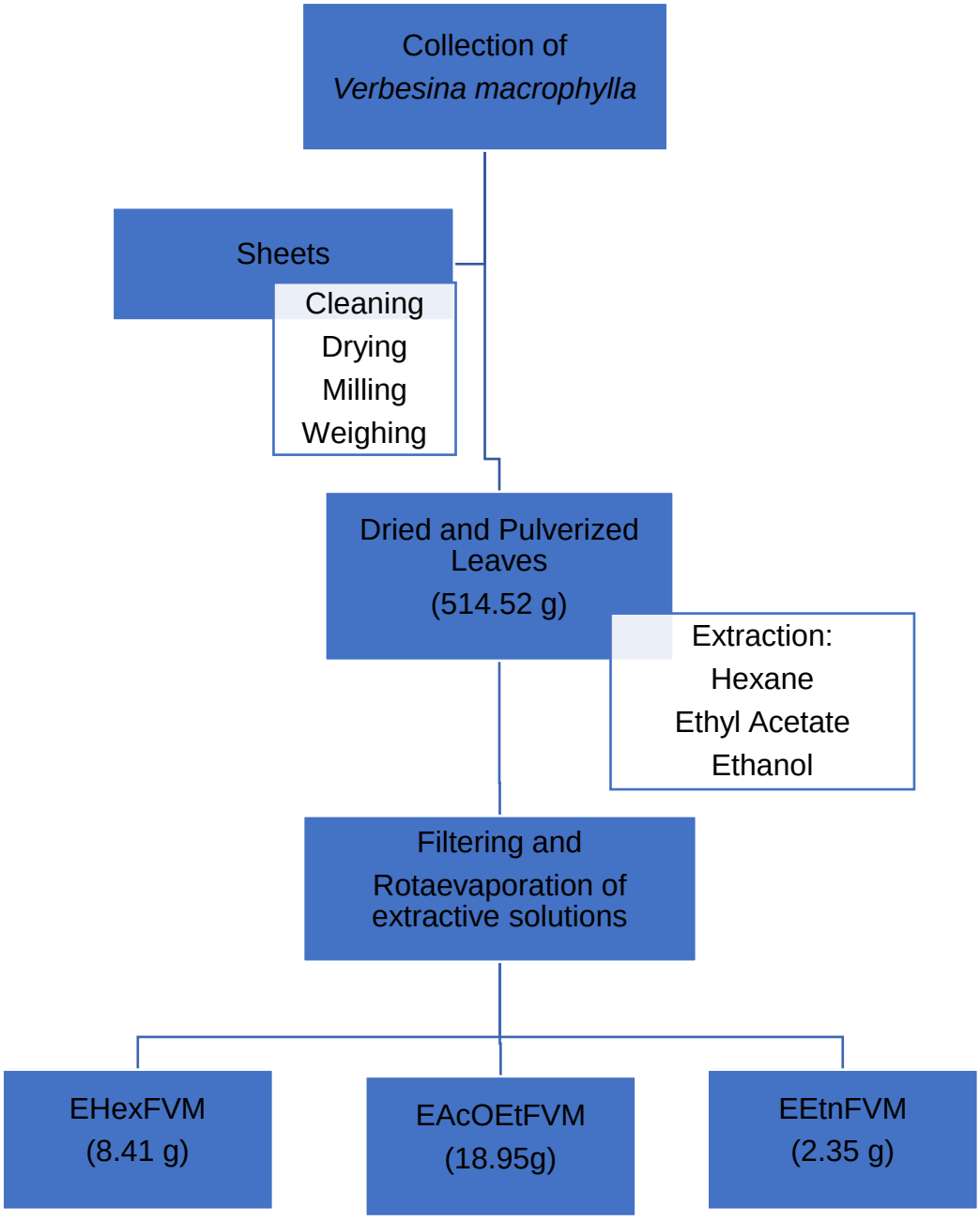
The leaves were washed, then dehydrated in an oven with circulating air at an average temperature of 40°C for five days, after complete drying they were crushed. The dried and pulverized material from *Verbesina macrophylla* weighed 514.52 g.

3.3 Obtaining extracts

The dried and pulverized plant material was subjected to cold extraction using hexane, ethyl acetate and ethanol in increasing order of polarity. This procedure consisted of keeping the material inside a glass container immersed in solvent, stirring occasionally, and after a period of 72 hours it was filtered. This process step was repeated three consecutive times for each solvent used to maximize extraction.

The extractive solutions obtained were concentrated with the aid of a rotary evaporator at an average temperature of 45°C. After complete evaporation of the solvents, crude hexane, ethyl acetate and ethanolic extracts were obtained. The resulting extracts were weighed and coded and named: EHexFVM (Hexane Extract from *Verbesina macrophylla leaves*) EAcOEtFVM (Ethyl Acetate Extract from *Verbesina leaves macrophylla*) and EEtnFVM (Ethanolic Extract of *Verbesina leaves macrophylla*) Figure 4 shows the route followed to obtain these extracts .

Figure 4: Scheme of obtaining hexane, ethyl acetate and ethanolic extracts from *Verbesina macrophylla* (Vm) .



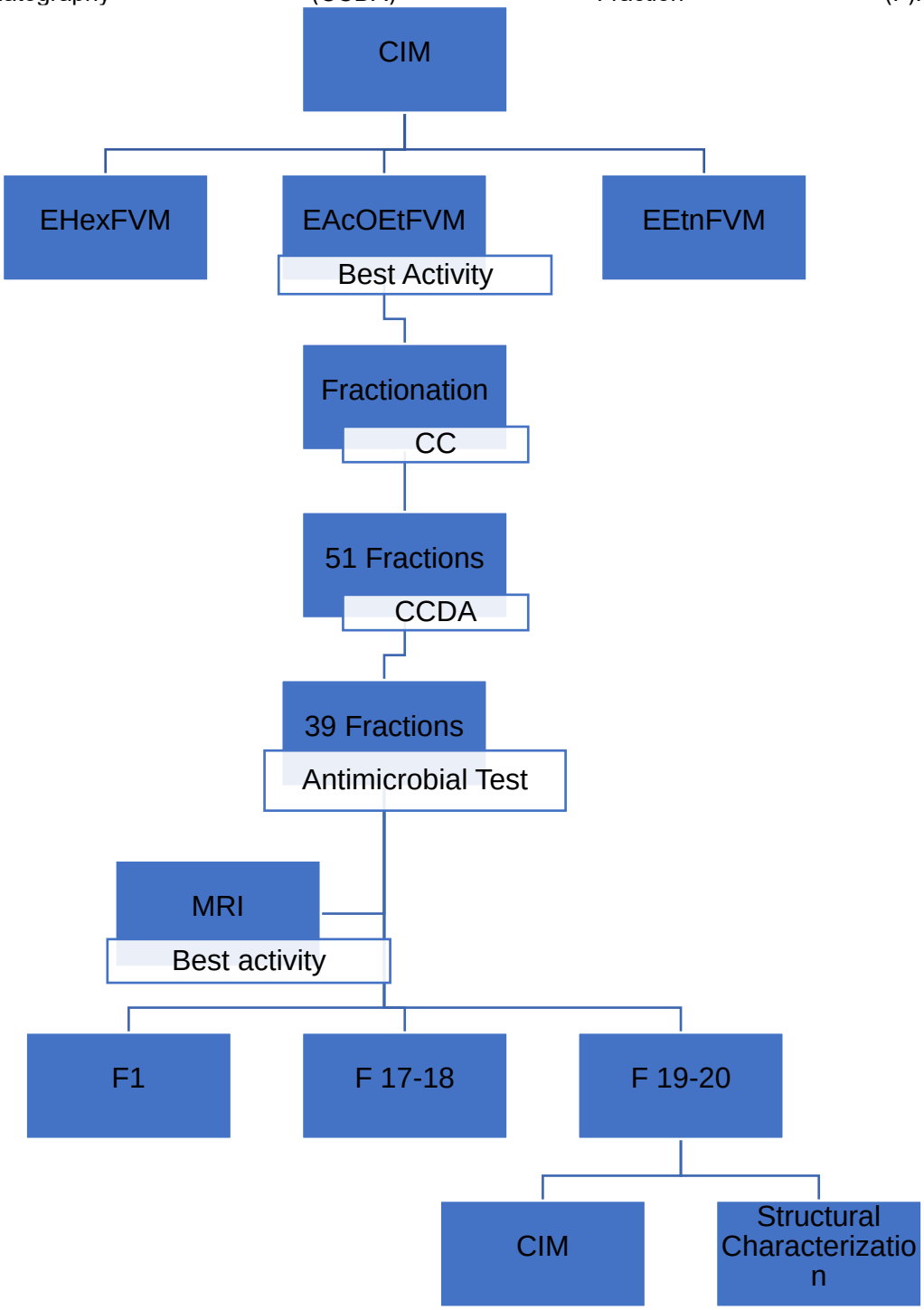
Source: Prepared by the author (2022)

3.4 Isolation and purification of chemical constituents

The isolation and purification of the chemical constituents of *V. macrophylla leaves* were guided by bioassays, also taking into account the greater mass availability

of the extract. At this stage of the work, the procedures presented in Figure 5 were carried out.

Figure 5: Isolation and purification process of the chemical constituents of *Verbesina macrophylla* (Vm). Minimum Inhibitory Concentration (MIC); Column Chromatography (CC); Analytical Thin Layer Chromatography (CCDA) Fraction (F).



Source: Prepared by the author (2022)

3.4.1 Chromatographic analyzes

The purification and isolation of the chemical constituents of *V. macrophylla* were carried out using chromatographic methods: Column Chromatography (CC) and Analytical Thin Layer Chromatography (ACC).

Column chromatography consists of a cylindrical glass tube in a vertical position, using Silica Gel 60 (MACHEREY – NAGEL) as the stationary phase (particles with 0.063-0.2mm/ 70-230 mesh) and for the mobile phase the solvents hexane, ethyl acetate and methanol alone or in systems with an increasing polarity gradient.

Analytical Thin Layer Chromatography was used to analyze the fractions obtained by CC. These chromatographies were carried out on glass plates. The fixed phase was prepared with a suspension of Silica Gel 60 (MACHEREY – NAGEL) w/TLC, UV 254 nm, in distilled water. After preparation, the chromatoplates were dried in the open air and activated in an oven at 120°C for two hours.

The substances on the chromatoplates were revealed through exposure to an ultraviolet radiation lamp at two wavelengths (254 and 366 nm) in a Mineralight device, model UVGL – 58 and by impregnating the plates in chambers saturated with iodine vapors.

3.4.2 Chromatographic fractionation of the crude extract of Ethyl Acetate from the leaves of *Verbesina macrophylla.*

For this procedure, an aliquot of 14.4 g was removed from the ethyl acetate extract, which was subjected to a chromatographic column and the partition process was carried out with solvents of different polarities, starting with hexane, followed by ethyl acetate and methanol, alone or in systems of different proportions.

51 fractions of 100 ml each were collected, then labeled and coded as follows: *Verbesina macrophylla* Fraction 1 (Vm-F1), Vm-F2 ... Vm-F51. The fractions were concentrated in a rotary evaporator and analyzed in CCDA. Table 1 demonstrates the grouping of fractions that was carried out according to their retention factors (Rf's).

Table 1: Chromatographic fractionation of the ethyl acetate extract from the leaves of *Verbesina macrophylla* (Vm) . Hexane (Hex); Ethyl Acetate (AcOEt).

FRACTION	ELUENT	PROPORTION	REUNITED
1-3	Hex	100	
4-7	Hex:AcOEt	99:1	2-6

8-14	Hex:AcOEt	98:2	7-9; 11-12; 13-14;
15-17	Hex:AcOEt	97:3	15-16
18-20	Hex:AcOEt	96:4	17-18; 19-20
21-23	Hex:AcOEt	95:5	21-22
24-41	AcOEt	100	23-24
42-51	Methanol	100	

Source: Prepared by the author (2022)

3.4.3 Structural characterization of isolated chemical constituents

The characterization of the chemical constituents isolated from *V. macrophylla* was carried out through the analysis of spectra obtained from Nuclear Magnetic Resonance and the results found were compared with literature data.

The experiments were carried out in the Organic Chemistry laboratory of the Federal University of Bahia (UFBA) . To obtain the NMR spectra, VARIAN-NMR-SYSTEM 500 MHz spectrometers were used. The solvent used to dissolve the samples was Deuterated Chloroform ($CDCL_3$) .

shifts (δ) were expressed in parts per million (PPM) and coupling constants (J) in Hz . The diversity of signals in MRI ^1H were outlined according to the convention: s (simplet), sl (wide simplet) d (doublet), dd (double doublet), t (triplet) and em (multiplet).

In vitro biological assays

3.5.1 Assessment of antimicrobial activity

The experiments to evaluate the antimicrobial activity of the crude hexane, ethyl acetate and ethanolic extracts, as well as the fractions obtained from the leaves of *V. macrophylla* were carried out at the Experimental Biology Laboratory (LABEXP) , located at the State University of Bahia (UNEB).). The method used to carry out the tests was the broth microdilution technique, in 96-well microplates, based on the CLSI/NCCLS M7-A6 document (NCCLS, 2012).

The bacterial strains were initially seeded in Petri dishes containing Mueller Hinton Agar, using the plate exhaustion technique (HENRY, 1995). In the experiment, the culture medium used for bacteria was Nutrient Broth and for fungi Yeast Broth and Malt/Malt Broth. For this assay, the following microorganisms were used: Gram-positive: *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 1024), *Bacillus cereus* (CCT 0096), *Staphylococcus aureus* (ATCC 6538). Gram-negative: *Escherichia coli* (ATCC 94863) and *Pseudomoas aeruginosa* (ATCC 25619). The fungal strains used were *Aspergillus niger* (ATCC 16404), *Candida glabrata* (ATCC 00720) and *Candida albicans* (ATCC 18804).

3.5.1.2 Preparation of the sample stock solution

The stock solution for each extract was prepared at a concentration of 2000 µg/mL. The diluent used was Dimethyl Sulfoxide (DMSO) at 20% v/v in water. Homogenization was carried out with the aid of a tube shaker.

3.5.1.3 Preparation of the microorganism suspension

The suspension of each microorganism tested was prepared by removing an aliquot of the recently cultivated biomass, with the aid of a platinum loop, and transferred to a test tube containing 10 mL of saline solution. After homogenization, this suspension was compared with the turbidity of a Barium Sulfate ($BaSO_4$) solution , corresponding to 0.5 on the McFarlend scale. The suspension of the microorganism was diluted in culture medium, so that 100 µL of this suspension was transferred to a Petri dish containing 10 mL of the medium, so that it could then be distributed into the microplate wells.

3.5.1.4 Microdilutions

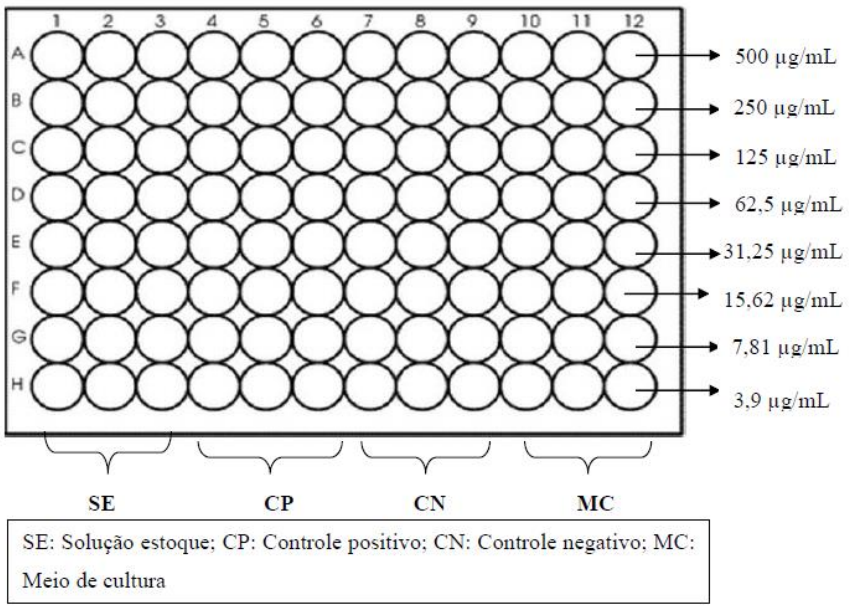
Microdilutions were carried out by distributing 100 µL of the appropriate culture medium for the microorganisms under test, using a multichannel micropipette, into the wells of the plate. The sample stock solution was tested in triplicate, with 100 µL of it being distributed at a concentration of 1000 µg/mL in wells A1 to A3. As a positive control, 100 µL of the antibiotic Chloramphenicol 500 µg/mL were used for the assay

with bacteria and cyclopiroxolamine 500 µg/mL for fungi, deposited in wells A4 to A6. For the negative control, 100 µL of 5% v/v DMSO in distilled water was used in wells A7 to A9. Wells A10, A11 and A12 received only culture medium, to confirm the sterility of the plate.

Serial dilutions were performed with a dilution factor of two, obtaining the following sample concentrations: 500; 250; 125; 62.5; 31.25; 15.62; 7.81 and 3.9 µg/mL (Figure 6), with the aid of a multichannel micropipette, initially homogenizing (3x) the solutions from the wells in row A and subsequently transferring 100 µL to row B and from there to C, and so on until the last row (H), with 100 µL being discarded at the end of the dilution.

In the final stage of serial dilution, 100 µL of the bacterial suspension was distributed into all wells of the plate, obtaining a final volume of 200 µL in each well. Soon after, the microplate is covered and incubated in an oven for 24 hours at 37°C. After this period, the results were read.

Figure 6: Schematic of the 96-well plate for determining the MIC .



Source: Prepared by the author (2022)

3.5.1.5 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration was interpreted as the lowest concentration of the antimicrobial agent that completely inhibited the growth of microorganisms in the wells, as detected by the naked eye by observing the turbidity

of the wells when there is bacterial and/or fungal growth (NCCLS, 2012), as well as such as through the MTT test [3-(4,5-dimethylthiazol- -2yl)-2,5-diphenyl tetrazoline bromide] one of the most used methods due to its sensitivity to detect mitochondrial viability and, consequently, cell viability (MOSMANN, 1983).

3.5.1.6 Determination of the antimicrobial activity of fractions obtained from the crude extract of Ethyl Acetate

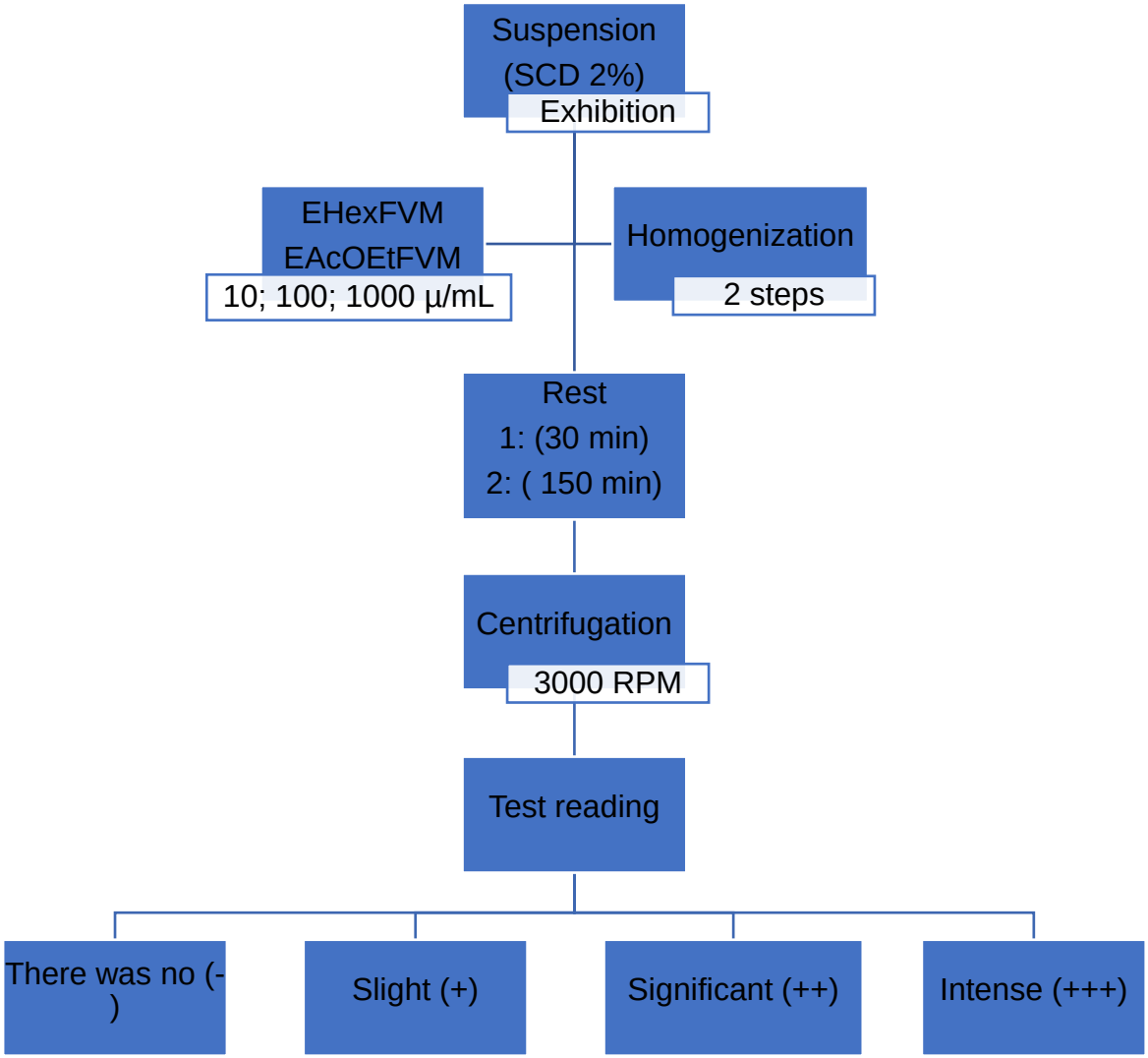
The analyzes were carried out following the NCCLS standard (2012). Initially, all fractions were tested at a concentration of 1000 µg/mL without serial dilutions being carried out. After reading the results, fractions with positive antimicrobial activity were selected and then subjected to new tests to determine the minimum inhibitory concentration in the extract.

3.5.2 Cytotoxicity test with red blood cells

The determination of hemolytic activity was carried out according to the methodology of the Brazilian Pharmacopoeia (2010), as shown in Figure 7. This test consists of checking the cell's metabolism when it is exposed to a certain substance. In the procedure, extracts in Hexane and Ethyl Acetate were added at concentrations of: 10, 100 and 1000 µg/mL, to a 2% suspension with defibrillated sheep blood (Laborclin ®). Subsequently, the suspension was mixed slowly, for one minute, in two stages. After the first stirring, the mixture rested for 30 minutes, after further homogenization it was kept at room temperature for another 150 minutes. Then the samples were centrifuged at 3000 rpm for 5 minutes.

The degree of hemolysis was qualitatively assessed by the reddish tone in the supernatant obtained after centrifugation. Symbols were assigned to the intensity of hemolysis, where one cross (+) indicates slight hemolysis, two (++) significant hemolysis and three (+++) indicates that there was intense hemolysis. In this test, distilled water was used as a positive control while saline solution and dimethyl sulfoxide (DMSO) were used as a negative control. Both received the same procedures used in the test samples.

Figure 7: Procedure for determining hemolytic activity. Defibrillated sheep blood (SDC) ; Revolutions per minute (RPM).

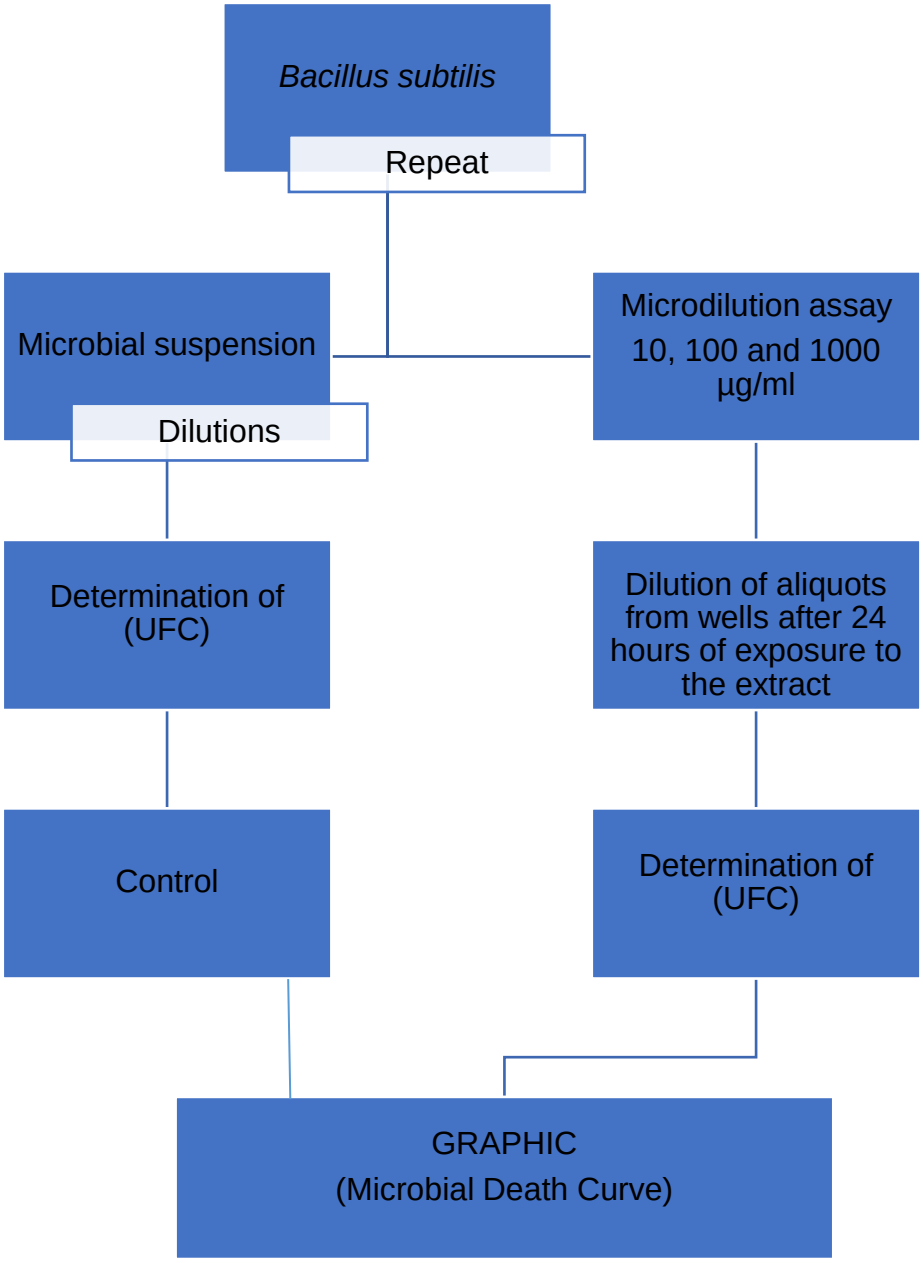


Source: Prepared by the author (2022)

3.5.3 Effect of the Vm-F19-20 fraction on the kinetics of microbial growth (time-kill).

Figure 8 shows the procedures carried out in this experiment, which was carried out according to the methodology described by Klepser et al, 1998. To carry out the test, strains of *Bacillus subtilis were used* , harvested according to the plate exhaustion technique (HENRY, 1995) .

Figure 8: Flow to obtain the effect of the VM-F19-20 fraction on the kinetics of microbial growth (time kill) .



Source: Prepared by the author (2022)

3.5.3.1 Determination of colony forming units (CFU) of the initial suspension

Initially, 4 test tubes were listed. 9.990 ml of saline solution was placed in tube 1 and 990 µL was placed in the remaining tube. Afterwards, 10 µL of the previously prepared microbial suspension was removed to be placed in tube 1, homogenizing

(3x), 10 µL was removed from this tube to be placed in tube 2 and so on until the fourth and last.

Soon after, the diluted suspensions were seeded in triplicate. To do so, 100 µL was removed from tube 2 and applied to the surface of a Petri dish containing Muller Hilton Agar. This procedure was carried out with tubes 3 and 4. The suspension was spread on the surface of the medium with the aid of a Drigaslky loop. . After sowing, the plates were placed in the oven at a temperature of 37 °C and after 24 h the colonies were counted.

3.5.3.2 Microdilution test

The stock solution was prepared by weighing 2 mg of the sample and solubilizing it in 500 µL of DMSO obtaining a concentration of (4.0 mg/mL). The experiment was carried out in 96-well microplates and the culture medium used was Nutrient Broth. Samples were analyzed at the following extract concentrations:

- 1000 µg/ml, obtained by adding 200 µL of the culture medium to 200 µL of the stock solution in an eppendorf;
- 100 µg/ml, obtained by adding 320 µL of the culture medium to 80 µL of the stock solution in an eppendorf;
- 10 µg/ml, obtained by adding 392 µL of the culture medium to 8 µL of the stock solution in an eppendorf;

µL of each sample was distributed in triplicate into the Wells of the Eliza Plate and 100 µL of the microbial suspension was added shortly after. The plate was placed in an oven at 37 °C and removed after 24 hours.

3.5.3.3 Determination of colony forming units after microdilution

Initially, 9 Petri dishes containing Mueller Hinton Agar were prepared. They were later identified in triplicate, indicating the concentrations of the extract that were analyzed in the experiment (10, 100 and 1000µg/ml). After 24 hours of incubation of the Eliza plate, in which the microdilution test had been carried out (described in topic 3.5.3.2), the test was read, and then the contents of the wells were seeded, so that the

content of the wells could be evaluated. growth of bacterial colonies after exposure of the microbial suspension to the VM-F19-20 fraction.

This methodological step consisted of previously distributing 990 µL of saline into 18 Eppendorf-type tubes, then 10 µL were removed from each of the wells of the triplicates (test) of the Eliza plate. Then, this aliquot was diluted twice as follows: in a first eppendorf containing 990 µL of saline, the 10 µL removed from the well was deposited, this content was homogenized and then 10 µL was transferred to a second eppendorf. Only after the second dilution, 100 µL of the content was removed and seeded in Petri dishes.

After sowing, the plates were placed in an oven at a temperature of 37°C. After 24 hours, the colony-forming units were counted , and then the approximate number of microorganisms was determined, after having been exposed to the VM-F19-20 fraction at different concentrations.

3.5.3.4 Statistical analysis

The microbial growth kinetic curve was plotted by log10 UFC/mL as a function of a 24-hour interval and the studied concentrations of the phytoconstituent. The tool used to process the data obtained after the experimental procedure was the GraphPadPrism 5 software. In this program it was possible to generate a logarithmic function which gave rise to the graph that illustrates the behavior of the bacterial strain *Bacillus subtilis* after exposure to the VM-F19- fraction. 20.

4 RESULTS AND DISCUSSION

4.1 Preliminary tests of crude hexane, ethyl acetate and ethanolic extracts of *Verbesina macrophylla* (Cass). Blake (Asteraceae)

4.1.2 Antibacterial activity

The Minimum Inhibitory Concentration (MIC) of crude extracts in hexane, ethyl acetate and ethanol from *V. macrophylla* leaves against bacterial strains is shown in Table 2.

It is possible to observe that none of the extracts showed activity against the Gram-negative strains *Pseudomonas aeruginosa and Escherichia coli* , at concentrations lower than 500 µg/mL. The hexane and ethyl acetate extracts were active against standard strains of Gram positive bacteria: *Bacillus cereus* , *Staphylococcus aureus* , *Bacillus subtilis* and *Micrococcus luteus* with minimum inhibitory concentrations that varied between 500 µg/mL and 31.25 µg/mL. As for the ethanolic extract, it did not show activity against *S. aureus,* however its effect could be observed against *B. cereus* and *B. subtilis* at a concentration of 500 µg/mL, and against the Gram-positive *Micrococcus luteus* , with MIC of 7.81 µg/mL. The microorganisms that are susceptible to these extracts are potentially pathogenic to humans and are considered agents responsible for common infectious conditions.

Table 2: Antibacterial activity of hexane, ethyl acetate and ethanol extracts of *Verbesina macrophylla* (Vm) leaves . Micrograms (µg) ; milliliters (mL); greater than (>); ethyl acetate (EtOAc).

Group	**Species**	**MIC of Extracts (µg/mL)**			**Control**
		Hexane	EtOAc	Ethanol	Chloramphenicol
Gram-positive	*B. cereus*	125	62.5	500	3.9
	S. aureus	250	500	>500	3.9
	B. subtilis	125	31.25	500	3.9
	M. luteus	125	31.25	7.81	3.9
Gram-negative	*P. aeruginosa*	>500	>500	>500	125
	E. coli	>500	>500	>500	3.9

Source: Prepared by the author (2020)

In this context, it is known that most bacteria of the genus *Bacillus* are considered pathogenic in humans (BATT, 2014). *Bacillus cereus* is responsible for cases of food poisoning, which can result in two types of syndromes: Emetic, which is triggered by bacterial cells growing in contaminated food, and manifests itself as nausea and vomiting approximately one to five hours after ingestion. And diarrheal syndrome, in which food poisoning arises from the presence of complex enterotoxins, infected individuals usually experience profuse watery diarrhea, abdominal pain, nausea and vomiting approximately 8 to 16 hours after contamination. *B. subtilis* is poorly recognized regarding its pathogenicity aspects and the occurrence of outbreaks in Brazil has not been described in the literature (RASKO et al., 2005; MELLEGARD et al., 2011).

Resistant strains of *Staphylococcus aureus* have emerged with each new antibiotic introduced in the treatment of pathologies attributed to it (SANTOS, 2007). Skin and soft tissue infections can reach from superficial regions to deeper tissues, with *S.aureus* the most common agent, which can affect community or hospitalized patients (ROBERT; CHAMBERS, 2005). This microorganism poses great risks to immunocompromised patients, causing everything from benign chronic skin infections to systemic infections leading to death. Skin infections include simple folliculitis, impetigo, boils and carbuncles, which are harmful to the subcutaneous tissue and produce systemic effects, such as fever (CARVALHO et al., 2005; CAVALCANTI et al., 2005). Staphylococcal toxins trigger toxic epidermal necrosis and toxic shock syndrome. Furthermore, *S. aureus* is capable of inducing food infections through the production of exotoxins during growth in contaminated food (CAVALCANTI et al., 2005; LOWY 1998).

Micrococcus luteus is a bacterium that constitutes the normal microbiota of the skin, however, in patients with compromised immunity, it can produce skin infections (SMITH et al., 1999). It is rarely classified as pathogenic in humans, but infectious diseases have been attributed to this bacterium, including septic arthritis (WHARTON et al *.,* 1986), meningitis (FOSSE et al., 1985) and endocarditis (COLEBUNDERS et al *.,* 1985) .

The results found in this work corroborate the data reported by De Veras et al. (2021), in which the essential oil from the leaves of *V. macrophylla* showed antimicrobial activity against *S.aureus ,* with an MIC of 512 µg/mL, while in this study

the values varied between 500 µg/mL for the ethanol extract and 250 µg /mL in hexane. In line with these data, other species of the genus Verbesina *also* showed activity against *S.aureus* : *V. negrensis* (MORA et al., 2015) and *V. encelioides* (TORIBIO, 2005; 2012).

There is no consensus in the literature on acceptable inhibition values for natural products when compared to standard antibiotics. However, Aligianis et al . (2001), proposed a classification for plant materials based on MIC results, considering the following: strong inhibition – MIC up to 500 µg/mL; moderate inhibition - MIC between 600 and 1500 µg/mL and weak inhibition - MIC above 1600 µg/mL. According to this classification, the three extracts tested have a strong inhibition against bacteria that were sensitive to them.

It is notable that the inhibitory activity of *V. macrophylla* extracts was lower than that promoted by the standard antibiotic indicated by the NCCLS, tested under similar methodological conditions, probably due to the fact that the antibiotics used as controls are substances with a high degree of purity. , in contrast, crude extracts have a wide variety of chemically distinct compounds.

The ethanolic extract, when compared to the others (hexane and ethyl acetate), in addition to not showing activity against *S. aureus,* inhibited the bacteria *B. cereus* and *B. subtilis* at a higher concentration. According to Nascimento et al. (2006), and Bayoub et al. (2010), the use of different solvents in the preparation of the extracts may have interfered with their biological activity, as the ethanolic extract has a high capacity to inhibit bacterial growth, due to the excellent extraction of polar active principles from plants, which will reflect on the antibacterial action. The reports by Mora et al. (2013), for example, demonstrated the antimicrobial activity of the ethanolic extract of *Verbesina negrensis* against *S. aureus* and *P. aeruginosa.*

growth inhibition of P. aeruginosa and *E. coli* bacteria when exposed to the tested plant extracts can be explained according to Contrucci et al. (2019), due to the lower susceptibility of Gram-negative bacteria to the action of antimicrobials, which highlights a major challenge for public health. Some of the mechanisms of this resistance are due to its own cellular structure, such as the lipopolysaccharide coating that is present on the bacteria's outer membrane, preventing the diffusion of active ingredients (ALVES et al., 2022). Biochemical mechanisms such as those provided by beta-lactamase enzymes that hinder the action of beta-lactam antibiotics, such as penicillins, also confer resistance to these microorganisms (MOTA et al., 2018).

Therefore, based on these authors, it can be stated that it is expected that the phytoconstituents present in the hexane, acetate and ethanol extracts of *V. macrophylla* used in this assay will also have difficulty inhibiting pathogens with these characteristics.

It is still possible to observe in Table 2 that the positive control for the bacterial strain *Pseudomonas aeruginosa* presented an MIC value equal to 125 µg/mL, this being the highest concentration found when compared with the control values obtained for all other bacteria tested, which corresponded to 3.9 µg/mL. In relation to these data, one can reflect on the findings in the literature, which report on the major problem of endemicity and multidrug resistance of clinical isolates of the bacterium P. aeruginosa *,* together with high rates of mortality and morbidity (HEIMESAAT et al., 2019).

Currently, therapeutic options for the intervention of infections caused by *P. aeruginosa* are limited, often restricted to the use of carbapenems (BUEHRLE et al., 2017). Another therapeutic option would be polymyxins, however, the mechanism that confers the bactericidal action of this drug remains unknown (HORCAJADA et al., 2019). This demonstrates the complexity of combating resistant pathogens such as the Gram-negative bacterium *P. aeruginosa.*

In cases where there was no inhibitory activity of the extracts obtained from the *V. macrophylla leaf* at the concentrations established for this biological assay, it is important to highlight that the presence of antimicrobial substances in the plant organ in question cannot be ruled out. For Fennel et al. (2004), this result can be attributed to the fact that the variations relating to the determination of the Minimum Inhibitory Concentration are influenced by many factors, among them it is possible to mention the technique applied, the strain used in the test, the origin of the plant and the period where it was collected, whether fresh or dried plants were used in the preparation of the extracts, as well as the amount of extract established for the test.

4.1.3 Antifungal Activity

Table 3 shows the MIC of crude extracts in hexane, ethyl acetate and ethanol of *V. macrophylla* against opportunistic fungi of clinical importance. It is possible to note that none of the extracts showed antifungal potential at concentrations up to 500 (µg/mL).

Table 3: Antifungal activity of hexane, ethyl acetate and ethanol extracts of *Verbesina macrophylla* (Vm) leaves. Micrograms (µg); milliliters (mL); greater than (>); ethyl acetate (EtOAc).CiclopiroxOlamine (C. Ola).

Group	Species	MIC of Extracts (µg/mL)			Control
		Hexanic	EtOAc	Ethanol	Glue
Yeastform	*C. albicans*	>500	>500	>500	15.62
	C. glabrata	>500	>500	>500	15.62
Filamentous	*A. niger*	>500	>500	>500	62.5

Source: Prepared by the author (2022)

No records of the antifungal activity of crude extracts of *Verbesina macrophylla were found in the literature* to be compared with the present study. However, De Veras et al. (2021), describe that the essential oil of *V. macrophylla* has antifungal activity against *Candida albicans.* This activity is possibly linked to the major compounds found in the species' essential oil, and the fungus' greater susceptibility to these substances. In this context, the work carried out by De Veras et al. (2021), also showed the presence of substances such as germacrene D, bicyclogermacrene and caryophyllene constituting the essential oil of *V. macrophylla*, with these compounds also described in other species of the same family, and which showed antifungal activity against *C. albicans.*

Data from the literature reveal a variety of plant extracts with anti- *Candida* action, from plants belonging to the Asteraceae family (NETO; MORAIS, 2003). However, there are few reports regarding antifungal activity with species belonging to the genus *Verbesina*, among them the work of Toribio (2005), which analyzed the antimicrobial activity of the dry methanolic extract of *V. encelioides* (Cav.) and in contrast to The present study obtained the inhibition of the yeast *C. albicans.*

Given this scenario, it is worth reflecting on the difficulty in obtaining new antifungal agents and the importance of studies for this purpose. In this sense, Garcia (1999) comments that fungi have developed multiple mechanisms to hinder the action of antifungal agents, such as altering the permeability site and the mechanism of action.

Currently, most of the drugs available on the market have side effects, questionable efficacy against re-emerging fungi, or quickly develop resistance, making a new generation of antifungal medications essential. In this way, studies are oriented

towards the investigation of agents that act selectively on the fungal cell without interfering with any biochemical system of the host (ZACCHINO, 2001).

It is important to highlight that the absence of antimicrobial activity of *V. macrophylla* extracts for the fungi tested in this study, the presence of antimicrobial substances in the extracts cannot be ruled out, so this fact may be related to several factors such as: the mode of extraction the amount of components present, as well as factors that influence the secondary metabolism of the vegetable in question. New tests are suggested under different methodological conditions.

4.2 Chromatographic fractionation of the ethyl acetate extract from *Verbesina macrophylla.*

The ethyl acetate extract (EtOAc) demonstrated consistent antibacterial activity in the preliminary biological test, as can be illustrated in Table 2, it also presented the highest yield when compared to the other extracts produced, yielding 18.95 grams, while the hexane extract weighed 8.41g and ethanolic 2.35 g. These data supported the choice of the EtOAc extract for chromatographic fractionation to be carried out and the phytochemical and biological study of its fractions to be carried out.

Thin Layer Chromatography (TLC) is a very efficient method for separating and quantifying antimicrobial compounds in a chromatogram of a crude or semi-pure extract, enabling the isolation of the active compound guided by bioassay. CCD is configured as a physical separation method, in which the substances to be separated are distributed between a fixed phase (silica gel), with a large surface area called the stationary phase, and another in which a fluid elutes through it, called the stationary phase. mobile. In the mobile phase, solvents with increasing polarities are used (CECHINEL FILHO et al., 2000).

Table 4 presents the data obtained after the EtOAc extract was subjected to Column Chromatography, using solvents in increasing order of polarity (hexane, ethyl acetate and methanol). It can be seen that the fractionation resulted in 51 samples, which were analyzed using the thin layer chromatography technique. The fractions that showed the same chemical shift (RF) in CCD were grouped (Table 4). After complete evaporation of the solvent, the samples were weighed, labeled and refrigerated so that biological activity tests and phytochemical studies could later be carried out.

Table 4: Chromatographic fractionation data of the ethyl acetate extract obtained from *Verbesina macrophylla* (Vm) with mass given in milligrams. Fraction (F); milligram (mg); Ethyl Acetate (EtOAc).

Chromatographic fractionation of EtOAc extract			
Fraction (Vm)	mg	**Fraction**	mg
F1	72.7	**F33**	16.3
F2 - 6	196	**F34**	10.2
F7 - 9	2034	**F35**	8.5
F10	230	**F36**	9
F11 - 12	1328	**F37**	8.9
F13 - 14	453.2	**F38**	8.4
F15 - 16	376	**F39**	11
F17 - 18	1744.1	**F40**	10.6
F19 - 20	1841	**F41**	12.2
F21 - 22	736.4	**F42**	10.8
F23 - 24	698.6	**F43**	127.2
F25	356.4	**F44**	1914.8
F26	1325.7	**F45**	237.8
F27	534	**F46**	24.9
F28	137.1	**F47**	8
F29	36.4	**F48**	3.9
F30	21.4	**F49**	3.5
F31	21.3	**F50**	2.3
F32	14.8	**F51**	1.6

Source: Prepared by the author (2022)

4.3 Biological activity of fractions obtained from the crude ethyl acetate extract from *Verbesina macrophylla.*

4.3.1 Antibacterial activity of fractions

Table 5 presents data on the antibacterial activity of fractions of the crude ethyl acetate extract against Gram-positive and Gram-negative bacteria. The antimicrobial action is only noted against Gram-positive bacteria, with *M. luteus being* the most susceptible bacteria, as 17 fractions inhibited its growth. The microorganisms *B. cereus*

and *B. subtilis* were sensitive to 11 fractions, while *S. aureus* to only three. The concentration of the extract fractions used in the test was 1000 µg/mL, following the MIC classification criteria proposed by Webster et al. (2008), this value is considered satisfactory.

Table 5: Antibacterial activity in 1000 µg/mL of the fractions obtained from the ethyl acetate extract, from *Verbesina macrophylla* (Vm). Micrograms (µg); milliliters (mL); fraction (F); presence of activity (+); absence of activity (-).

EtOAc Extract Fractions (1000 µg/mL)

Fraction (Vm)	**Gram-positive**				**Gram-negative**	
	B.cereus	*S. aureus*	*B. subtilis*	*M. luteus*	*E. coli*	*P. aeruginosa*
F1	-	-	-	+	-	-
F2 - 6	+	+	+	+	-	-
F7 – 9	-	-	-	+	-	-
F10	+	-	+	+	-	-
F11 - 12	+	-	+	+	-	-
F13 - 14	+	-	+	+	-	-
F15 - 16	+	-	+	+	-	-
F17 - 18	+	+	+	+	-	-
F19 - 20	+	+	+	+	-	-
F21 - 22	+	-	+	+	-	-
F23 - 24	+	-	+	+	-	-
F25	+	-	+	+	-	-
F26	+	-	-	+	-	-

F27	-	-	-	+	-	-
F28	-	-	-	-	-	-
F29	-	-	-	+	-	-
F30	-	-	-	-	-	-
F31	-	-	-	+	-	-
F32	-	-	+	-	-	-
F33	-	-	-	+	-	-
F34	-	-	-	-	-	-
F35	-	-	-	-	-	-
F36	-	-	-	-	-	-
F37	-	-	-	-	-	-
F38	-	-	-	-	-	-
F39	-	-	-	-	-	-
F40	-	-	-	-	-	-
F41	-	-	-	-	-	-
F42	-	-	-	-	-	-
F43	-	-	-	-	-	-
F44	-	-	-	-	-	-
F45	-	-	-	-	-	-
F46	-	-	-	-	-	-
F47	-	-	-	-	-	-
F48	-	-	-	-	-	-
F49	-	-	-	-	-	-
F50	-	-	-	-	-	-
F51	-	-	-	-	-	-

Source: Prepared by the author (2022)

As shown in Table 5, the first fractions were the most active, this fact indicates that the components responsible for the antimicrobial action can be extracted in low polarity, since these fractions were removed during the CCD method using pure hexane and hexane/acetate systems. ethyl. Regarding the fractions obtained with polar solvents (ethyl acetate and methanol), these showed practically no activity, only Vm-F32 and Vm-F33 were capable of inhibiting *B. subtilis* and *M. luteus* respectively.

According to Bresciania et al. (2004), and Carvalho et al. (2001), non-polar solvents such as hexane enable the extraction of steroid groups (estimagmasterol, sistosterol), coumarins, oleanoic acid esters, sesquiterpene lactones and terpenoids (kauranic acids). In this sense, it is worth highlighting that some of these groups have already been reported in phytochemical studies of the species *Verbesina macrophylla.* Furthermore, the scientific literature mentions that several microorganisms are inhibited by the action of terpenes, whose mechanism of action of this activity is not completely elucidated, however it is believed that the rupture of the cell membrane by lipophilic compounds may be involved in this process (COWAN, 1999; DORMAN; DEANS, 2000; WILKENS, 2002).

The results found indicate that Gram-positive bacteria are more susceptible to the components present in *V. macrophylla* when compared to Gram-negative bacteria. This chemical profile of selectivity against Gram positives is not restricted to compounds found in plants, but rather a common phenomenon observed among many antimicrobials (BASILE et al*.,* 2000). Other studies with fractions of plants belonging to the Asteraceae family have already reported antimicrobial activity against Gram-positive strains, for example: *Acmela brasiliensis* Spreng (SARTORI, 2005), *Verbesina negrensis* Steyerm (MORA et al, 2013) *Baccharis dracunculifolia* DC (DE ABREU; ONOFRE, 2010).

No antimicrobial action was observed against the bacteria *Escherichia coli* and *Pseudomonas aeruginosa*. This fact can be justified by the fact that the outer membrane of Gram-negative bacteria presents a barrier to the penetration of many antibiotic molecules, and the periplasmic space encompasses enzymes, which have the capacity to lyse foreign molecules inserted in this space (DUFFY; POWER, 2001; SARTORI et al., 2003).

4.3.2 Minimum inhibitory concentration (MIC) of active fractions Vm-F17-18 and Vm-F19-20

Table 6 demonstrates the results of the Minimum Inhibitory Concentration found for the Vm-F17-18 and Vm-F19-20 fractions of *Verbesina macrophylla*, against Gram-positive bacterial strains.

The reason why these fractions were selected for determining the MIC is the fact that the growth of all Gram-positive bacteria preliminarily tested at a concentration

of 1000 µg/mL was inhibited, as well as because the Nuclear Magnetic Resonance spectrum , demonstrated a high degree of purity, even pointing out a great similarity in the composition of the samples.

Table 6 shows a pronounced antibacterial activity of the fractions, being capable of inhibiting the growth of microorganisms at low concentrations, with MIC varying between 2.5 and 3.9 µg/mL. It is worth mentioning that the result obtained for *S. aureus* and *B. cereus* surpassed the standard antibiotic used in the test.

Table 6: Minimum inhibitory concentration (MIC) in µg/mL of the active fractions Vm-F17-18 and Vm-F19-20, obtained from the ethyl acetate extract, from Verbesina macrophylla (*Vm*) against Gram-positive bacteria. Micrograms (µg); milliliters (mL); greater than (>) Chloramphenicol (Clr).

		MIC of Active Fractions (µg/mL)		
Group	**Species**	**F17-18**	**F19-20**	**Clr Control**
Gram-positive	*B. cereus*	2.5	2.5	3.9
	S. aureus	2.5	2.5	3.9
	B. subtilis	3.9	3.9	3.9
	M. luteus	3.9	3.9	3.9

Source: Prepared by the author (2022)

These results corroborate data from studies previously carried out with species of the Asteraceae family (to which *V. macrophylla belongs*), where the most nonpolar fractions showed activity against Gram positive bacteria (DE ABREU ; ONOFRE, 2010; DE MATOS, 2001; DA SILVA et al., 2002).

Through the analysis of NMR data from fractions Vm-F17-18 and Vm-F19-20 (Table 11) it was possible to suggest that 70% of the samples are composed of the terpene 6-ObEp-coumaroyl-4a-hydroxyeudesmane (Vm A), previously reported by Bohlmann et al. (1980), and then by Maia (2011), and 25% by the compound 6-ObZp-coumaroyl-4a-hydroxyeudesmane (Vm B) (Figure 24), previously described only by Maia (2011), so that this is the second report of this substance in the species *Verbesina macrophylla* and the third of its isolation in the literature. Previously, the compound had been isolated only from the species *Verbesina virginica* (XU et al., 2010).

Therefore, it can be stated that these secondary metabolites are responsible for the marked antibacterial activity observed. This study corroborates data from the

literature, which expose other substances already isolated from species of the Asteraceae family that showed biological activity. Al-Dabbas et al., (2005) studying the ethyl acetate extract of the whole aerial parts of *Varthemia iphionoides* (Compositae) *,* reported the isolation of a eudesmane sesquiterpene (seline-4,11(13)-dien-3-on- 12-oic) which exhibited potent antibacterial activity against (*Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Escherichia coli, Bacillus cereus* and *Salmonella enteritidis*); ten sesquiterpene lactones were isolated from the aerial parts of *Centaurea spinosa* , with antimicrobial activity against Gram-positive bacteria being observed (SAROGLOU et al., 2005); substances isolated from *Epaltes Mexicana* Less. inhibited the growth of Gram-positive and negative bacteria (KATO, 1996).

Around 1,000 natural eudesmanoids have been described in species of the Asteraceae family, with distinct oxygenation and cleavage patterns. The biological activity of eudesmane-type sesquiterpenoids in Asteraceae has been the focus of many phytochemical, pharmacological and synthetic studies. These compounds exhibit a wide range of biological activities that include plant growth regulatory, insect antifeedant, antifungal, antitumor and antibacterial actions, so that interest in relating structure and oxygenation patterns to function has increased (WU et al., 2006).

In this context , Flores (2010) mentions that this varied biological activity allowed many sesquiterpenes to be isolated and characterized. The author also states that few compounds isolated from species of the *Verbesina genus* have been studied regarding their biological activity. Therefore, it is important to highlight that the present study is the first record of the antibacterial effect of substances isolated from *Verbesina macrophylla*.

4.3.3 Classification of the antimicrobial action of fractions Vm-F17-18 and Vm-F19-20

A considerable amount of research around the world has proven the microbiological and microbicidal properties of plant products. *In vitro* biological assays are usually carried out in susceptibility or sensitivity tests to study, evaluate and confirm the type of action of the sample (SOUZA et al., 2003). As carried out in this work.

The classification of antimicrobial action was determined by removing an aliquot from the inhibition well, MIC of the fractions, for each microorganism and consecutively inoculating in sterile medium. The concentrations of the fractions that did not

demonstrate any microbial growth in 24 hours were classified as bactericidal and the concentrations that showed growth in the MIC at the same time were classified as bacteriostatic (WALSH, 2003).

Table 7 presents the classification results regarding the bactericidal or bacteriostatic action of Fractions Vm-F17-18 and Vm-F19-20. It is noted that for the strains *Bacillus cereus* and *Staphylococcus aureus* the action was bactericidal, so that at the end of 24 hours of experimentation, there was no growth of colonies, thus characterizing this effect, while for Bacillus *subtilis* and *Micrococcus luteus* the fractions demonstrated to be bacteriostatic .

The bacteriostatic action of plant extracts does not destroy bacteria, however it prevents their multiplication and as a consequence reduces the possibility of mutations occurring (PHILLIPSONS, 2001). Therefore, carrying out studies like this is extremely important to motivate other work that has as its perspective the identification of the specific property of the plant extract in terms of its action.

Table 7: Classification regarding the antimicrobial action (bactericidal or bacteriostatic) of the active fractions Vm-F17-18 and Vm-F19-20. Fraction (F).

Statement Action Classification		
Species	Fractions (Vm)	
	F-17-18	F19-20
Bacillus cereus	bactericidal	Bactericide
Bacillus subtilis	bacteriostatic	bacteriostatic
Staphylococcus aureus	bactericidal	Bactericide
Micrococcus luteus	bacteriostatic	bacteriostatic

Source: Prepared by the author (2022)

4.3.4 Antifungal activity of fractions

Fungi are extremely interesting because they are capable of developing on different surfaces and colonizing environments, animals, plants and humans (SELINTRENNIKOFF, 2001). In the last 30 years, the occurrence of fungal infections has become increasingly common, especially in immunocompromised patients, and has therefore become an important public health problem, which worsens when cases of microbial resistance emerge (ANDRADE et al., 2017). According to Lazzarini et al. (2018), there are estimates that more than 300 million individuals are diagnosed with

serious fungal infections worldwide, of which the pathogens causing infections are mainly from the genera *Cryptococcus, Candida, Aspergillus and Pneumocystis.*

Given this scenario, the fractions obtained from the crude ethyl acetate extract were analyzed against yeast-like (*Candida albicans and Candida glabrata*) and filamentous (*Aspergillus niger*) fungal strains, which are clinically important and difficult to treat due to the emergence of multidrug resistance. Among the 38 fractions, microorganisms were exposed to 34, due to insufficient mass of the extracts to carry out the test. Therefore, fractions Vm-F48, Vm-F49, Vm-F50 and Vm-F51 were not tested.

Yeast-like fungi are very common in hospital infections, among them, the genus *Candida*, is recognized for being the cause of infections such as candidiasis or candidosis, the damage caused can be mild, acute or chronic, manifesting itself superficially or deeply. Its clinical presentation varies according to the patient's immunity (FREIRE et al*.,* 2016).

C. albicans stands out due to the high incidence in cases of candidiasis, whose frequent exposure of this species to antifungals favored the emergence of multi-resistant strains, however infections caused by non- *albicans Candida species* have increased dramatically (LÓPEZ-MARTÍNEZ, 2010). In this sense, the literature reports that approximately half of the occurrences of candidosis are caused by non- *albicans species*, so that *C. glabrata* already represents the second most isolated fungus in blood samples (GÓMEZ et al., 2010; OREN; PAUL, 2014; SAMPAIO; PAIS, 2014).

Infection by *C. glabrata* is mainly endogenous, since this species can colonize the skin and mucous membranes, with transmission also occurring through infected material, healthcare professionals or even other patients. The reduction in commensal bacterial flora in the gastrointestinal tract after administration of broad-spectrum antibacterials also facilitates the proliferation of yeast in the digestive tract, increasing the risk of yeast passing through translocation into the bloodstream, through the intestinal epithelium (PEMÁN; SALAVERT, 2013).

Aspergillus are opportunistic filamentous fungi, capable of causing allergic syndromes, such as allergic bronchopulmonary aspergillosis; they can colonize cavities, creating fungal balls in the lungs or nasal sinuses; cause invasive infections, such as disseminated pulmonary aspergillosis and AI. Depending on host conditions, infections can be localized or disseminated (DENNING, 1998; MARR et al*.,* 2002). *Aspergillus niger* is commonly isolated in cases of otitis externa (COLLEE et al., 1993).

It is also a secondary etiological agent in bacterial otitis, and can cause lung disease in patients with a compromised immune system. The presence of calcium oxalate crystals in microscopic samples is a useful feature for diagnosing *Aspergilus niger infection*, even in the absence of conidia (PERSON et al, 2010).

Table 8 presents the results obtained for the antifungal activity of *V. macrophylla fractions*. It is notable that the yeast-like fungus *Candida glabrata* was the only fungal strain not susceptible to any of the tested fractions. Vm-F1 and Vm-F2-6 showed activity against *C.albicans* and *A. niger* , while Vm-F7-9 and Vm-F10 were active only for *C* . *albicans.* The inhibition of the fractions is considered satisfactory (WEBSTER et al., 2008). The others (Vm-F11 ... Vm-F47) did not inhibit fungal growth at a concentration of 1000 µg/mL.

Table 8: Antifungal activity in 1000 µg/mL of the fractions obtained from the ethyl acetate extract from *Verbesina macrophylla* (Vm). Micrograms (µg); milliliters (mL); fraction (F)*;* presence of activity (+); absence of activity (-).

Antifungal Activity of Fractions (Vm) of EtOAc Extract (1000 µg/mL)

Fraction	**Yeastform**		**Filamentous**
	Candida albicans	*Candida glabrata*	*Aspergillus niger*
F1	**+**	**-**	**+**
F2 - 6	**+**	**-**	**+**
F7 – 9	**+**	**-**	**-**
F10	**+**	**-**	**-**

Source: Prepared by the author (2022)

When comparing the results of the antifungal activity of the fractions with those obtained in the tests in which the crude extracts were tested against the same fungal strains, it was observed that only the fractions were active. This result is in line with the reasoning of Cechinel Filho et al. (2001), and Malheiros et al. (2001), when they state that the analysis of active compounds is much more complex and prolonged, since the active substances commonly present in a smaller proportion in the species are those that present the best biological effects.

Another important observation to be made is that among all the fractions tested, only four fractions showed antifungal activity, while 12 demonstrated antibacterial action. When comparing these results, it appears that the number of active fractions for bacteria is three times greater than for fungi. This discrepancy can also be observed in the case of antimicrobial drugs already available on the market.

Considering these facts, Silva (2016) states that currently, there is a variety of antimicrobial medications that can be used to treat fungal infections, however, this "diversity" becomes relatively small, compared to the availability of drugs used in fungal therapy. bacterial infections. Góes (2009), states that this fact can be attributed to the eukaryotic nature of fungal cells, as well as the impasse in finding unique targets not shared with human hosts. These factors have even increased the pressure to search for new antifungal agents among natural products, which, in addition to being effective, are less toxic than those currently in use (KHAN; AHMAD; CAMEOTRA, 2013). The enormous chemical diversity, intrinsic cellular permeability and bioactive specificity of natural antimicrobial substances represents a promising source of new drugs to treat infectious diseases (JIANG et al., 2008).

Candida glabrata is noted. Regarding this strain Gołaś et al. (2014), reports that this is an emerging fungus among non- *albicans* species, being capable of developing resistance to Azoles and spreading as a result of the use of fluconazole and itraconazole as preferential and empirical prophylactic treatment, often indiscriminately. Tscherner et al. (2011), draws attention to the need to develop genetic studies of *C. glabrata* , in addition to investigating the mechanisms of pathogenicity, to understand what led this species to transition from commensal to pathogen, in order to improve established therapies.

Table 8 also shows the antifungal action of four fractions against *C. albicans*, and two against *A. niger*. It is important to comment that given the emergence of these pathogens, and reports of the emergence of resistance to traditional antifungals, the isolation of compounds that contribute to combating these microorganisms is of paramount importance. Given this scenario, Heard et al., (2021) reflects that natural products with antifungal properties emerge as therapeutic options. It can be an alternative and/or be combined with other pharmacological agents (ALVES et al., 2019). Mainly seeking to control resistant and infectious fungi (HELAL et al., 2019).

According to Nuclear Magnetic Resonance analysis, the antifungal activity observed in the test can be attributed to components such as fatty acids. Several fatty

acids are known to have antibacterial and antifungal properties (RUSSEL, 1991). However, this study reports for the first time the antifungal activity of fatty acids present in *Verbesina macrophylla*.

The results obtained in this work are in agreement with data found in the literature. Cantuária (2018), identified in Eremanthus erythropappus (DC) MacLeish (Asteraceae) specifically in the hexane fraction, compounds that belong to the class of acids and fatty acid esters, responsible for prominent antifungal activity; Moreno et al. (2013), evaluated palmitic acid against the *Candida albicans strain,* and obtained an MIC of 500 µg/mL for this molecule. Other studies have also associated fatty acids with antifungal activities (DA CUNHA 2006; SINGH et al., 2002; KIM et al., 2003*;* SACCHETTI et al., 2005).

Reda and Carneiro (2007), state that the Asteraceae family is still little studied with regard to the oil content and the composition of these components, however it is known that they present a predominance of linoleic acid, as already observed in traditional oilseeds such as *Carthamustinctorius* (75.9%) (GRIECO; PIEPOLI, 1967) and *Helianthusannuus* (68.4%) (ERBA; BAYDAR, 2007). Asteraceae has a variety of unusual fatty acids, such as *cis* or *trans unsaturated ones* with double bonds at positions 3 or 5, or conjugated, acetylene, epoxylated or hydroxylated (TSEVEGSUREN et al*.,* 1999, 2000). The existence of unusual fatty acids in these species confers economic importance, as some of them have therapeutic effects and are sold as herbal medicines (γ-linolenic) (TSEVEGSIREN; AITZETMÜLLER, 1996).

Thus, as this work demonstrated that the antifungal action of the fractions came from fatty acid components, it is extremely necessary that more detailed studies be carried out with the active fractions of *V. macrophylla* that presented this activity, in order to carry out the isolation and the chemical characterization of the samples.

4.4 Cytotoxicity in red blood cells of Hexane and Ethyl Acetate extracts

Vegetables have a diversity of intrinsic biological properties, celebrated for their applications in traditional medicine since ancient times. However, despite this custom, it is necessary to consider this use, medicinal plants are not a synonym for harmlessness. Opposite to the belief that, if the natural medicine does not do any good, it cannot do any harm, it is necessary to understand that the medicinal plant is a xenobiotic, that is, it is a chemically unknown product, and that it has therapeutic or

intoxicating properties, and that When introduced into the human body, it can undergo biotransformations, and in this way, can produce toxic products for the body (KHWAIRAKPAMA et al*.,* 2018; OLIVEIRA et al*.,* 2014).

In this context, the hemolytic activity assay has been widely used to determine the toxicity of plant extracts, precisely due to the need to investigate the risks and efficacy in human cells (ZOHRA; FAWZIA, 2014).

Hemolysis occurs when erythrocytes rupture through the destabilization of the cell membrane, causing the release of hemoglobin, affecting the functioning of vital organs such as kidneys, liver and heart (REZENDE et al., 2017). Thus, the use of the imminent biological activities of natural compounds must take into account their toxicological effects, however, this principle has made it impossible to apply several drugs (GHOSH et al., 2018; SILVA et al., 2017).

Faced with this concern, the present work investigated the cytotoxic activity of crude extracts in hexane and ethyl acetate from the leaves of *Verbesina macrophylla.* Samples were selected for testing taking into account the prominent biological activity shown in preliminary tests. Table 9 shows the results of the toxic effect of the extracts on red blood cells, so that slight hemolysis (+) was observed only for the hexane extract at its highest concentration (1000µg/mL).

Table 9: Cytotoxic activity in 10, 100 and 1000 µg/mL of hexane and ethyl acetate extracts of *Verbesina macrophylla* (Vm). Micrograms (µg); milliliters (mL); there was no hemolysis (-); slight hemolysis (+); significant hemolysis (++); intense hemolysis (+++).

	Cytotoxicity in RBCs		
Extract	10 µg/mL	100 µg/mL	1000 µg/mL
Hexane	-	-	+
Ethyl Acetate	-	-	-

Source: Prepared by the author (2022)

Verbesina macrophylla were not found in the literature, however, the study of the essential oil of this species carried out by De Veras et al. (2021), demonstrated that in all tested concentrations there was safety for its use as a drug, with hemolysis being less than 5%. Other findings in relation to extracts from species with therapeutic properties belonging to the Asteraceae family demonstrated that they are not toxic in the models used: *Dasyphyllum tomentosum* (Spreng.) (PAULA, 2014); *Praxelis*

clematidea (PEREIRA, 2022); *Eupatorium ballotifolium* (SOBRINHO et al., 2016); *Baccharis dracunculifolia* (DA SILVA FILHO et al., 2009) *Arctiumlappa, Mikaniclusta* (DE HARO MORENO, 2018).

On the other hand, there are studies of species that demonstrate the unviability of chemical compounds due to their toxicity, such as the macrocyclic trichothecenes present in *Baccharis coridifolia*, which have antiviral properties (GARCIA, 2002). *In vivo* toxicity tests in rabbits and rats showed that ingestion of *B. coridifolia* and/or purified trichothecenes developed toxic symptoms that, in most cases, resulted in the death of the animals, with diarrhea being the most common symptom (HABERMEHL et al., 1985; DOBEREINER et al., 1976). The study by Faria (2019) demonstrated a certain toxicity of the extract and phases of *Ageratum fastigiatum* used as an anti-inflammatory, analgesic and healing agent when used topically in alcoholic or hydroalcoholic preparations. *Eupatoriumba llotifolium* essential oil has anticancer and antifungal activities in low concentrations, however, in these concentrations it also has a hemolytic potential of 12.5% (SOBRINHO et al., 2016)

All these works demonstrate that toxicological safety is essential and must surpass the biological and/or pharmacological potential of extracts or compounds obtained from plants, with hemolytic activity being an important indicator of the impossibility of their application. Thus, the hexane and ethyl acetate extracts of *Verbesina macrophylla* showed toxicological safety, having expressed slight hemolysis, only at the highest concentration tested for the hexane extract.

4.5 Effect of the Vm-F19-20 fraction on the kinetics of microbial growth (time-kill)

Time-to-kill studies of antimicrobial agents are capable of providing a more dynamic assessment of the relationship between an antimicrobial agent and a given organism, and may have greater clinical applicability than static determinations of Minimum Lethal Concentration (MLC). For this reason, investigations focused on time of death have been widely used to evaluate the relative rate and extent of the bactericidal potential of many agents, in addition to pharmacodynamic characteristics and possible antagonism or synergy between agents administered simultaneously (PFALLER et al., 2004). This type of evaluation is exhausting, however, in addition to confirming the MIC determinations, it can provide the exact time of action in which antimicrobials cause the death of the bacteria evaluated (TEETHAISONG et al., 2014).

Using the time-kill method, it is possible to determine the death of a bacterial isolate over time by testing one or more antimicrobial agents under methodically controlled conditions. If the objective is to evaluate synergy or antagonism between two or more antimicrobials, the death curves will present a fixed concentration of each agent alone and together, being compared with those of the control without drugs and at established time intervals. When the method is used to analyze only one antimicrobial agent, (as in the case of this work), the death curves are plotted by testing multiples of the Minimum Inhibitory Concentration (MIC) (NCCLS, 1998).

Bacillus subtilis strain bacteria after exposure to the Vm-F19-20 fraction at different concentrations over 24 hours, identifying whether the activity of this fraction has bactericidal or bacteriostatic action., as well as whether this activity is concentration or time-dependent.

Table 10 presents the microbial count data, which were used to obtain Graph 1, which expresses the death kinetics of the Bacillus subtilis bacteria *and* illustrates the relationship between the three concentrations (10; 100; 1000 µg/mL) of the active fraction Vm-F19-20 and the number of bacteria resulting after a period of 24 hours of exposure to the phytoconstituent.

Bacillus subtilis count data for the three concentrations (10; 100; 1000 µg/mL) of the fraction (Vm-F19-20). Micrograms (µg); milliliters (mL); fraction (F).

Experiment Data			
Concentration (µg/mL)	No. Bacteria	Log. Concentration (x)	No. Bacteria (y)
0	16.3. 10^{8}	0	1630. 10^{5}
10	52.10^{5}	1	52. 10^{5}
100	12.10^{5}	2	12. 10^{5}
1000	6.10^{5}	3	6. 10^{5}

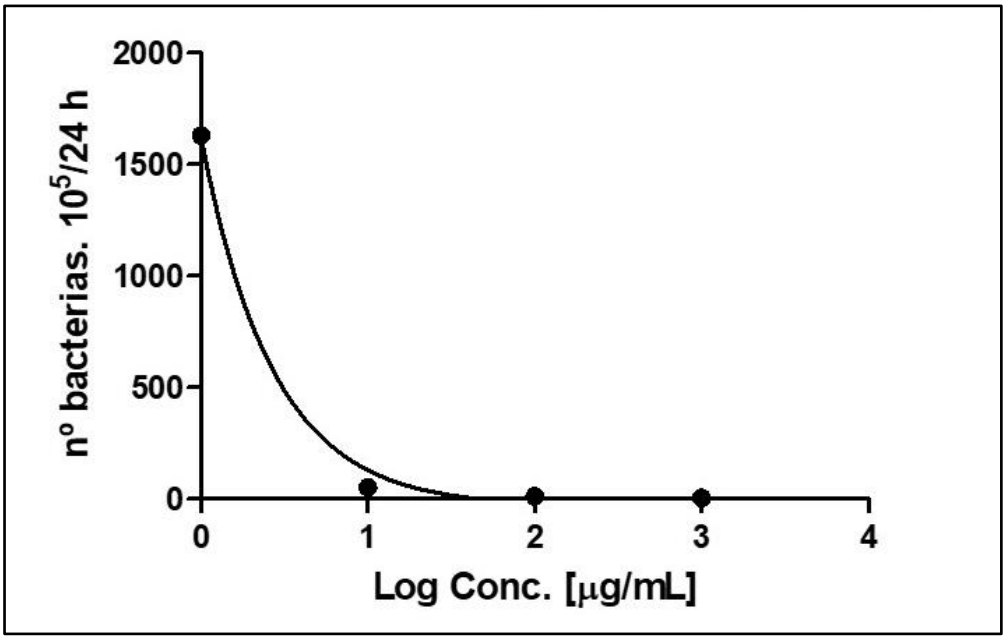
Source: Prepared by the author (2022)

The behavior pattern of the bacterial population of the *Bacillus subtilis species* can be represented according to the logarithmic function below:

$$Y = Bottom + (Top - Bottom)/(1 + 10^{(X - Log|C50)}).$$

Where X is the logarithm of the concentration of the fraction, and Y represents the number of bacteria after a period of 24 hours of exposure to the extract.

Graph 1: Microbial death curve (Log10UFC/mL) of the *Bacillus subtilis* strain , under action of the Vm-F19-20 fraction in three concentrations .



Source: Prepared by the author (2022)

Observing Graph 1, it is noted that the Vm-F19-20 fraction presented a bacteriostatic effect for the *Bacillus subtilis strain*, as there was no reduction greater than or equal to 3 log10UFC/mL (99.9%) from the initial inoculum in the MIC at all concentrations tested, over a 24-hour period. As the concentration of the fraction increases, the number of bacteria resulting after UFC counting is lower, so that when subjected to a concentration of 1000 µg/mL, approximately 6.10 ^{5} bacteria were found, at 100 µg/mL12. 10^{5} and 10 µg/mL 52.10 ^{5} . Therefore, a great influence of the Vm-F19-20 fraction on the growth of bacteria is observed when observing the control (0 µg/mL), which presents an approximate number of 1630. 10 ^{5} bacteria.

Thus, the analysis of Graph 1 suggests that due to the death kinetics, both at lower concentrations and at the highest concentration of the fraction, there is bacteriostatic activity dependent on the concentration, since the increase in this potentiates the antibacterial activity. It is important to highlight that cytotoxicity studies of the fraction must be carried out to evaluate the impact of this phytoconstituent at high concentrations.

No studies were found in the literature that evaluated microbial growth parameters related to the antimicrobial efficiency of a crude extract or fraction extracted from *Verbesina macrophylla* . Despite the scarcity of data for this type of study, the test results are promising, since there is the possibility that in association with a standard antibiotic the antibacterial activity can be enhanced. In this context, it is reported by Stefanovic et al. (2012), that different substances combined increase the chances of containing an infection, and that the use of extracts from bioactive plant species, semi-synthetic derivatives and isolated pure substances increase the *in vitro effectiveness* of antimicrobials used against a diversity of microorganisms.

Dutra (2016), demonstrated in his studies that combinations of methanolic extracts of *P. granatum, P. guajava and A. occidentale* associated with standardized antibiotics showed an enhancement of the antimicrobial effect against Gram-positive bacteria, suggesting a synergistic action between them. ; Morais-Braga et al. (2012), describe that *Lygodium venustum* has secondary metabolites that can be used in association with antibiotics, and this synergistic effect was observed between the fraction obtained from the extract in ethyl acetate and the antibiotic amikacin against *E. coli* and *S. aureus* .

Given this possibility, and the promising results obtained in the test, it can be suggested that further studies of the Vm-F19-20 fraction of *Verbesina macrophylla* should be carried out. Thus, the Checkerboard Method *, which* is characterized by being a microdilution test that evaluates the MIC of drugs alone and in combination, can be carried out to investigate whether the association of this fraction with standard antibiotics results in synergism, antagonism or indifference.

4.6 Characterization of the chemical constituents of *Verbesina macrophylla*

The figures below illustrate the Nuclear Magnetic Resonance spectra obtained from fractions from the crude extract in ethyl acetate of *V. macrophylla* . Spectral data were acquired on a Varian INOVA 500 spectrometer, operating at 500 MHz for H ^{1} .

Figure 9: H[1] NMR spectrum of the Vm-F1 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

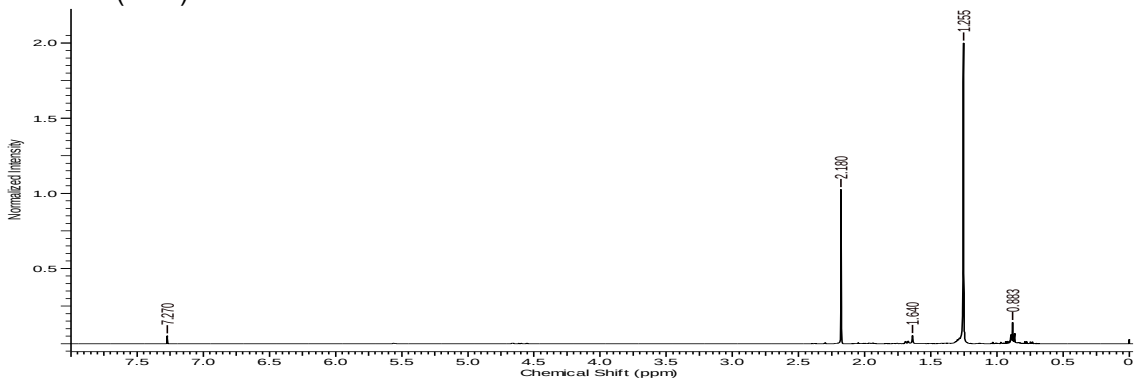


Figure 10: H [1] NMR spectrum of the Vm-F2 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

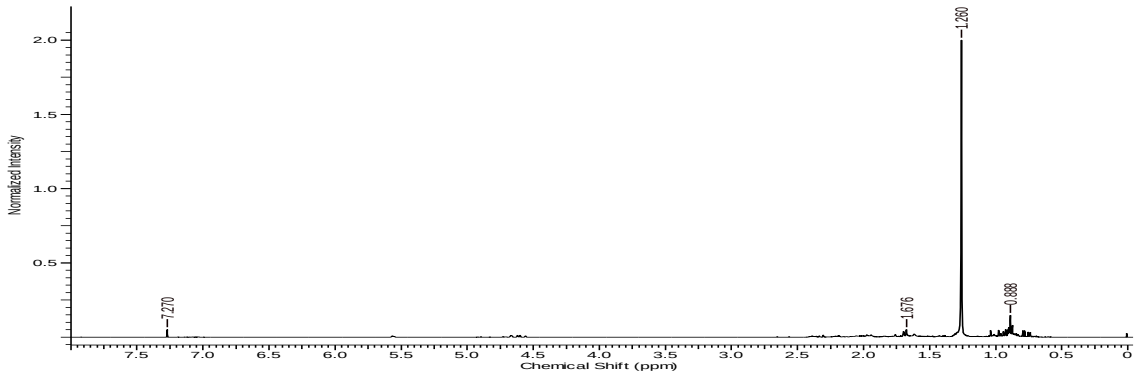


Figure 11: H[1] NMR spectrum of the Vm-F10 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

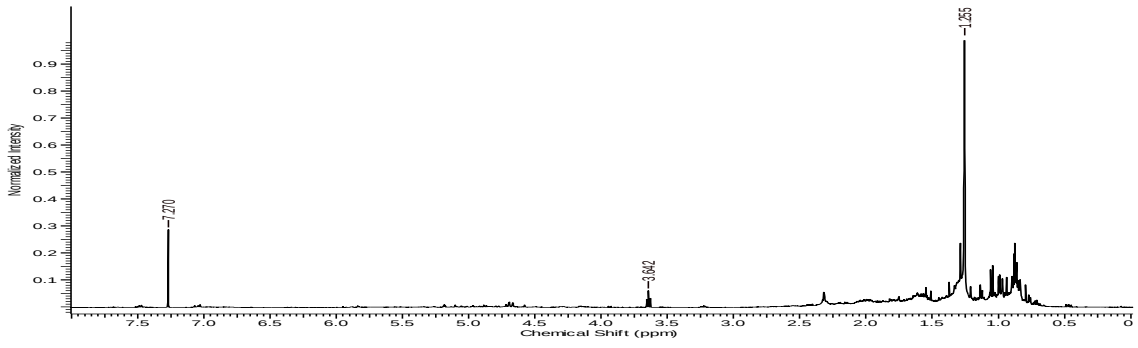


Figure 12: H^1 NMR spectrum of the Vm-F11-12 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

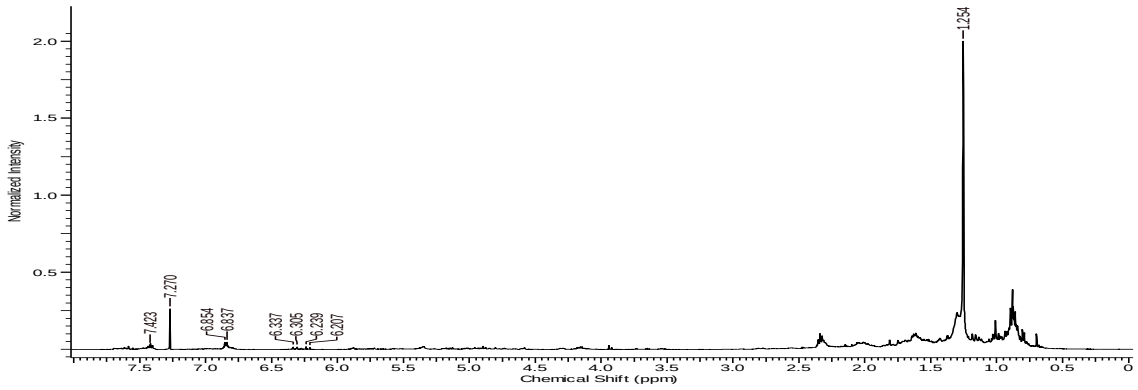


Figure 13: H^1 NMR spectrum of the Vm-F13-14 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

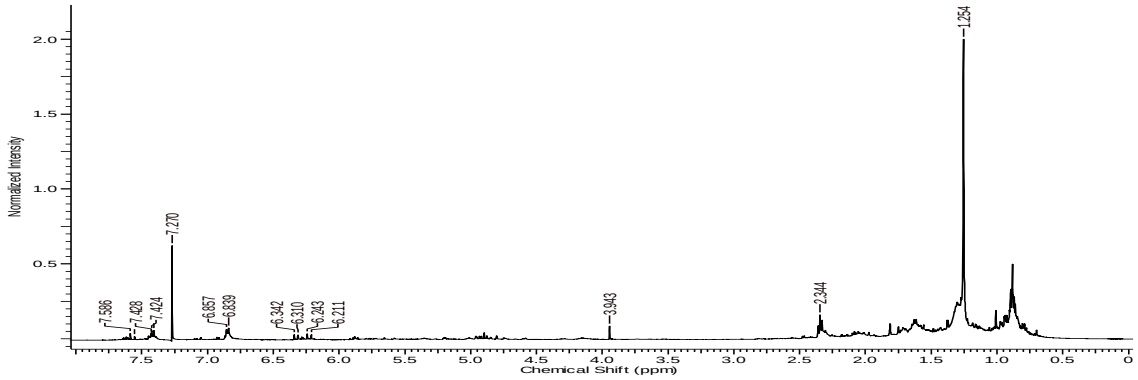


Figure 14: H^1 NMR spectrum of the Vm-F15-16 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

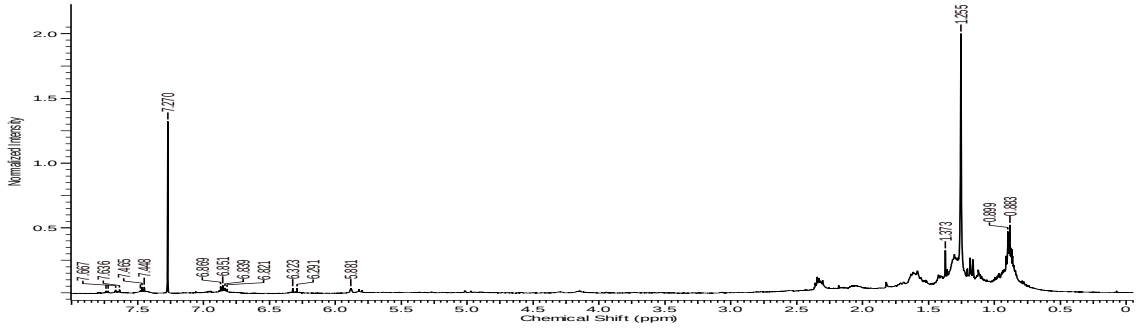


Figure 15: H^1 NMR spectrum of the Vm-F17-18 fraction of the ethyl acetate extract of *V. macrophylla* (Vm).

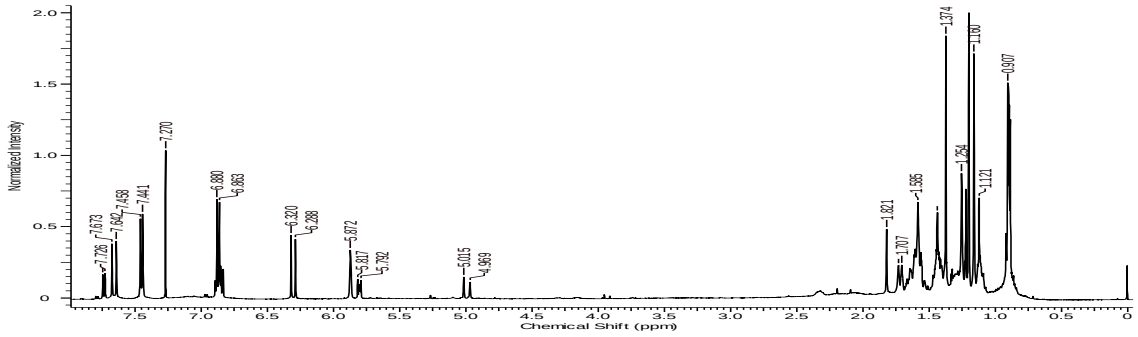


Figure 16: H^1 NMR spectrum of the Vm-F19-20 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

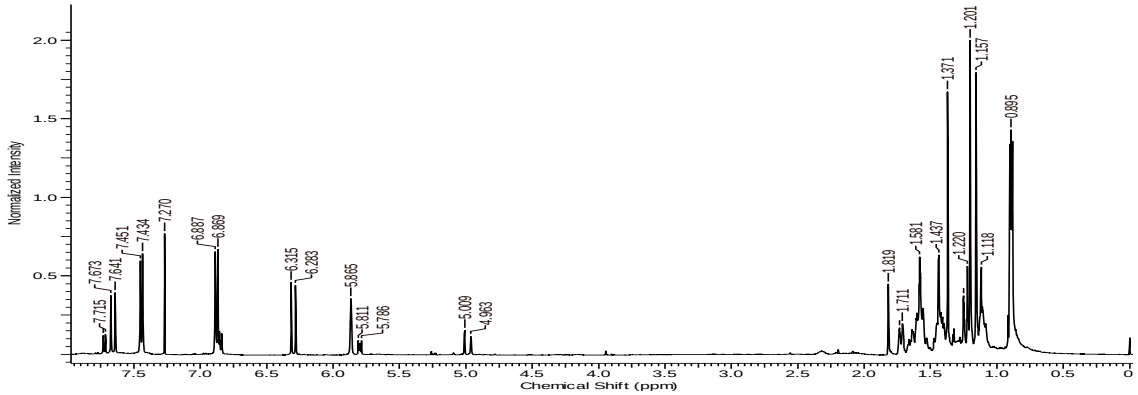


Figure 17: H^1 NMR spectrum of the Vm-F21-22 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

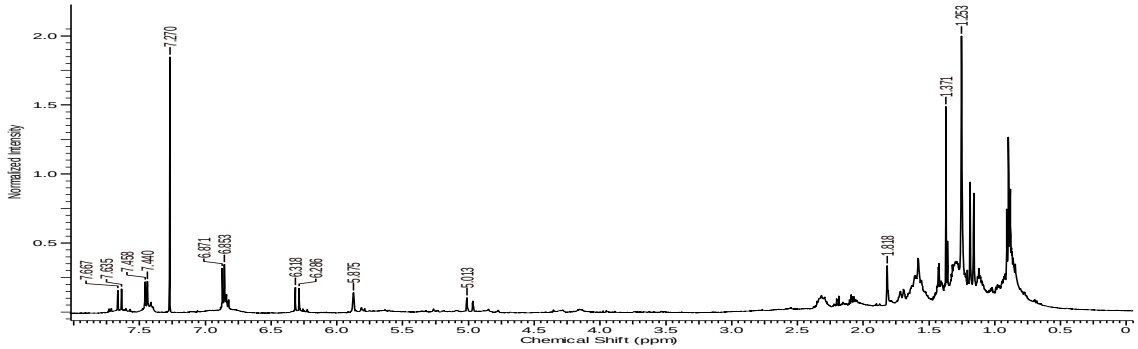
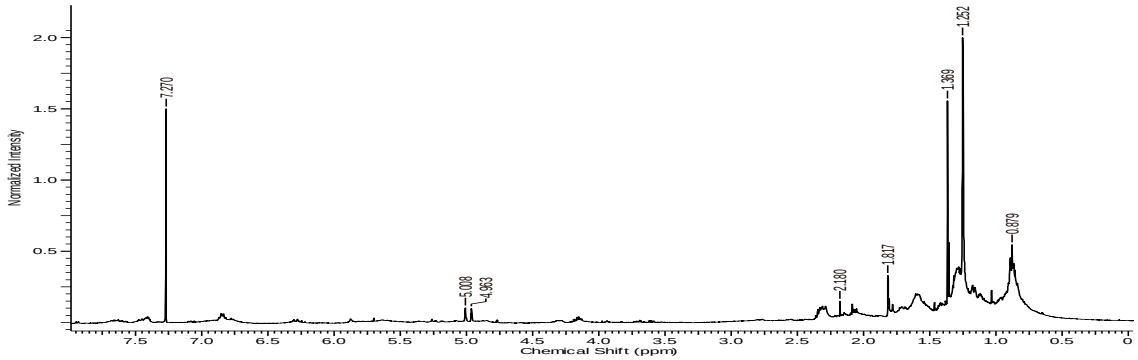


Figure 18: H^1 NMR spectrum of the Vm-F23-24 fraction of the ethyl acetate extract of *Verbesina macrophylla* (Vm).



H^1 NMR spectra (Vm-F1 - VmF10) present a set of signals that suggest the predominance of fatty acid-type components. Vm-F1 and Vm-F2, with a higher degree of purity, appeared as a white and amorphous solid. From Vm-F11 to Vm-F16, signals appear, between δ 6.2 and 7.4 ppm , typical of compounds that have an aromatic group. To a lesser extent, a set of signs suggestive of the presence of components of a terpenic nature, between δ 2.34 and 0.82.

Between fractions Vm-F17 to Vm-F22, there is a set of very similar signals, with a predominance of aromatic and terpene component signals, but with a higher degree of purity in the Vm-F19-20 fraction. Therefore, it was the sample chosen to carry out new NMR experiments. Represented by the H^1 NMR spectra (Figure 19), and their magnifications (19.1 - 19.3) and by the NMR spectra: C^{13} , DEPT, HSQC and HMBC (Figures 20, 21, 22 and 23). The spectrum of the Vm-F23-24 fraction showed a low degree of purity. It shows signs at δ 5.08 and 4.96 of hydrogen bonded to olefinic carbon , present in a terpenic component, with the possible presence of a fatty contaminant (δ 1.25).

Figure 19: H [1] NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer, operating at 500 MHz for H [1] and 125 MHz for C [13] .

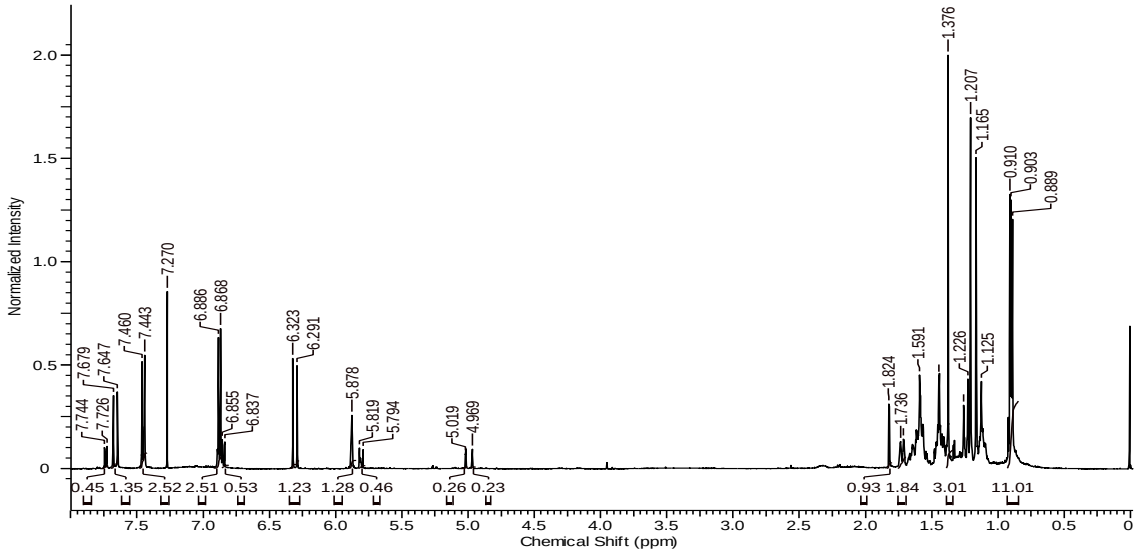


Figure 19.1: Magnification of the H [1] NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer, operating at 500 MHz for H [1] and 125 MHz for C [13] .

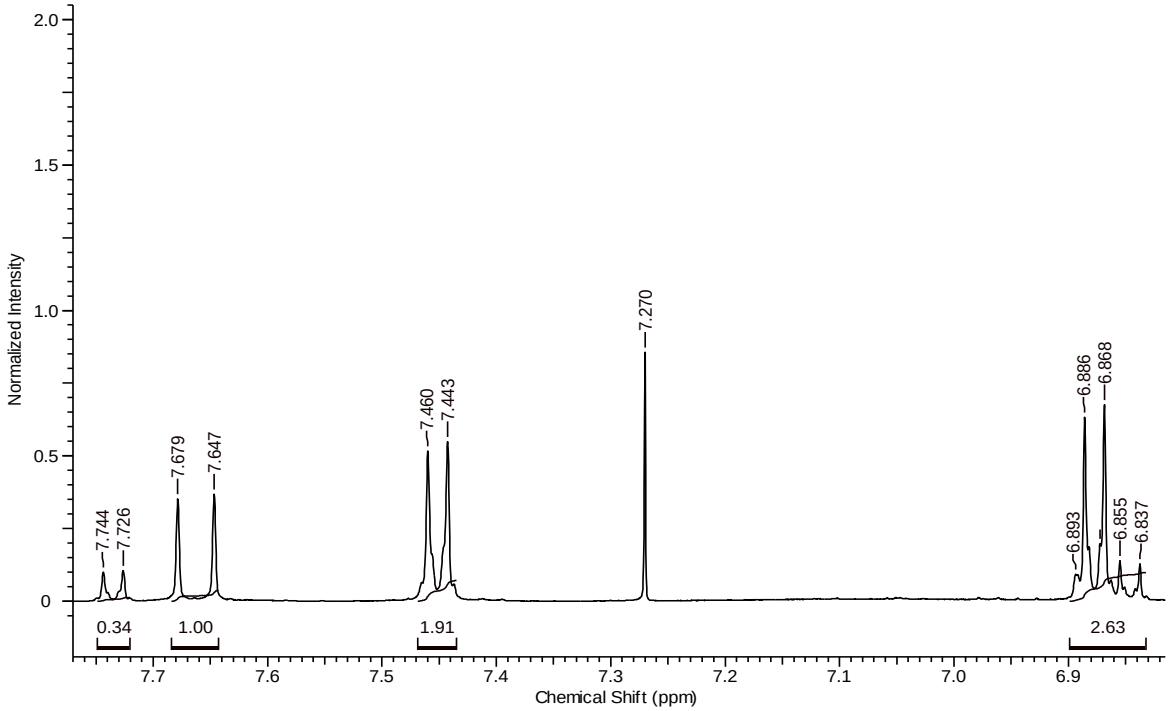


Figure 19.2: Magnification of the H [1] NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer, operating at 500 MHz for H [1] and 125 MHz for C [13] .

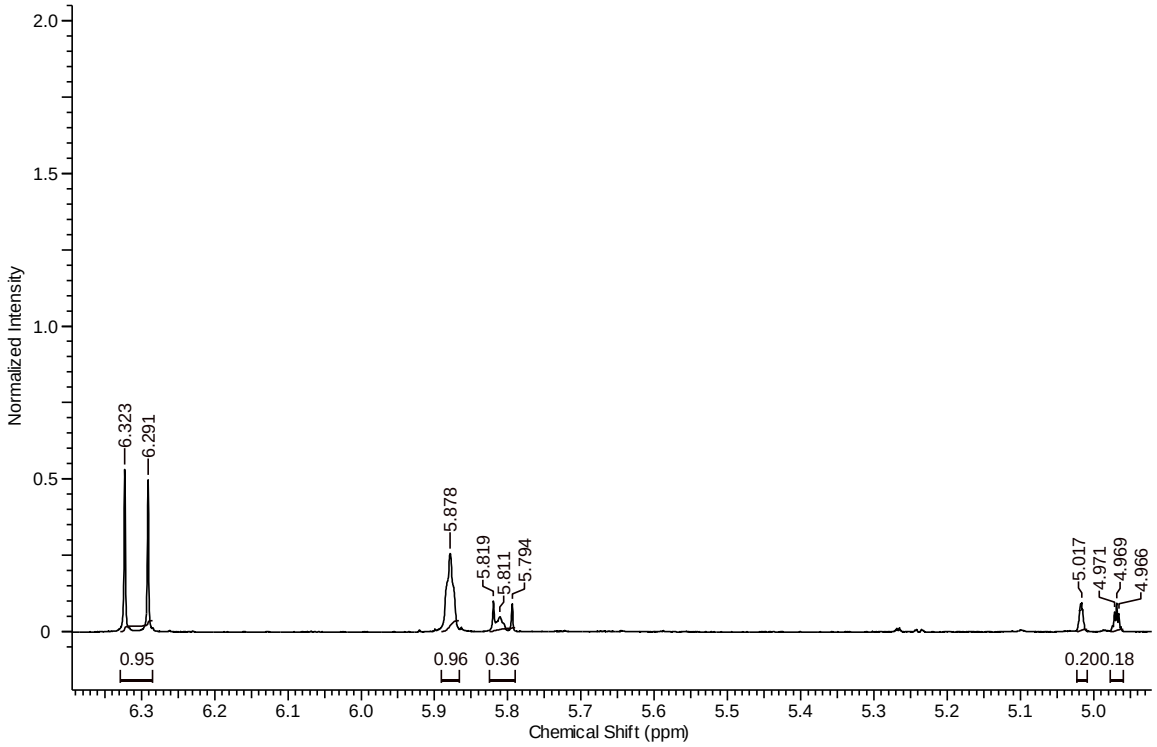


Figure 19.3: Magnification of the H [1] NMR spectrum of the fraction (Vm-F19-20) (figures. 19 -19.2) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer. Operating at 125 MHz.

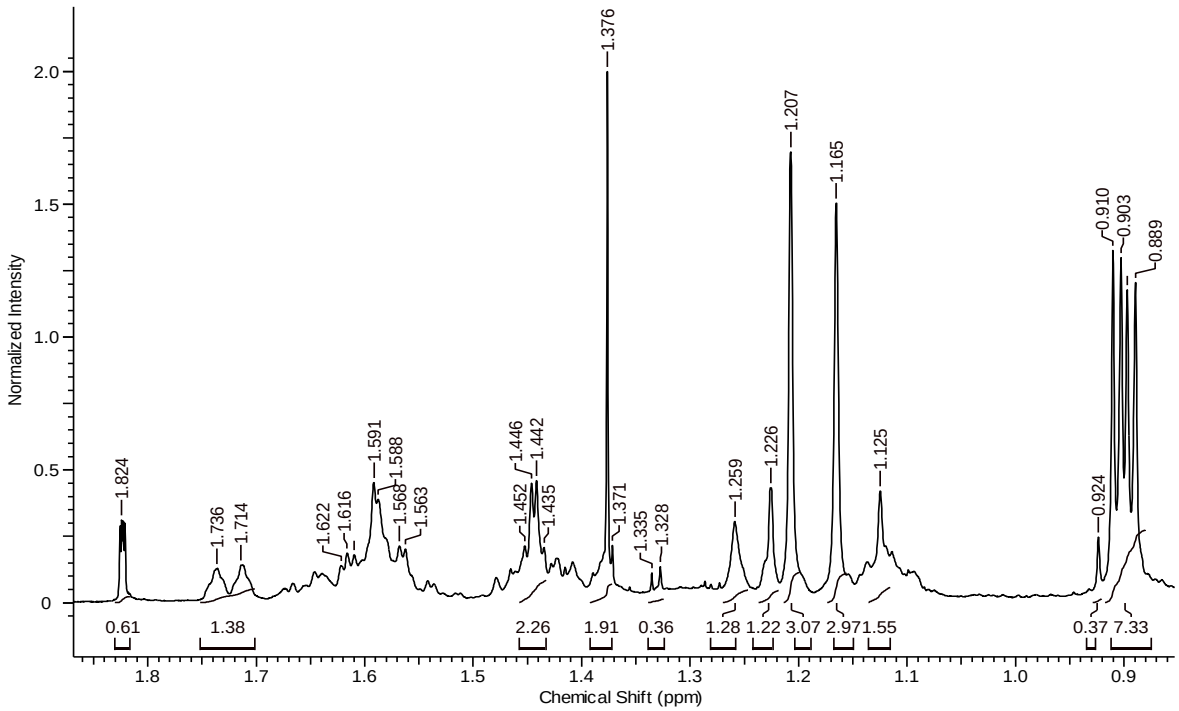


Figure 20: C^{13} NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer. Operating at 125 MHz.

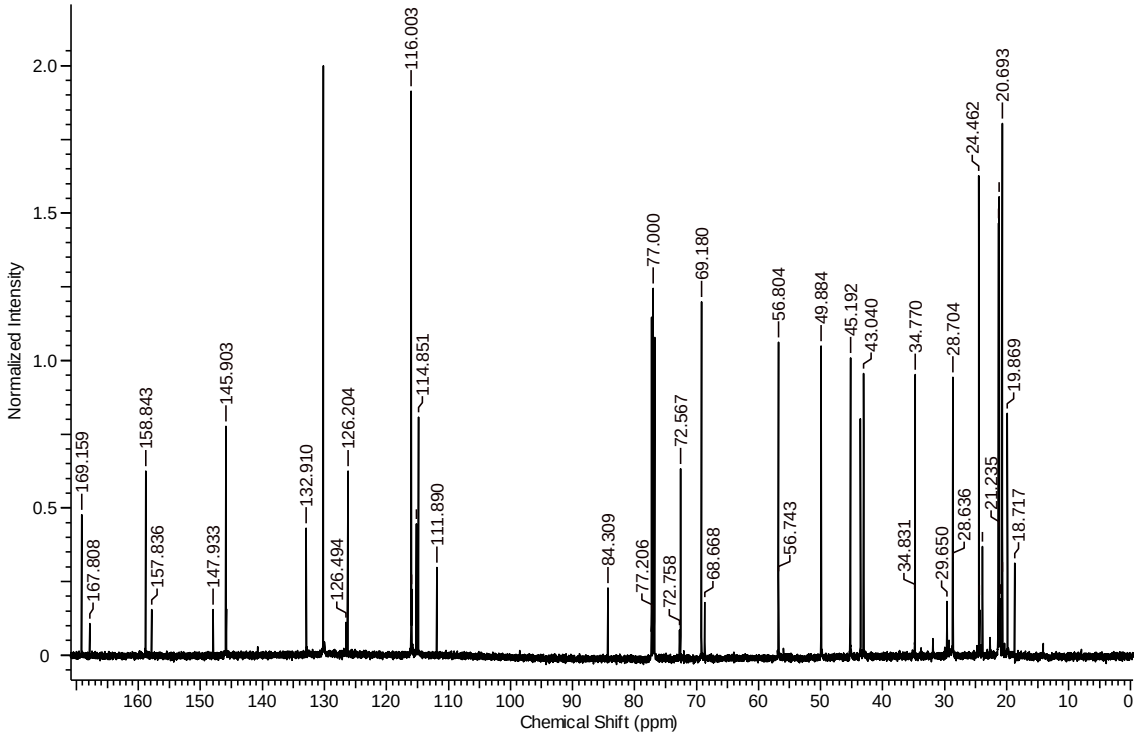


Figure 21: DEPT NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer. Operating at 125 MHz.

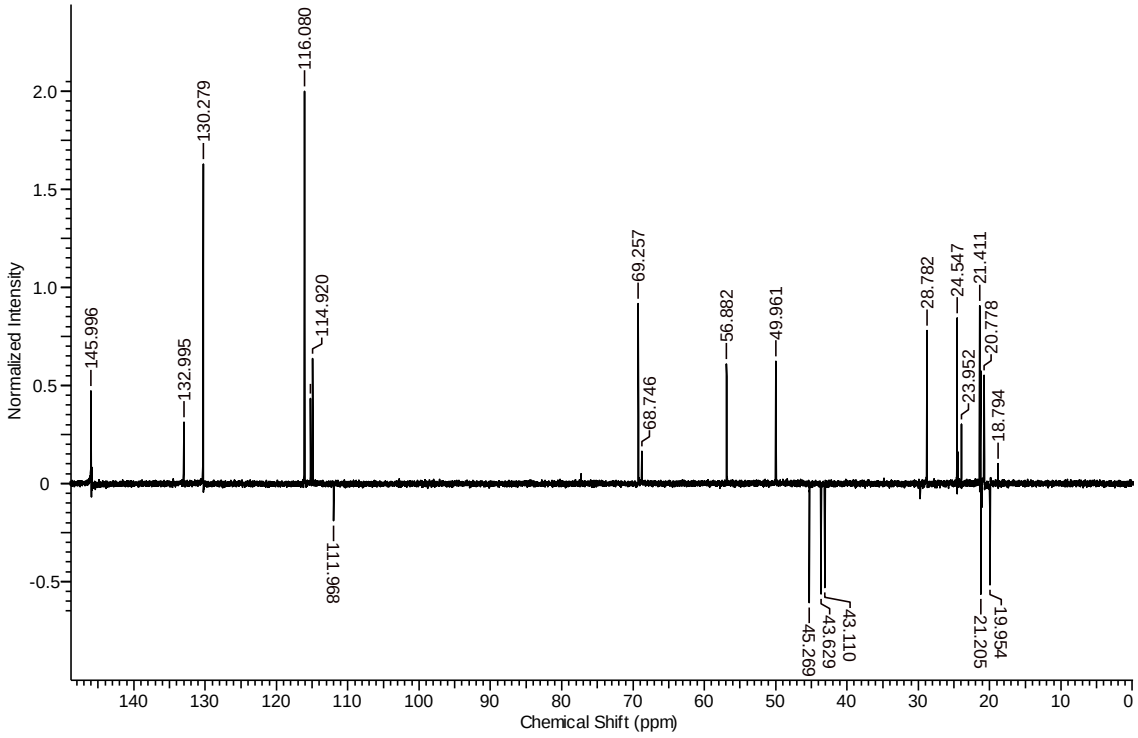


Figure 22: HSQC NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer. Operating at 125 MHz.

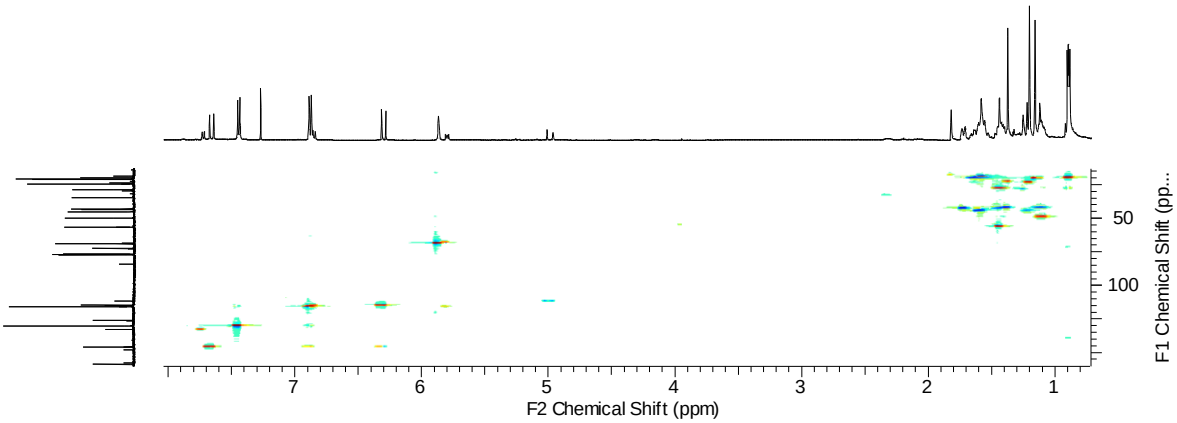


Figure 23: HMBC NMR spectrum of the fraction (F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer. Operating at 125 MHz.

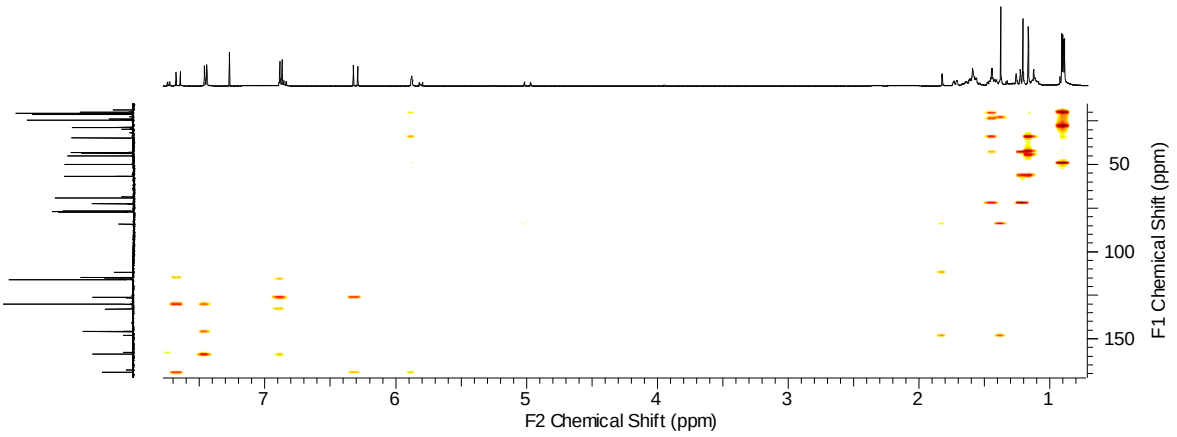
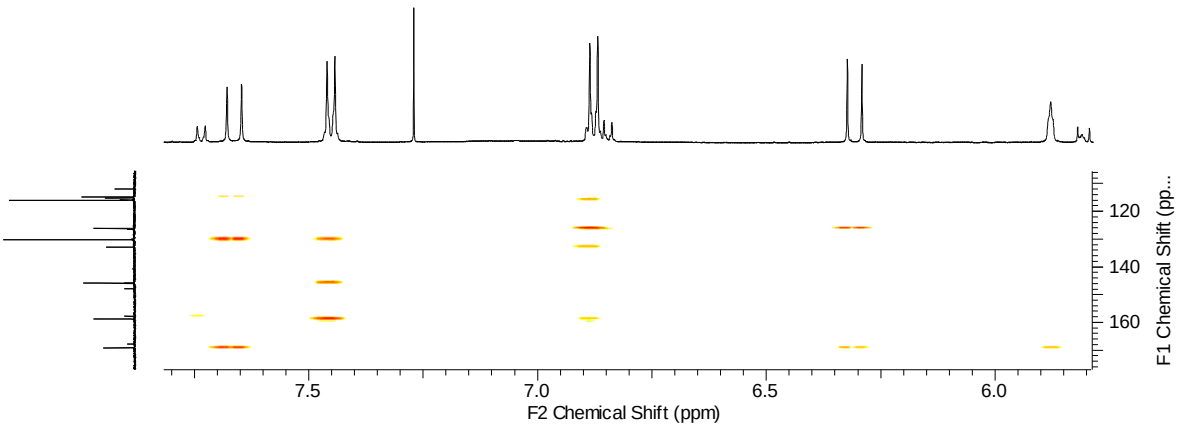


Figure 23.1: Magnification of the HMBC NMR spectrum of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm). Spectral data were acquired on a Varian INOVA 500 spectrometer. Operating at 125 MHz.



H^1 NMR spectrum (Figure 19) of the Vm-19-20 fraction shows a set of signals, characteristic of the presence of an aromatic component and an olefinic double component as a structural part of the molecular constituents, located between δ 5.80 and 7, 65 ppm . Between them it is observed that there is a difference in intensity. The integration of the signals revealed that the majority component corresponds to approximately 70% of the sample, characterized by the signals: 7.65 (d) integrating for 1H (16.0 Hz); 7.45 (d), integrating for two H (8.5 Hz); 6.87d (8.5Hz, 1H); 6.32 d (16.0 Hz); 5.87 sl and the lowest proportion, close to 30%, for the signals: 7.73 d, integrating to 1H (12.0 Hz); 6.88 (d), integrating for two H (8.5 Hz); 6.84d (8.5Hz, 1H); 5.80 d (12.0 Hz); 5.81 sl . In the region of least protection between δ 0.88 and 1.82, a set of characteristic signals of methyl, methylene and methine hydrogens suggests the presence of a group with a terpenic skeleton .

C^{13} NMR spectrum (125 MHz, $CDCl_3$) (Figure 20), the presence of 56 signals was verified. Which, in comparison with the DEPT spectrum (Figure 21), allowed us to determine the presence of 11 signals referring to non-hydrogenated carbons, 11 methinic , 13 methylene and 07 methyl carbons . Analysis of the data in Table 11, together with the correlations observed in the HSQC / HMBC spectra (Figures 22 and 23, respectively) and the comparison with the literature (Table 11), (BOHLMAN et al., 1980; XU et al. , 2010) allowed us to suggest that the Vm-F9-20 fraction is a mixture of the isomers 6-O-β-Ep-cumorail-4α- hydroxyeudesmane (Vm-A), with approximately 70% and 6-O-β -Zp-cumorail-4α- hydroxyeudesmane (Vm -B) with approximately 30% (Figure 24).

Figure 24: Major components of the fraction (F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm).

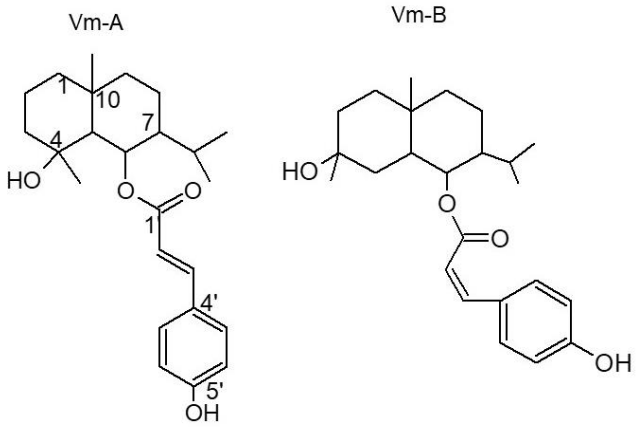


Table 11: NMR spectral data of H [1] , C [13] and HMBC of the components of the fraction (Vm-F19-20) of the ethyl acetate extract of *Verbesina macrophylla* (Vm) compared with literature data.

Sample data					(BOHLMANN, 1980) / (XU et al., 2010).		
A			**B**		**A**		**B**
W	C1 [3]	H1 –	C1 [3]	H1 –	C1 [3]	H1 –	C1 [3]
1	45.19	1.57	45.19		45.5		45.5
two	19.86	1.86	19.84		20.1		20.1
3	43.04	1.72	43.04		43.9	1.69	43.9
4	72.56	----	72.75	------	72.7	------	72.7
5	56.80	1.44	56.74	1.37	57.1	1.43 m	57.1
6	69.18	5.86	68.66		68.9	5.86 br	68.9
7	49.88	1.11	49.85		50.1		50.1
8	21.12	1.48	21.01		21.2		21.2
9	43.56	1.44	43.56		43.3		43.3
10	34.77	---	34.83	------	35.0	-------	35.0
11	28.90	1.44	28.63		28.8	1.43 m	28.8
12	21.33	0.90d	21.23		21.5	0.92d	21.5
13	21.18	0.89 d	21.12		21.4	0.90d	21.4
14	20.69	1.15	20.65		20.8	1.16s	20.8
15	24.46	1.20	24.37		24.5	1.19s	24.5
1'	169.15	----	167.80	------	168.0	------	168.0
two'	114.85	6.30d [16.0Hz]	115.16	5.78d [12.0Hz]	115.4	6.31d	115.4
3'	145.90	7.65d [16.0Hz]	145.70	6.86d [12.0Hz]	146.1	7.65d	146.1
4'	126.20	---	126.49	-----	126.5	------	126.5
5'	116.02	7.44d [8.5Hz]	115.16	7.72 [8.5Hz]	115.9	7.46d	115.9
6'	130.20	6.88d [8.5Hz]	132.91	6.85 [8.5Hz]	133.2	6.85d	133.2
7'	158.84	----	157.83	-----	157.8	-----	157.8

8'	130.20	6.88d [8.5Hz]	132.91	6.85 [8.5Hz]	133.2	6.85d	133.2
9'	116.02	7.44d [8.5Hz]	115.16	7.72 [8.5Hz]	115.9	7.46d	115.9

Source: Prepared by the author (2022)

5 CONCLUSION

The present work made it possible to expand scientific knowledge related to the secondary metabolism and biological activity of the species *Verbesina macrophylla* (Cass). Blake (Asteraceae), contributing to the appreciation of Brazil's flora.

The phytochemical investigation carried out with the crude extract of Ethyl Acetate from the leaves of *Verbesina macrophylla,* using the Hexane and Ethyl Acetate fraction as a base, allowed the isolation of two substances already described in the literature (6-ObEp-coumaroyl-4a -hydroxyeudesmane and 6-ObZp-coumaroyl-4a-hydroxyeudesmane), whose biological activity was reported for the first time in this study.

The crude extracts in Hexane, Ethyl Acetate and Ethanol were active against Gram-positive Bacteria and against filamentous and yeast-like fungal strains.

From the data obtained in the cytotoxicity test, there was no hemolysis for the crude Ethyl Acetate extract, while the Hexane extract showed slight hemolysis only at the highest concentration tested, however, it is important to highlight the need for more studies on human blood cells *In vitro* in addition to *in vivo studies* need to be carried out to confirm the toxicological profile of the extracts.

When evaluating the biological activity of the 51 fractions obtained from the Ethyl Acetate extract, a better antibacterial and antifungal effect is observed in the nonpolar fractions. However, chemical analyzes were unable to identify all the compounds present in the samples due to the great complexity of carrying out phytochemical studies. Therefore, new analyzes are necessary in order to isolate and characterize these compounds, some of which are even present significantly in fractions that showed good activity, as it is important to associate the chemical constituents of the fractions with the observed biological activities.

The compounds found in the Vm-19-20 fraction are related to the biological activity observed in preliminary tests with the crude ethyl acetate extract, indicating a possible application against bacterial infections, and could serve as a basis in the

future for developing medications for this purpose, new studies of this fraction are necessary for better biological analysis regarding this activity.

The NMR data demonstrated that the metabolites responsible for the antifungal activity of the fractions are mainly fatty acids. Therefore, new studies are suggested to characterize these substances, as well as to carry out new biological analyzes regarding this activity.

According to the kinetics of microbial death, the FVM19-20 fraction presents a concentration-dependent bacteriostatic effect for the *Bacillus subtilis strain,* as the increase in concentration increases the non-viability of bacterial cells.

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