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Facultad de Ingeniería en Telecomunicaciones, Informática y Biomédica
Departamento de Ingeniería Biomédica

University of Antwerp
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Neuropharmacological activity of leaf extract of Plectranthus neochilus Schltr. grown in Cuba

Dissertation submitted in fulfilment of the requirements for the degree of

Doctor in Biological Sciences

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Doctor in Biomedical Sciences (Ph.D)

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Neurofarmacologische activiteit van bladextract van *Plectranthus neochilus* Schltr. gekweekt in Cuba

Proefschrift ingediend ter vervulling van de eisen voor de graad van

Doctor in de Biologische wetenschappen

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world.”

Louis Pasteur

To my children: Frank Ernesto and Gabriel

To my parents and sister

To my professors, Julio C. Escalona Arranz

and

Wim Vanden Berghe

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SUMMARY

SUMMARY

Plectranthus neochilus Schltr. is a plant recently introduced in Cuba and its cultivation has spread throughout the island due to its ethnopharmacological benefits that are different from those reported internationally. In order to clarify such incongruence, the present study had the general objective of evaluating the neuropharmacological activity of *P. neochilus* leaves extracts cultivated in Cuba. The ethnobotanical study was based on Tramil methodology modified and included a bibliometric analysis. The quality parameters of the leaf and derived extracts were determined, according to the Public Health Branch Norm 309 and 312 respectively. *In vivo* assays were used for the evaluation of neuropharmacological activity; gene expression studies were performed on whole brain samples of mice from the *in vivo* study using RT-QPCR techniques. The bibliometric analysis confirmed that the use and scientific interest of the international community on this plant are mainly focused on its antimicrobial effects. The ethnobotanical study showed that this plant is consumed in Cuba alone or mainly with benzodiazepines to induce or enhance its sedative and hypnotic effects. The pharmacognostic evaluation showed a low yield of essential oils, the absence of glandular trichomes and the presence of metabolites with a high degree of oxidation. Among the metabolites identified were rosmarinic acid as the major compound, flavonoids and abietane-type diterpenes, 15 of them informed for the first time for the species, and have been reported, to be active on the Central Nervous System. The neuropharmacological evaluation showed that the extracts modified the behavior and motor coordination of mice. Gene expression study showed activation of the dopamine/opioid system as well as concomitant inhibition of metabolic-energetic, inflammatory and GABAergic signaling pathways. It confirms the traditional sedative-hypnotic use of the species in Cuba, different from the rest of the world, and provides the first scientific evidence on the possible pharmacological mechanism of action.

SAMENVATTING

SAMENVATTING

Plectranthus neochilus Schltr. is een plant die onlangs in Cuba werd geïntroduceerd. De teelt ervan heeft zich over het hele eiland verspreid vanwege de etnofarmacologische gezondheidsvoordelen. Opmerkelijk zijn de internationale verschillende traditionele gebruiken tegen ontstekingen (buiten Cuba) of bij slaapproblemen (in Cuba). Om eventuele slaapverwekkende eigenschappen van deze plant wetenschappelijk te verifiëren, werden in dit doctoraatsonderzoek de neurofarmacologische eigenschappen bestudeerd van extracten van *P. neochilus*-bladeren die in Cuba worden gekweekt en bereid volgens GGD-kwaliteitsnorm 309 en 312. In vivo assays werden gebruikt om neurofarmacologische activiteit te evalueren; genexpressiestudies werden uitgevoerd met hele hersenmonsters van muizen uit de in vivo studie met behulp van RT-QPCR-technieken. Eerst werd een etnobotanische bibliometrische studie uitgevoerd, gebaseerd op de Tramil-methodologie. Deze analyse bevestigde dat het gebruik en de wetenschappelijke belangstelling van de internationale gemeenschap voor deze plant vooral gericht zijn op de antimicrobiële effecten. Echter, in Cuba wordt deze plant alleen of voornamelijk met benzodiazepinen geconsumeerd om de kalmerende en hypnotische effecten te induceren of te versterken. De farmacognostische evaluatie vervolgens, toonde een lagere opbrengst aan etherische oliën in de Cubaanse cultivar, evenals de afwezigheid van glandulaire trichomen en de aanwezigheid van metabolieten met een hoge mate van oxidatie. Onder de geïdentificeerde metabolieten bevonden zich rozemarijnzuur als de belangrijkste verbinding, flavonoïden en diterpenen van het abietane-type, waarvan de meeste voor het eerst voor de soort werden geïnformeerd en waarvan is gemeld dat ze actief zijn op het centrale zenuwstelsel.

Neurofarmacologische evaluatie toonde aan dat de extracten het gedrag en de motorische coördinatie van de muizen veranderden. De studie van genexpressie toonde de activering van het dopamine / opioïde systeem, evenals de gelijktijdige remming van de metabolisch-energetische, inflammatoire en GABAerge signaleringsroutes. Het traditionele sedatieve hypnotische gebruik van de soort in Cuba, anders dan de rest van de wereld, wordt bevestigd en het eerste wetenschappelijke bewijs over het mogelijke mechanisme van farmacologische werking wordt geleverd.

RESUMEN

RESUMEN

Plectranthus neochilus Schltr. es una planta de reciente introducción en Cuba y su cultivo se ha extendido por toda la isla debido a sus beneficios etnofarmacológicos que son diferentes a los reportados internacionalmente. Para esclarecer tal incongruencia, el presente estudio se trazó como objetivo general evaluar la actividad neurofarmacológica de los extractos de la hoja de *P. neochilus* cultivadas en Cuba. Se parte del estudio etnobotánico, según metodología Tramil con modificaciones e incluyó un análisis bibliométrico. Se determinaron los parámetros de calidad de la hoja y de extractos derivados, de acuerdo a la Norma Ramal de Salud Pública 309 y 312, respectivamente. Para la evaluación de la actividad neurofarmacológica se emplearon ensayos in vivo; los estudios de expresión de genes se realizaron con muestras de cerebro completas de ratones procedentes del estudio in vivo mediante técnicas de RT-QPCR. El análisis bibliométrico confirmó que el uso y el interés científico de la comunidad internacional sobre esta planta, se centran principalmente en sus efectos antimicrobianos. El estudio etnobotánico demostró que esta planta se consume en Cuba sola o principalmente con benzodiazepinas para inducir o potenciar sus efectos sedantes e hipnóticos. La evaluación farmacognóstica evidenció un bajo rendimiento de aceites esenciales, la ausencia de tricomas glandulares y la presencia de metabolitos con un alto grado de oxidación. Entre los metabolitos identificados, están el ácido rosmarínico como compuesto mayoritario, los flavonoides, y diterpenos tipo abietanos; 15 de ellos son informados por primera vez para la especie y han sido reportados como activos sobre el Sistema Nervioso Central. La evaluación neurofarmacológica mostró que los extractos modificaron el comportamiento y la coordinación motora de los ratones. El estudio de la expresión génica, demostró la activación del sistema dopamina/opioide, así como la inhibición concomitante de las vías de señalización metabólico-energética, inflamatoria y GABAérgica. Se confirma el uso tradicional sedante hipnótico de la especie en Cuba, diferente al resto del mundo y se aportan las primeras evidencias científicas sobre el posible mecanismo de acción farmacológico.

LIST OF ABBREVIATIONS

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Ach: Acetylcholine

AChE: Enzyme acetylcholinesterase

Anova: One-way analysis of variance

ACN: Acetronyl

APA: American Psychiatric Association

ATCC: American Type Culture Collection

BBB: Blood-brain barrier

BLA: Basolateral amygdala

BV2: Mouse microglial cells

cAMP: Cycle adenosine monophosphate

COX-2: Cyclooxygenase type 2

CNS: Central Nervous System

CRD: Centre square duration

CSE: Centre square entries

D1: Dopaminergic receptors family D1 and D5

D2: Dopaminergic receptors family D2.D3 and D4

DA: Dopamine

DLE: Dried leaves ethanol extract

DLW: Dried leaves water extract

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DSM: Diagnostic and Statistical Manual of Mental

EBP: Extended binding pocket

EO: Essential oil

EOY: Yield values

FA: Formic acid

FBMN: Feature-Based Molecular Networking

FBS: Inactivated foetal bovine serum

FLD: Fresh leaves water extract

FPN: Fecal pellet number

G: Grooming

GABA: Gamma-aminobutyric acid

GAD: Generalized anxiety disorder

Glu: Glutamate

GNPS: Global Natural Products Social Molecular Networking

HMGB1: High mobility group box 1

HPLC-DAD: High performance liquid chromatography with photodiodo-array-detection

Iba1: Ionized calcium-binding adaptor

ICD: International Classification of Diseases

ICIMAR: Institute of Marine Sciences

IDT: Integrated DNA Technologies

iNOS: inflammatory enzymes

LPS: Lipopolysaccharide

LR: Line crossing

L190(ECL2): Extra-cellular loop residue

MAO: Enzyme monoamine oxidase

MAPK: Mitogen-activated protein kinase

MDD: Major depressive disorders

Nad: Noradrenaline

nr3c1: Nuclear Receptor Subfamily 3 Group C Member 1

NS: Nervous system

OXt: Oxytocin

OBP: Orthosteric binding pocket

PA: Panic disorder

PC12: Rat adrenal medulla

pH: Potencial hydrogen

PNS: Peripheral Nervous System

PTSD: Depression, post-traumatic stress disorder

R: Rearing

REM sleep: Rapid eye movements sleep

ROS: Reactive oxygen species

RPMI-1640: Roswell Park Memorial Institute Medium

RT-qPCR: Real-Time Quantitative Reverse Transcription

SAD: Social anxiety disorder

SD: standard deviation

SM: Selective mutism

SP: Specific phobias

TIC: Total ion chromatogram

TRAMIL: Traditional Medicine in the Islands

U: Urination

UPLC-DAD-MS/MS: Ultrahigh-performance liquid chromatography-diode array

WHO: World Health Organization

List of genes

ACTB	Actin beta
ATP2A1	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1
B2M	Beta-2-microglobulin
BDNF	Brain-derived neurotrophic factor
CASP8	Caspase-8
CASP9	Caspase-9
CHRM2	Muscarinic acetylcholine receptor M2
CHRNA1	Acetylcholine receptor subunit alpha
CLCN1	Chloride channel protein 1
CREB1	Cyclic AMP-responsive element-binding protein 1
CSFR3	colony stimulating factor 3 receptor
CXCR2	C-X-C Motif Chemokine Receptor 2
DRD1	Dopamine receptor D1
DRD3	Dopamine receptor D3
DRD4	Dopamine receptor D4
GABBR1	Gamma-aminobutyric acid type B receptor subunit 1
GABRA1	Gamma-aminobutyric acid receptor subunit alpha-1
GABRA4	Gamma-aminobutyric acid receptor subunit alpha-4
GABRA5	Gamma -aminobutyric acid (GABA) A receptor, subunit alpha 5
GABRG2	Gamma -aminobutyric acid receptor subunit gamma-2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HSPA1B	Heat shock protein family A (Hsp70) Member1B
HTR1A	5-hydroxytryptamine receptor 1A
HTR2A	5-hydroxytryptamine receptor 2A
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1
NRF1	Nuclear Respiratory Factor 1
OPRD1	Delta-type opioid receptor
OPRL1	Opioid receptor-like 1
OPRM1	Opioid Receptor, Mu 1

PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
S100B	Protein S100-B
SELL	Selectin, lymphocyte
SIRT3	NAD-dependent protein deacetylase sirtuin-3
SOD1	Superoxide dismutase [Cu-Zn]
TLR3	Toll-like receptor 3
TNFA	tumor necrosis factor

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CHAPTER I

CHAPTER I. Introduction and research objectives

I.1 Introduction

During evolution, the human nervous system (NS) developed into an extraordinarily complex and highly specialised structure, capable of regulating and executing multiple processes. It is composed by two main functional structures: the Central Nervous System (CNS) and the Peripheral Nervous System (PNS). The CNS control the physical movements, the integration, and correlation of different types of sensory information, and the secretion of hormones, while PNS is the responsible for transmitting nerve impulses to the thirty-one pairs of spinal nerves, communicating the brain with the rest of the body.

Within the wide range of CNS disorders, close association have been observed for insomnia and anxiety, which reinforce each other. This implies that the existence of persistent insomnia generally induces states of anxiety and, in turn, persistent states of anxiety generate insomnia. Epidemiological studies estimate that the relationship between the appearance of sleep disturbances and anxiety disorders can reach up to 42%[1].

International statistics indicte that more than one-third (up to 50%) of the worldwide adult population suffers from sleeping problems 50%[2]; ranking insomnia as the most common symptom of mental distress[3]. Those values change between regions and instruments/classification criteria, but most of them agree that gender (female) and age (elderly) are the most common somatic conditions associated with insomnia. Others highlight the biopsychosocial factors as vulnerability, due to lower-income or limited education. Some co-morbidities such as diabetes, hypertension, heart diseases, chronic pain, psychiatric disorders and cancer have been associated as well[4].

On the other hand, anxiety disorders affect 10 to 25% of the total population. Experiencing physiological or moderate anxiety can help us to stay focused, to be cautious, or to face challenges, to support stress resilience. Only an imbalance of this

normal response evolves into a pathological status, so-called anxiety disorders. Those worries or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of activity[5]. The prevalence of anxiety disorders also varies by culture, with rates in European/Anglo-Saxon cultures almost double those in African ones. Prevalence rates also vary by gender, with women statistically more likely than men to develop an anxiety disorder at some point in their lives. With the emergence of the COVID-19 pandemic, an environment was created in which many triggers of poor mental health were exacerbated and anxious disorders became the sixth leading cause of worldwide disability[6].

The high prevalence of insomnia and anxiety, their significant association with a deterioration in the quality of life, the increased likelihood for developing behavioural and mood disorders, as well as the enhancement of other co-morbid pathologies represents an increased economic cost to society (reduced productivity, absenteeism, care consumption, increased work and domestic accidents), and has become a health concern.

Different psychological and pharmacological approaches, alone or in combination, have been used to treat insomnia[7] and anxiety[8]. However, within all the possible treatments for each discomfort, two of them stand out as common to both pathologies: cognitive-behavioural therapy (as a psychological treatment), and benzodiazepine intake (selective pharmacological therapy)[9]. This seems logical considering the strong relationship between the appearance of sleep disturbances and anxiety disorders (up to 42%) and its reciprocal induction. As a consequence, preclinical tests for both disorders show much similarity, making it difficult to define a specific test for the evaluation of a specific activity, giving place what is known as Preclinical Behavioural Tests. Nonetheless, both kinds of therapies are expensive, and due to the early onset of symptoms, chronicity, and high rate of associated comorbidity, it is essential to develop new safe and effective alternative treatments based on medicinal plants.

The traditional folk use of medicinal beverages to treat insomnia and anxiety disorders is ancestral, being transmitted from generation to generation, making phytopharmaceuticals a fundamental resource of Natural and Traditional Medicine Systems. That is why the research on the medicinal plants used in developing countries for treating those central nervous systems ailments emerged as a viable cost-effective alternative to face up the deficiencies of conventional drugs[10].

*P. neochilus*is is an aromatic and succulent species belonging to the *Lamiaceae* family. It is endemic to South Africa and Namibia and is commonly known as "blue coleus" or "lobster flower" by the Angloamericans, "muskietbossie" by the Africans and "boldo-rasteiro" by the Portugues[11]. It is traditionally used in African and Brazilian folk medicines to treat skin diseases, respiratory ailments, digestive disorders, liver failure, and dyspepsia[12,13]. Pharmacologically, it has been shown to be a potent antimicrobial due to the presence of its essential oils[14,15], besides its characteristics as a phytoremediator[15], antioxidant[13], cytotoxic[16] and α-glucosidase inhibitor[17].

The recent introduction of the species *P. neochilus* in Cuba has been recognized by the Enviromental, Sciences and Technology Ministry in 2016[18]. Its cultivation has rapidly expanded throughout the island due to its ethnopharmacological benefits mainly to treat anxious and sleep disorders, which seem to be different from what is worldwide reported[19]. Due to the poor description of the ethnobotanical and pharmacological properties of *P. neochilus* since its introduction in Cuba, the purpose of this work is the characterisation of the ethnobotanical use, the determination of the phytochemical composition and the evaluation of the neuropharmacological effect leaves extracts of cultivars growing in Cuba. In addition, the scientific evidence obtained will contribute to the formulation of recommendations for the effective of this plant by the Cuban population. Under this premise, the following research poses the following scientific problem:

Scientific problem: The fragmentary information on the traditional medicinal use of the extracts from leaves of the species *P. neochilus* in Cuba and the absence of

experimental scientific evidence of its neuropharmacological activity limit its effective use.

Hypothesis: The neuropharmacological evaluation of the extracts from leaves of *P. neochilus* will provide the first scientific evidence supporting its ethnopharmacological use, drawing the first steps for a future natural treatment.

According to the above-mentioned, the **General Objective** of this research was:

General Objective: To evaluate the neuropharmacological activity of leaf extracts from *P. neochilus* cultivars in Cuba.

For this general objective, three **Specific Objectives** were defined:

1. To characterize the ethnobotanical use of the species (Chapter IV).
2. To determine the quality parameters of the drug and leaf extracts of the species (Chapter V).
3. To determine the neuropharmacological activity of leaves extracts of the species (Chapter VI).

I.2- Novelty of the study

The following results, which constitute a first report in the national and international context, emerge as the most relevant scientific news of this research:

1. The first ethnobotanical characterisation of *P. neochilus* leaves growing in Cuba, highlighting (regardless of the geographical area studied) a common use as a sedative, hypnotic, analgesic, and anti-inflammatory. The sedative and hypnotic effects of the plant are described for the first time.
2. The combined and articulated use of ethnobotanical and bibliometric studies allowed to demonstrate that the traditional medicinal use of the species *P. neochilus* throughout Cuba for sedative purposes differs from that reported in the rest of the world.
3. It is reported that the species *P. neochilus* that grows in Cuba may undergo some morphoanatomical changes, with a direct impact on the low yields in the

production of essential oils, and on the oxygenated chemical nature of its non-volatile components.

4. The presence of 15 new non-volatile compounds identified in the leaf of the species is reported.
5. A preclinical pharmacological scientific study is carried out allowing justified the neurobehavioral effects of the traditional consumption of the leaf of the species by the Cuban population, proposing a possible mechanism of action and thus validating its ethnopharmacological use.

I.3- Practical relevance

P. neochilus leaves are commonly used by Cuban inhabitants to treat neurobehavioural disorders, although so far there is no scientific evidence for its neuropharmacological effects. Within this thesis, we found that in Cuba, the species is used for a different purpose than its traditional use reported in other countries worldwide. Presumably, climat specific adaptive mechanisms have been evolved for the species with impact in their morphoanatomic structures and the chemical composition of secondary metabolites. In this respect, different chemical patterns in Cuban cultivars support its neuropharmacological ethnobotanical a use, supported by observational preclinical tests in mice as well as brain molecular markers determined by QPCR-RT gene expression. By demonstrating the modification of dopamine and opioid regulatory systems, as well as several anti- and pro-inflammatory intermediates, this research start to resolve a molecular mechanism of action, highlighting its promising value as a source of bioactive metabolites with therapeutic interestfor the development of phytomedicines with potential neuropharmacologial clinical use.

I.4- Social relevance

Inhabitants of low incoming countries are common users of their natural recourses; a proven fact even recognized by the WHO. Since its recent introduction of *P. neochilus* in Cuba, it has been extensively used to amelerioate neurological complains. That is why, investigations focused to demonstrate scientific proof for its neuropharmacological effectiveness and safety creates new opportunities for recommendations towards

national public policies, aiming or discouraging its empirical use and at the same time contributing to enhance the Cuban natural cultural heritage.

CHAPTER II

CHAPTER II. Behaviour disorders and Natural Products

II.1.- Nervous System. Overview

During evolution, the nervous system (NS) has evolved into an extraordinarily complex and highly specialised structure, capable of regulating and executing multiple and diverse processes. It enables the control and performance of vegetative functions, the perception and processing of sensory stimuli, motor response and emotions, as well as being able to learn and store memory. For the development of these functions, the NS requires a complex molecular cellular organisation which regulates its effector system.

The Nervous System is divided into two main parts: The Central Nervous System (CNS) that is structured by the brain and the spinal cord and the Peripheral Nervous System (PNS) consisting of the nerves that branch out from the spinal cord and are extended to all parts of the body.

The basic unit of the nervous system is the nerve cell or neuron. A neuron has a cell body and special extensions called axons and dendrites. Bundles of axons, called nerves, are found throughout the body. Axons and dendrites allow neurons to communicate, even over long distances[20]. There are different types of neurons that control or perform different activities. Motor neurons transmit messages from the brain to the muscles to generate movement. Sensory neurons detect light, sound, smell, taste, pressure, and heat, sending messages about these elements to the brain[21].

Interneuronal communication takes place via an electrical signal along its axon. At the end of the axon, the electrical signal is transformed into a chemical signal and neurotransmitters are released into the synaptic space where the chemical synapse occurs between an axon and the dendrite tip of another neuron. This chemical signal is re-converted into an electrical signal that travels through the neuron and undergoes the same conversion processes as it travels to neighbouring neurons[22].

The nervous system also includes non-neuronal cells, called glia. Microglias are the resident immune cells of the brain and are located within the brain parenchyma behind

the blood-brain barrier. They originate from mesodermal hematopoietic precursors and are slowly turned over and replenished by proliferation in the adult central nervous system. Glial cells perform many important functions that make the nervous system drives properly. Glial cells support neurons, have a protective function, participate in the movement of nerve impulses, repair and restore neuronal function, remove dead neurons, and regulate the release and uptake of neurotransmitters[23].

Neurotransmitters are excellent chemical messengers and represent the main way of communication between the different structures of the NS playing four main functions: Excitatory (acetylcholine (ACh), glutamate (Glu), dopamine (DA), noradrenaline (NAd), and adrenaline (Ad)); inhibitory (gamma-aminobutyric acid (GABA), serotonin (5-HT), and Dopamine); neuromodulators (DA, Ach, 5-HT, histamine, and NAd); and neurohormones (Oxytocin (Oxt), and vasopressin (ADH) as main[24]. On the other side, neurotransmitter receptors can be divided into two fundamental groups: ionotropic receptors, also called ligand-gated ion channels, and metabotropic receptors, which are typically G protein-coupled receptors. The ionotropic receptor family (N-methyl-d-glutamate, kinase-quisqualate, nicotinic cholinergic, glycine, and GABA), consists of ion channels that open when bound by the neurotransmitter, which facilitates a fast response by altering the influx of chloride, sodium, potassium or calcium causing excitatory or inhibitory membrane potentials. In the metabotropic ones (5-HT, α and β-adrenergic, and DA), the neurotransmitters interact with G proteins and activate another molecule (second messenger such as cAMP) which regulates the intracellular signal transduction, activation of protein kinases, and ion channels permiability. The responses mediated by a second messenger system are slower and allow a more delicate regulation of the rapid response of ionotropic neurotransmitters[25].

II.2.- Central Nervous System Generalities

The function of the Central Nervous System (CNS) is to control physical movement, the integration, and correlation of different types of sensory information, and the secretion of hormones. It consists of the brain and spinal cord. The brain is composed of the

cerebrum, cerebellum, and brainstem which are within the skull. On its side, the spinal cord is located inside the spinal canal connected to the brain through the occipital foramen of the skull.

The brain receives information through our five senses: taste, smell, sight, touch, and hearing, often several of them at the same time. Gathering these messages and assigning them a meaning; and then store that information in our memory. The brain controls our thoughts, memory and speech, the behavioural movements of our arms and legs, and the functioning of many organs in our body. The Cerebrum, the largest part of the brain, is composed of the right and left hemispheres. It performs higher functions such as interpreting touch, vision and hearing, as well as speech, reasoning, emotions, learning and fine control of movement. The cerebellum: lies beneath the cerebrum. Its function is to coordinate muscle movements and maintain posture and balance. By its sides, the brainstem acts as a relay centre connecting the brain and cerebellum to the spinal cord. It performs many automatic functions such as breathing, heart rate, body temperature, sleep-wake cycles, digestion, sneezing, coughing, vomiting, and swallowing.

In the same way, the brain is divided into two halves: the right and left hemispheres. They are linked by a bundle of fibres called the corpus callosum that transmits messages back and forth. Each hemisphere controls the opposite side of the body, without implying that all their functions are shared. The cerebral hemispheres, in turn, have some fissures that divide the brain into lobes. Each hemisphere contains 4 lobes: frontal, temporal, parietal, and occipital. Each lobe can be divided, again, into areas that serve very specific functions. It is important to highlight that each lobe of the brain does not function alone, working under complex relationships between the lobes and hemispheres of the brain. Within the brain are pathways called white matter tracts that connect areas of the cortex to other areas. Messages can travel from one gyrus to another, from one lobe to another, from one side to anotherand deeper structures within the brain.

The brain has other important parts such as the hypothalamus, which is involved in controlling behaviours such as hunger, thirst, sleep and sexual response, body temperature, blood pressure, emotions, and hormone secretion. Connected to the hypothalamus, the Pituitary gland (known as the 'master gland'), controls other endocrine glands in the body. It secretes hormones that promote bone and muscle growth, control the sexual development, and respond to stress. On its side, the Pineal gland regulates the body's internal clock and circadian rhythms by secreting melatonin[26].The thalamus serves as a relay station for almost all information to and from the cortex and involves pain sensation, attention, alertness and memory; while the basal ganglia work with the cerebellum to coordinate fine movements. At last, the Limbic system acts as centre of our emotions, learning, and memory.

By its side, the spinal cord is a long cylindrical structure located in the spinal canal, and responsible for transmitting nerve impulses to the thirty-one pairs of spinal nerves. The brain communicates with the body through the afferent functions, in which sensations are carried from the trunk, neck, and four limbs to the brain, and the efferent functions, in which the brain commands the effector organs to perform a response action. It also consists of three membranes that surround it concentrically: the pia mater, the arachnoid, and the dura mater[27].

This strong connection between all the anatomical structures from the brain and spinal cord is responsible for the so-called normal mental and motor behaviour, which is affected in various mental and behavioural disorders.

II.3.- Mental health and Anxiety disorders

Mental health, as an indivisible part of health, contributes significantly to a quality of life and full social inclusion. Mental disorders constitute a major economic and social burden because of the disability they produce, dysfunctionality on personal well-being, social relationships, and productivity at work.

There are several classification systems, but the most recognized are: the International Classification of Diseases (ICD-10)[28] created by the WHO and the Diagnostic and Statistical Manual of Mental Disorders (DSM -V)[5] prepared by the American Psychiatric Association (APA).

According to the DSM-V, mental disorders are classified as:

- Neurodevelopmental disorders.
- Substance /Medication-Induced Psychotic Disorder.
- Bipolar disorder and related disorders.
- Substance / Medication-Induced Depressive Disorder.
- Anxiety disorders.
- Obsessive-compulsive disorder and related disorders.
- Acute stress disorder.
- Sleep-wake disorders.
- Substance / Medication-Induced Sleep Disorder.
- Neurocognitive disorders.

II.3.1.- Sleep-wake disorders

The sleep-wake disorders enclosed in a single term of insomnia include all the subjective perceptions that generate difficulty with sleep initiation, duration, intermittence, consolidation, and quality, which occurs despite adequate conditions for sleep, generating a daytime impairment. Due to the different ways of perception, different classifications, and scales exist to determine it. There are also multiple psychometric instruments based on these classifications: the Mini Sleep Questionnaire, Insomnia Severity Index, Pittsburgh Sleep Quality Index, Athens Insomnia Scale, and Lebanese Insomnia Scale. As a consequence of the different classifications, , the data regarding the prevalence of the global population remain variable and depend on the applied method[29].

Despite those disagreements, insomnia is classified as the most common symptom of mental distress[3]. Although its significance is frequently underestimated, misevaluating

the facts that large period of insomnia multiplies the risks for the appearance of other medical disorders, accidents, alcohol/drug abuse, and psychiatric illnesses. This critical panorama becomes even worse when insomnia co-occurs with other co-morbilities but specifically other mental distress such as major depression and anxiety. Patients with insomnia suffer from daytime impairments, reduced quality of life, and low levels of treatment adherence; increasing healthcare utilization and costs. Some reports show that almost 90 % of insomnia-related costs can be attributable to work absences and reduced productivity[7].

II.3.2.- Anxiety disorders

Anxiety is the most common and universal of emotions, a reaction of tension without apparent cause, more diffuse and less focused than fears. It is a normal emotional state that constitutes a habitual response to different stressful daily situations. It becomes pathological when it exceeds the adaptive capacity causing significant discomfort with symptoms that affect the physical, psychological and behavioural levels. The main symptom of anxiety disorders is excessive fear or worry. Anxiety disorders can also make it difficult to breathe, sleep, be quiet, and concentrate. The specific symptoms depend on the type of anxiety disorder you have[30].

There are several types of anxiety disorders: Generalized anxiety disorder (GAD), Panic disorder (PA), Social anxiety disorder (SAD) also called social phobia, Specific phobias (SP) like heights or flying, Selective mutism (SM), and Medication-induced anxiety disorder as main.

Anxiety is a multifactorial disease, with associated morbidity that makes its diagnosis more complex. Some of the causes may be genetic since the disorders can be hereditary. They may also be associated with chemical factors. It is suggested that anxiety disorders may be related to faulty circuits in the brain that control fear and emotions, environmental stress (stressful events that have been seen or experienced like abuse and neglect in childhood, death of a loved one, etc.). Other causes associated with anxiety disorders can be abstinence, drug use, and dependence on long-term therapy

with psychotropic drugs. Among the risk factors associated with anxiety disorders highlights having another mental disorder, such as sleep-wake disruption, depression, post-traumatic stress disorder (PTSD), stressful or negative life events, and constant worry about your health or that of a loved one[31,32].

Different psychological and pharmacological approaches, alone or in combination, have been used to treat insomnia[7] and anxiety[8]. However, within all the possible treatments for each discomfort, two of them stand out as common to both pathologies: cognitive-behavioural therapy (as a psychological treatment), and treatment with benzodiazepines (elective pharmacological therapy)[9]. There is a strong relationship between the appearance of sleep disturbances and anxiety disorders (up to 42%) and its reciprocal induction. As a consequence, neurobiology, neurochemistry, and genetic markers as well as the preclinical tests used to evaluate the incidence of any substance/drug are also similar. This makes it difficult to select a specific marker or test for the evaluation of a specific activity, giving place to what is known as general preclinical behavioural markers and tests.

II.3.3.- Neurobiology, neurochemistry, and genetic markers of insomnia and anxiety disorders.

Knowledge of the neurobiology, its neurochemical underpinnings, and the molecular basis of insomnia and anxiety disorders remain limited. Nowadays is accepted that the hypothalamus is the most important brain part in regulating sleep-wake functions. Sleep onset and maintenance are generally controlled by GABAergic neurons located in the preoptic area of the hypothalamus. These GABAergic neurons induce sleep by inhibiting the cells involved in arousal functions, and at the same time inhibiting the cholinergic neurons irradiates in the basal forebrain, deactivating the cortex activity. Also, serotonin, histamine, and norepinephrine cells are inhibited by GABAergic neurons, remaining “off” during REM sleep and playing a distinct role in the sleep-wake cycle. Histamine has a main role in the control of arousal but a limited role in the muscle tone

control, whereas norepinephrine and serotonin affect both muscle tone and arousal but are not as tightly linked to the waking maintenance state as histamine is[33].

Molecular and electrophysiological studies have demonstrated that neurotransmitter receptor expression and/or function are sensitive to sleep loss. The up regulation of the inhibitory GABAA receptor subunits could happen as a result of the prolonged discharge of GABA neurotransmitters in the basal forebrain. Furthermore, attenuation of 5-HT1A autoreceptor function could be explained by receptor desensitization or down-regulation with a consequent diminution of serotonin liberation. As happened with the 5-HT1A receptor, cholinergic, histamine, and norepinephrine receptors can be down-regulated as well[34].

Mood and anxiety disorders are characterized by a variety of neuroendocrine, neurotransmitter, and neuroanatomical disruptions. The degree of interconnectivity between neurotransmitters liberated in the limbic circuits, brain stem, and higher cortical brain areas seems pivotal for appearing these symptoms. The pathophysiology of generalised anxiety disorder is significantly affected by low levels of GABA. GABA A receptors are particularly concentrated in the medial prefrontal cortex, amygdala, and hippocampus, all of which are involved in anxiety and fear responses[27].

Studies showed that the regulation of GABAergic and serotonergic synaptic pathways are involved in anxiolytic mechanisms, being identified potential compounds activating anxiolytic mechanisms of GABAA and 5-HT receptor expressions by RT-qPCR techniques[35,36]. Other mechanisms explored to arrive at this result are protein-protein interaction analysis, gene clustering, and gene ontology[35].

As well as for insomnia, in anxiety disorders not only GABA and serotonin are involved, but also dopamine which acts modulating a cortical brake and the amygdala, have an important influence on the trafficking of impulses between the basolateral nucleus and central amygdaloid nucleus, which activatae non-overlapping populations of D1-and D2-dopaminergic receptors located in these structures. Behaviourally, intra-amygdala

infusion of D1 agonists and antagonists elicit anxiogenic and anxiolytic effects, respectively, in conditioned and unconditioned models of fear/anxiety, suggesting an anxiogenic role of D1 receptors in the amygdala[37].

The effects of D2 agonists and antagonists suggest that anxiogenic or anxiolytic effects can be obtained, depending on the nature of the threat experienced by the animal. It is suggested that dopamine D1 and D2 receptors in the amygdala may have a differential role in the modulation of anxiety. It is stated that D1 receptors may be involved in danger recognition, whereas D2 receptors have a role in establishing adaptive responses to cope with aversive environmental stimuli[37].Weger and Sandi suggestedthat vulnerability to stressful situations in individuals with elevated levels of anxiety could be caused by epigenetic alterations in neurotransmitter and neuroendocrine systems and mitochondrial function[38,39].

So far, only a few association studies have been performed among patients with anxious disorder phenotype, leaving us without a consistent conclusion about vulnerable genes with this disease. The association of a general anxiety disorder with a variation of the 5-hydroxytryptamine receptor 1A (5-HTR1A) gene is partially mediated by co-morbidity with major depression[40].

The intensive search for genetic predictors of treatment has revealed a few genes, including the serotonin receptor 2A (HTR2A) gene, dopamine D3 receptor (DRD3), nuclear receptor subfamily group C member 1 (NR3C1), and phosphodiesterase 1A (PDE1A), as potential markers[41-46].Regardless of the behavioural disorder, some neurotransmitters appear as common within brain regulation distress. GABA, dopamine, and serotonin are the most frequently involved, but also others like histamine, adrenergic, and cholinergic ones can also participate. That is why searching the influence of any drug or substance on the CNS trends includes the exploration of those neurotransmitter system receptors[35].

II.3.4.- Behavioural disorders and neuroinflammation

The interaction between the immune system and the central nervous system (CNS) provides insight into possible mechanisms of how the immune system influences our brain, resulting in different behavioural disorders[47]. Cytokines play an important role in CNS functions such as neurotransmitter metabolism, neuroendocrine function, synaptic plasticity, and neural circuits related to mood. As soluble substances are released within the different brain cells (e.g., microglia, astrocytes, oligodendroglia, and neurons), modulating the neuronal activity in specific brain regions such as the amygdala, hippocampus, hypothalamus, and cerebral cortex. As consequence, their deregulation could lead to multiple behavioural disorders such as anxiety, depression, sleep-wake, and cognitive dysfunction[48].

Much evidence suggests that systemic inflammation triggers a neuro-inflammatory response characterised by sustained microglial activation[49], with microglia being the main source of cytokines in the inflamed central nervous system. Functionally, microglial activation is defined as the release of pro-inflammatory cytokines such as interleukin-1, tumour necrosis factor, and interleukin-6[50].

Microglial cells also produce chemokines that attract monocytes to the brain[51] and monocytes in turn produce pro-inflammatory cytokines[52]. These increased levels of pro-inflammatory cytokines in the brain may exert direct and indirect neurotoxic effects which are somatically expressed as multiple behavioural disorders.

There is enough observational and experimental data (clinic, and preclinic as well) showing that sleep disturbances and anxiety can be related to systemic markers of inflammation such as reactive protein C, interleukin 6, and NFK-β factor[53]. This relationship is likely bidirectional: systemic inflammation may mediate the association between sleep dysfunction and adverse clinical outcomes as anxiety[54]. However, a more prolonged period of awakening and/or anxiety, triggers the release of both proinflammatory and anti-inflammatory cytokines, representing a compensatory

mechanism to disrupted circadian rhythms and this inbalance may lead to greater inflammation overall[55].

Recently, Heng-Zhi et al., investigated the effect of LPS-induced neuroinflammation on the synaptic and non-synaptic plasticity in the basolateral amygdala (BLA) in a mouse model of inflammation induced by lipopolysaccharide (LPS) injection. The results showed that LPS treatment led to the activation of microglia and the production of proinflammatory cytokines in the BLA. Furthermore, LPS treatment increased excitatory synaptic transmission due to the enhanced presynaptic glutamate release[56]. Additionally, LPS administration increases the immunoreactivity of the ionized calcium-binding adaptor (Iba1) (a marker of microglial cells) in BLA[57], as well as produces proinflammatory cytokine in the amygdala[58].

Since inflammatory condition can modulate synaptic activity involved behavioural disorders like sleep-wake and anxiety disorders, the gene expression and/or protein release of those markers should be monitored. This is particularly recommended when conducting studies in the exploration of the molecular pathways associated with the activity induced by drugs and natural extracts.

II.3.5.- Preclinical observational methods for assessing anxious behaviour

With the continuous drug discovery pipelinesin the pharmaceutical industry, the number of new drugs designed and/or introduced in clinical practices increases, there is a growing need for preclinical pharmacological platformsfor the evaluation of these products ensuring their viability and therapeutic safety. The most common experimental biomodel used in preclinical pharmacology are rodents, due to their strong similarity with humans in many of the systems explored, as well as their easy way of handling and reproduction. In such a case, the assessment of behavioural changes induced after the administration of any compound is usually designed on rodent models based on observational estimates.

In the observational estimates, the collected information is based on direct observation, which is recorded by experts in short periods, ensuring the fidelity and reproducibility of the method. They usually take into account the rodent's motor activity, behavioural changes, coordination, sensory/motor reflexes, body temperature, as well as urination, and defecation frequency[59].

Throughout the history of preclinical trials, there has been described many observational tests to evaluate the behavioural changes induced by a testing drug. The most used include the Open Field Test, the intermittent observations method, the hole board test, and the combined Open Field Test.

The Open Field Test consists of the evaluation of aspects related to the behaviour of mice in open spaces, such as rearing, grooming, involuntary reflexes, as well as motor activity, locomotion, and locomotion speed[59]. As with all methods, each group of mice is repeatedly observed only for a short time period of one hour and compared with a control group by a "blind" observer; being the necessity of a skilled and trained observer for reproducible results the main method's limitation[59]. The Open Field Test provides reliable results for sedative and or excitatory drugs and compounds with central depressant and/or stimulant activity.

The method of intermittent observations was described by Ther in 1953 and was designed to study stimulant and inhibitory drugs on the CNS[60]. The test is performed with 3 mice since this condition implies a special social situation of the experimental animals. This method provides reliable results for sedative drugs and compounds with central depressant activity, such as antihistamines, neuroleptics, and hypnotics.

The hole-board test is frequently used to assess mouse behaviour related to curiosity or exploration, and consists of measuring how many times the mouse pokes its nose into holes in the open field[59]. Benzodiazepines tend to suppress nosepocking at relatively low doses and was explained by Boissier et al. [61], and Boissier and Simon[62].

The combined open-field test includes a simultaneous determination of locomotion and curiosity levels by a modification of the hole-board test by a photo-ray system. It was proposed by Weischer et al. as a relatively simple test, although less frequently used than the previous one[63]. One of the challenges of the method is to correctly dissociate the exploratory behaviour and the locomotion by the expert. It has been well described that for the best-known stimulants, reduced exploratory behaviour corresponds with an increase in locomotion. Also, depending on the dose, tranquilizers can reduce exploration without affecting locomotion[59].

Other preclinical tests are more focused on the evaluation of muscle coordination. One of the most used is the Inclined Plane test, originally developed to test curare-like agents (*Anomospermum grandiflora*)[64], This test was subsequently used to test compounds related to muscle relaxation and to differentiate neuroleptics from other centrally active drugs. This method proved to be a simple assay to evaluate muscle relaxant activity. Nevertheless, although the tests satisfy the sensitivity and relative potency criteria, the effects of anxiolytics are not clearly differentiated from neuroleptics or neurotoxic compounds[59].

Another well-used method is the "chimney test", which was introduced by Boissier et al. as a simple test for tranquilizing and muscle relaxant activity estimation[65]. This test can be used as a complementary test of others with more prevalence and acceptance[59].

The Grip strength test is used to assess muscle strength or neuromuscular function in rodents, which can be influenced not only by sedative drugs and muscle relaxant compounds but also by toxic agents.This test allows to distinguish between a central relaxation or a toxic effect of some substance on neuromuscular function by simultaneous observation of animals under normal conditions[59].

At last, the most used and accepted test is the Rotarod method. Proposed in 1956 by Dunham and Miya, infers that the skeletal muscle relaxation induced by the compound assessed affects the rodent's ability to remain on the rotating rod[66]. Many central

depressant drugs are active in this test, such as benzodiazepines as well as neuroleptics such as chlorpromazine or haloperidol. Therefore, the test does not distinguish between anxiolytics and neuroleptics, but it can assess muscle relaxant potency in a wide number of compounds. In addition, the test has been used in toxicology to test for neurotoxicity[59].

In general, most of the research evaluating the behavioural effects induced by a testing drug or plant extracts includes at least one test from each category: Observational exploratory behaviour and muscle relaxing tests, being the Open Field Test and Rotarod tests are the most used in each category respectively.

II.3.6.- Medicinal Plants used to treat behavioural disorders

Herbal beverages are worldwide used to treat various behavioural disorders. Among the most relevant plant species used stand out:

- ✓ *Valeriana officinalis* L.

Valerian roots are possibly the most studied species for its effects in relieving ailments that cause anxiety disorders and insomnia. Preclinical studies in rats and mice, combining observational exploratory behaviour and muscle relaxing tests have been used to prove its anxiolytic, sleep-promoting and antidepressant activity. The elevated plus maze observational method was used to evaluate its anxiolytic, sleep-promoting, and antidepressant activity; whereas the forced swimming, the horizontal wire, and the Rotarod tests were explored to define the myorelaxant and/or motor coordination and behaviour properties[67]. Valeric acid, as the main and most active metabolite of the plant, was also explored forits capacity regulates the genetic expression of some brain genes using molecular RT-PCR techniques. Both, preclinical and molecular experiments demonstrate that valeric acid inhibits the enzymatic catabolism-decomposition of gamma-aminobutyric acid (GABA) in the brain, resulting in sedation. This sedative effect of valerian extracts is also associated with the β3 subunit of the GABAA receptor[68].

- ✓ *Passiflora sp.*

Different clinical trials have confirmed the anxiolytic and sedative effects of at least six species of *Passiflora* genus: *P. alata* Curtis*., P. caerulea* L., *P. edulis* L., *P. foetida* L., *P. incarnata* L*., P. ligularis* Juss. Studies suggest that the active compounds (alkaloids such as harmano, harmol, harmine and flavones such as apigenin, leutonin, quercetol) of the species stimulate an increase in GABA production, indicating that the effects reported for the genus act by a GABAergic mechanism rather than a serotonergic effect[69]. The aerial parts (flowers, leaves, and stems) are the most commonly used and can be administered in infusions, tinctures and/or as dried material in tablets and capsules.

- ✓ *Piper methysticum* G. Forst.

A kava kava leaf, a Polynesian-origin species is used to treat anxiety, stress, and insomnia. In high doses, it behaves as an anaesthetic and hypnotic agent[70,71]. Its main compounds are kavalactone derivates, which in preclinical trials demonstrated its effect through the GABAergic route since combined doses of kavain and diazepam improved the GABA activity. Its effects have been confirmed in humans, demonstrating a decrease in anxiety levels in patients with General Anxiety Disorders, especially in participants with a high level of anxiety according to the Diagnostic and Statistical Manual of Mental Disorders[72].

- ✓ *Galphimia glauca* Cav.

The *Galphimia glauca* Cav is a tropical plant from Central America whose aerial parts are used to treat anxiety and sleep disorders. Their nor-secotriterpenes (galfimins) have been described as inhibitors of dopaminergic activity. However, these compounds may also interact with the serotonergic system, which could partially explain their anxiolytic effects described above[73]. Herrera et al. tested the anxiolytic properties of this species using the Elevated Plus Maze (EPM) test with different sets of mice. They found that the fraction rich in galfimine A and galfimine B showed an anxiolytic effect, due to an interaction of galfimine B with the serotonergic system in the dorsal hippocampus, which modulates the induced response of 5-HT1A receptors in an allosteric manner

without affecting the GABAergic system[74]. Avilés et al. performed another preclinical trial with galfimine A in mice and observed an anxiolytic-like effect, without a sedative effect[75]. An overall molecular mechanism was proposed by Santillán et al. who observed that galfimines also act on the dopaminergic system but not on the GABAergic system[76]. In a clinical trial, Romero-Cerecero et al. observed the same anxiolytic effect in a study with patients who received extract, sertraline, and alprazolam; observing a reduction in the anxiety levels without somnolence, thus confirming the dopaminergic hypothesis as the mechanism of action of galfimina B[77,78].

- ✓ *Withania somnifera* L.

Also known in traditional folk medicine as the Indian ashwagandha bufera and ginseng, *Withania somnifera* root is a popular product used in Ayurvedic medicine[79]. Its biologically active chemical compounds include isopeletierine alkaloids, anaferine, steroidal lactones such as withanolidines and withaferins. This species is known for its effects as an anxiolytic and for its ability to decrease stress, with reductions in cortisol levels[80,81]. Fuladi et al. suggested that ashwagandha could be used as an adjuvant to selective serotonin reuptake inhibitors in patients with General Anxiety Disorders[82].

- ✓ *Melissa officinalis* L.

Melissa leaves are used in folk medicine as a sedative-hypnotic agent to treat insomnia and stress[83]. Their leaves are rich in essential oils and flavonoids (quercitrin, rhamnocitrin, luteolin). Other polyphenolic compounds (hydroxycinnamic acid derivatives, especially caffeic acid, rosmarinic acid, and protocatechuic acid), triterpenes (oleanolic acids and ursolic), tannins, and sesquiterpenes have been informed as well[84-87]. Preclinical evidences support its effects based on the inhibition of GABA[88]. A clinical trial with healthy volunteers proved its effects on the reduction of anxiety symptoms and insomnia in patients with mild to moderate anxiety[89].

- ✓ *Matricaria chamomilla* L.

Chamomile flowers has been used as a traditional herbal remedy for its calming effect. Although there are many varieties of chamomile, Roman (*Anthemis nobilis* L.) and German (*M. recutita* L.) are the most commonly used. *M. recutita* L. is considered the most potent variety and is the most frequently used to relieve depression and anxiety[90]. Preclinical studies suggest that its anxiolytic effect may be related to flavonoids isolated from chamomile, which may exert an anxiolytic and antidepressant effect through modulation of noradrenaline (NA), dopamine (DA), serotonin (5-HT), and γ-amino butyric acid (GABA)[91]. It is consumed as infusions, tinctures, and/or as dry material in tablets and capsules.

II.3.7- Medicinal Plants used to treat behavioural disorders in Cuba

Cuba has a long and extensive tradition in the use of medicinal plants, officially supported by a national program that approves the use of medicinal plants that grow in Cuban and that have proved to be safe and effective. Two of the aforementioned plants have been approved by the regulatory bureaux of the National Public Health System to be used in the sedative category: *Matricaria recutita* L. (Chamomile), and *Passiflora incarnata* L. A third species not included in the previous one mentioned: *Justicia pectoralis* L. (Linden) also belongs to this selected list of approved plants[92]. *Justicia pectoralis* L. is popularly known as a lime tree and/or woodpecker. Its aerial parts are characterized by the presence of coumarins, betaines, flavonoids, saponins, and amino acids, with coumarins as the main compounds. The presence of these polyphenol compounds has been suggested as responsible for their sedative action on the central nervous system[93-95].

Despite those approved plants, the population uses other plant extracts/beverages from other species like the leaves of *Mentha spicata* L. leaves (Yerba Buena), and *Moringa oleifera* Lam. (Moringa)[96]; and the aerial parts of *Lawsonia inermis* L. (Resedá), *Ocimum tenuiflorum* L. (Purple basil), and *P. neochilus*; being this last species the one that motivates the present investigation [19].

CHAPTER III

CHAPTER III. *Plectanthus neochilus* Schltr. Generalities

III. 1. *Lamiaceae* family and *Plectanthus* sp. Generalities

The accumulated knowledge about the use of plants to alleviate ailments or for disease treatment is a practice as old as human history. It evolved through human empiricism, considering the effects of plant species on human health. Whereas ethnopharmacology, cover the uses of plants with medicinal purposes, ethnobotany encompassalso as include plant use as a nutrient, decorative, fodder, folklore, wood and coal industries between others. Today, ethnopharmacology is the main branch of the ethnobotany since herbal medicines constitute for many countries a therapeutic alternative, sometimes regulated by public health systems.

Around ten percent of all vascular plants are used as medicinal plants, raising almost half a million species[97]. All the known civilizations since the origin of human being evolved to develop this form of medicine. The oldest medical text or report comes from ancient Mesopotamia, circa 2600 BC, describing approximately 1,000 plants and plant-derived substances, such as the juice of the poppy seed *Papaver somniferum* (Newman et al, 2000). The ancient Egyptian Ebers Papyrus, dating from 1550 BC, also contains about 700 natural agents such as *Aloe vera* (aloe), whiles the Corpus Hippocraticum (Greece, circa 460–377 BC) includes more than 400 natural agents and described their use. Many of these herbs and formulations are still used today[98].

Until the 18th century, the therapeutic properties of many plants, their effect on the human organism, and their method of treatment were known, but the active compounds were unknown. Actual medicine needs an industry producing pharmaceutical medicines largely based on the active principles of plants. However, for under developed countries without access to this modern synthetic pharmacological medicine, traditional folk medicine is continued based on the direct use of medicinal plants due to their low cost[99].

Studies on medicinal plants have boosted scientific research, intending to evaluate therapeutic properties, possible mechanisms of action, and the identification of the

chemical compounds responsible for them. The main reason is to provide evidence-based outputs that guarantee the safe and effective use of those medicinal plant-based remedies.

The Lamiaceae is a family of flowering plants with a cosmopolitan distribution containing about 236 genera and approximately 7200 species. The largest genera are *Salvia* (900), *Scutellaria* (360), *Stachys* (300), *Plectranthus* (300), *Hyptis* (280), *Teucrium* (250), *Vitex* (250), *Thymus* (220), and *Nepeta* (200). It belongs to the order Lamiales being one of the largest groups in the plant kingdom[100]. It is characterised by shrubs and herbaceous plants, therefore; an important source of ornamental, culinary, and medicinal herbs. Usually, they produce large quantities and a variety of essential oils; making it a group of exceptional ecological, ethnobotanical and floristic importance[101].

The genus *Plectranthus* L' Her. belongs to the tribe *Ocimeaem*, subfamily *Neptoideae,* and comprise around 300 herbaceous and subshrub species. They appear distributed mainly in the tropical and subtropical regions of Asia, and Africa[18], although it is also reported from the European and American continents[18,102]. Nevertheless, the largest reserve of this genus is located in South America, especially in Brazil[103]. The name *Plectranthus* is derived from the Greek words splektron (spur) and anthos (flower), as the flowers have spurs at their base. Due to their smell, some species of *Plectranthu*s are also called the King of Moths[102,104].

The taxonomic classification of the genus and their species has been compromised due to the lack of morphological criteria for correct identification. This has led to numerous taxonomic problems in the classification of species often misclassified and included in other genera such as *Coleus*, *Solenostemon, Englerastrum*, and *Isodon*[104].

The ethnobotanical uses are quite abundant, being around 85% of *Plectranthus* sp. informed with medicinal while other important uses are horticulture, and floriculture[102]. The most commonly used species as medicinal plants are: *Plectranthus amboinicus* (Lour.) Spreng, *Plectranthus barbatus* Andrews., *Plectranthus laxiflorus*

Benth., *Plectranthus mollis* (Aiton.) Spreng., *Plectranthus vettiveroides* (Jacob.) N.P, and *Plectranthus aegyptiacus* Forsssk. Its main traditional uses are to treat digestive, circulatory, and liver disorders, as well as skin diseases, respiratory and genito-urinary infections. Others are used to lower fevers and relieve headaches and muscular pains. At last, many of them are used to "calm the nerves" and for the treatment of intoxications and inflammatory processes[104].

The chemistry of the genus *Plectranthus* is still not well known. The main phytochemical constituents of the genus are diterpenoids, essential oils, and phenolic compounds[105]. Essential oils are responsible for the fragrance, flavour, and most of their medicinal properties according to the available scientific records. It is composed mainly of mono and sesquiterpenes. Other non-volatile terpenoid are diterpenoids such as abdanes, labdanes, abietanes, clerodane, and kauranes types, and triterpenoids such as: squalene, ambrein, lanosterol and amirins[106-108].

III. 2. *Plectranthus neochilus* Schltr.

The German taxonomist Friedrich Richard Rudolf Schlechter firstly described the species *P. neochilus* in 1896. It is endemic to South Africa and Namibia. Naturally grows in sandy to rocky areas from the Eastern Cape to Limpopo. It is an aromatic and succulent perennial herb; with blue and/or purple flowers, and grey-green leaves forming an attractive ground cover[11,109]. Grows in mainland Europe (Portugal)[13,110] and is now naturalised in the Americas and the insular Caribbean (Cuba)[18]. Within the American continent, the largest documented population of the species is in Brazil[111-113], followed by Venezuela[114]. With such as distribution, it is namely and recognized with many vernacular names highlighting: Blue Coleus or Lobster Flower for English spoken people, Muskietbossie for Afrikaans, Ibozane for Zulú, and Boldo, Boldo-rasteiro, Boldinho, o Boldo-Gambá by Portuguese.

III.2.1- Taxonomic classification

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Lamiales
Family	Lamiaceae
Genus	*Plectranthus* L'Hér
Species	*Plectranthus neochilus* Schltr.

Source: Méndez & Rifá, 2016

III.2.2- Characteristics and Morphology

P. neochilus is anatomically characterised by the presence of a quadrangular stem showing incipient secondary growth. The epidermis is uniseriate with polygonal cells in a frontal form, being more tangential than radial. It remains as the dermal system with an established phellogen. In the cortex, below the phellogen of the same organ, there is a continuous cord of collenchyma. Multi-layered cortical parenchyma with chloroplasts and small intercellular spaces can be also observed. The innermost boundary of the cortex is represented by a single layer of large parenchymatous cells, with tangential and radial walls which are impregnated with lipophilic substances. Vascular changes form the xylem inwards and the phloem outwards, being active mainly in the fascicular region and towards the xylem[18,115-117].

Leaves usually appear with wrinkled margins, perinervial venation, petiole with wedge-shaped base, membranous texture, and greenish-grey colour. Adaxial and abaxial leaf surfaces are pubescent, with orange glands on mainly in the abaxial surface. Leaves are also characterized by an obtuse and narrow apex, with acute base, and toothed margin; also presents diaceous and stomata[18,117]. Additionally, possesses a strong and characteristic aromatic odour with a bitter taste[118].

Comparative systematic investigations considered trichomes as relevant structure in morphodiagnosis analysis, informing both types: glandular and non-glandular trichomes. Glandular trichomes can be capitate and peltate types. Capitate ones are

numerous and can be unicellular or multicellular. On its side, peltate ones are scarcer and slightly sunken in the cauline epidermis. The secretion of the glandular trichomes is lipophilic. Non-glandular trichomes are simple, multicellular and uniseriate, consisting of three to ten cells, with an acute apex and possibly a dehydrated pedunculate cell[117,119-121].

Flowers appear as racemose inflorescences (70-150 mm), violet coloured, with bracts greenish with purplish tips, precociously deciduous, and angle at 25°. Distributed in 3 sessions, form whorls of six flowers dense at the top, flexible, and 5-15 mm to the bottom and erect pedicels. The calyx emerges long in fruit of 6 mm, with a tube slightly geniculate in the centre and expanded to the throat. The upper lips are bluish white, 2 mm long; while the lower ones boat-shaped (8-11 mm). Stamens arise united at base by 2-3 mm and thereafter, 8-11 mm long, curved with lower lip, slightly exerted. Style with 4-5 mm, slightly excerpted, nucleus ovoid and glabrous are other representative characteristics[18,116,122].

III.2.3Ethnobotanical usage

P. neochilus is one of those plants that belong to the worldwide arsenal of medicinal plants. It is traditionally used in rural communities of South Africa to relieve respiratory infections (chills, cough and runny or blocked nose). The leaves infusion and/or decoction it is consumed alone or together with another herb *Lippia javanica*, attenuating catarrhal manifestations[12] In Brazil, it is fresh leaves are used as infusion, decoction or aqueous extract by the population to treat skin infections, digestive disorders, liver failure, dyspepsia and respiratory ailments[103,117]. The use of fresh leaves in the form of infusion as anti-inflammatory and anti-infective agents was also reported by the Portuguese rural population[13,123]. In Cordoba, Argentina, the fresh leaves infusion is recognised by the urban and suburban population for treating stomach ailments and liver diseases[124].

In Venezuela, they have nickname it as "acetaminophen", because its leaves infusions became popular in the treatment of cases with Chikungunya fever; referring their

analgesic, cold and fever reducing properties[125,126]. The urban people of French Guiana likewise documented the presence and tradicional uses of *P. neochilus* to alleviate the headache, flu and decreasing the fever[127]. In Cuba, the species has acquired other nickname associated to a commercial drug: "Meprobamato", but it is also used as condiment, and ornamental herb[18,19].

Due to the ecological plasticity of the species, its ability to reproduce vegetatively growing as a perennial herb throughout the year, its high tolerance to climatic changes and high temperatures, and its leaves in form of an attractive ground cover; gained adepts for the horticulture. It was also informed as air purifier[102,128]and used in green roof technology; showing a decrease in canopy surface temperature, and contributing to a low heat flux exchange in these green roofs[129]. In addition, it appears to be an effective phytoremediator of water contaminated with the pesticide 2,4-dichlorophenoxyacetic acid[130] and the herbicide sulfentrazone[131].

III.2.4. Chemical composition and pharmacological activities

III.2.4.1Essential oil

The species *P. neochilus* characterised by the presence of essential oils, mainly monoterpenes and sesquiterpenes. The chemical composition of these oils might change depending on the climatic characteristics where the species grows. Lawal and Hutchings analysed for the species growing in South Africa its volatile compounds by GC and GC/MS; and reported the presence of monoterpenes, with citronellyl formate, linalool and isomentone as main compounds[132]. By its side, Motta et al., identified with the same techniques, mainly monoterpenes, with α-thujene, α-pinene , and β-pinene as majority for the species growing in Portugal[13]. Similarly, predominance of sesquiterpenes, with α and/or β-caryophyllene, trans caryophyllene, germacrene D, or caryophyllene oxide were reported for the species growing in different parts of Brazil[103,111,121,133,134].

The identification and quantification of the chemical compounds isolated from essential oils reaches great importance, as there were correlated to the main plant

pharmacological activities. The essential oil of *P. neochilus* leaves showed from potent to moderate antimicrobial activity against a broad strain of bacteria such as *Enterococcus faecalis*, *Streptococcus salivarus*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, *Streptococcus mitis*, *Lactobacillus casei*, and *Streptococcus* mutans, this last one with the highly susceptibility (MIC = 3.9 µg/mL)[111]. Additionally, the antifungal effect was reported with MIC = 125 µg/mL against *Rhizopus stolonifer*[133]. Other activities that have already been attributed to "boldo" essential oil were *in vitro* antiparasitic properties, which at 100 µg/mL killed the 100% of adult *Schistosoma mansoni* worms[103].

III.2.4.2- Non-volatile components

Generally, *P. neochilus* is traditionally used in the form of infusion, decoction of their fresh leaves and/or aerial parts. Nowadays there are few reports on their chemical composition, especially for the most used aqueous extracts. Previous studies have identified caffeic, ferulic, and coumaric acids in this solvent[130]. Also, rosmarinic acid was identified by Brito et al., in a traditional tea obtained by decoction[16]. In a hexane extract of their stems and leaves were found some triterpenoids such as friedelin, α-amyrin, sitosterol and stigmasterol as well as some fatty acids; while the ethanol extract is rich in crysimaritin[135]. Recently, Matias et al., using HPLC-DAD techniques determined the extraction capacity of different solvents (water, methanol, and acetone) and the supercritical CO_2 method in the quantification of chlorogenic, caffeic, rutin, rosmarinic acid and other secondary metabolites[110].

From the pharmacological point of view highpoints, the potent antibacterial activity against Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis,* and *Mycobacterium smegmatis*) of the methanolic extracts from leaves. Furthermore, acetone extracts of *P. neochilus* showed relevant antibacterial activity against gram-negative *Klebsiella pneumonia*, highlighting that this activity it is not necessarily exclusive of the plant essential oils[110]. Within the same line, the antifungal activity of other leaves methanolic extracts was demonstrated, exhibiting an inhibitory concentration equivalent to EC_{50}=20.51 µg/mL, and killing 100% *Candida krusei*[112].

Likewise, ethanolic extracts of fresh leaves of *P. neochilus* were evaluated for antiparasitic effects showing significant leishmanicidal activity IC_{50}< 20 µg/mL in *Leishmanial chagasi* species[136]. This evidence validates the traditional uses of these plants as anti-infective agents with independence of the place where the species was collected. The other pharmacological activity explored is the antioxidant capacity. Rijo et al. evaluated the radical scavenging activity of several extracts modifying solvents and methods of extraction. From the nine combinations, only those extract methanol based were active at concentrations of 100 µg/mL with DPPH inhibition ratios of 64.9 and 62.3 % for ultrasound and maceration extractive methods[137].

Similar results were obtained for the methanol extracts from the aerial parts of the plant by the same method[110]. Also, ethyl acetate extracts derived from leaves and stems of the species showed antioxidant activity at a concentration of 300 ppm, but their inhibition ratios of the DPPH radical did not exceeds 7 %, similar to was observed in the reducing power test[135].

Moreover, Viana et al. reported that methane and hexane extracts derived from the aerial parts of *P. neochilus* showed a marked inhibition of the enzyme acetylcholinesterase (AChE) emerging as a therapeutic alternative to treat neurodegenerative diseases[135]. Similarly, other study evaluated the effects of AChE inhibition at the muscle level, favouring gastrointestinal motility and thus the digestion process. The AChE inhibitory action of *P. neochilus* water extracts was estimated in IC_{50} 430 ± 50 µg/mL[16].

CHAPTER IV

CHAPTER IV. Characterization of ethnobotanic study of *Plectranthus neochilus Schltr.*

Modified version from the published article: Rodriguez-Ferreiro, A.O., Léon-Duharte, D., Polanco-Durán, G., Guisado-Bourzac, F., Ochoa-Pacheco, A., & Escalona-Arranz, J. C. (2020). Ethnobotany of *Plectranthus neochilus Schltr.* (Meprobamato) in Cuba. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas, 19(2), 236-246.

IV.1. Introduction

In underdevelopment countries, medicinal plants emerge as a valuable resource for their health systems. Even though there is no precise way to estimate the worldwide use of medicinal plants, the World Health Organization has calculated that approximately an 80% of the world's population uses traditional medicine in primary healthcare. In this estimation, phytotherapy is highlighted by its prevalence, using not only plant extracts but also their isolated compounds[138].

Ethno-botanic is the science studying how plants are used by humans with independency of their cultures, social rank, and type of society[139]. Its implementation allows documenting valuable information that in turn, becomes the basis of the necessary knowledge to develop new herbal remedies. As the information collected generally comes from ancestral uses, not only offers crucial evidence of the plant effect and way of use but also their riskiness associated with any type of harmful or toxicity. Its results, make it possible to design and establish programs for its rational use, as well as for its conservation, contributing in this way to a comprehensive species management, diminishing its exploitation/overuse impacts on biodiversity[140].

The use of plants to treat Central Nervous System disorders is quite common in the Cuban population. A study conducted by Heredia et al., in the North-eastern region of Cuba reflects that 16 different species are used for this purpose. Within these 16 medicinal plants, *Justicia pectoralis* Jacq. was the most used, agreeing with its scientific and ethno-botanic reports. The next species with attributes according the study was *P. neochilus* classifying as the second in the fidelity index parameter (85 %) and as the fourth for the total number of reports. From such a diversity of options and traditions, using plants to treat Central Nervous System disorders (16

species, including those recognized by the regulatory bureaux of the National Public Health System), it is remarkable and alarming from the scientific point of view that the recently introduced *P. neochilus* reaches such popularity. This panorama is aggravated when considering that this use given by the Cuban population does not agree with the ethnobotanical information available for this species in other latitudes. Under these facts, this study was aimed to investigate in a bibliometric study and a comprehensive and specifically designed ethnobotanical survey, the use that the Cuban population does to this plant species highlighting its coincidences and divergences.

IV.2. Material and Methods

IV.2.1 General overview

This study was divided into two parts: the first one was consistent in a descriptive and retrospective bibliometric analysis using the professional software Harzing´s Publish or Perish v5 and *Plectranthus neochilus* as keyword, while the second one consists of a descriptive and transversal ethno-botanic study about the specie considering the three main regions of Cuba.

IV.2.2 Bibliometric study

The bibliometric analysis was developed in two moments or periods: The first considering all the published articles until December 2018, and the second from January 2019 until September 2022. For both moments were used the tools that are related below:

- Software used: Harzing´s Publish or Perish v5
- Keywords used: *Plectranthus. neochilus*
- Database explored: Google academic
- Inclusion criteria: All hits papers with any of the following categories (Title, Keywords, Summary) written in English, Spanish or Portuguese languages; and hits papers dealing with ethno-botanic information, and/or chemical, and/or pharmacological experimentation
- Exclusion criteria: Research production of the author, and papers dealing with botanic information and/or other categories not related to ethno-botanic information, and/or chemical, and/or pharmacological experimentation.

- Output variables: Total number of papers, Plant collected area, and author´s countries (main and correspondence authors).

IV.2.3 Ethnobotanical study

The ethnobotanical study encompassed the period from January 2017 to May 2018 and included 500 surveys for each one of the three geographic regions of Cuba (see Figure IV.1), covering in total 1500 interviews. The Western Region was represented by inhabitants of the municipality "*Habana Vieja*"; the Central Region for the municipality of "*Camaguey*", while the Eastern region was represented by "*Santiago de Cuba*" municipality. These municipalities were included because of their higher population levels, as well as their consumption rates of medicinal plants. The study combined different qualitative and quantitative methods and techniques characteristic of this kind of research.

The fieldwork (interviews) was performed by three specialists with experience in the field of medicinal plants and community work, trained in communication techniques and collection of ethno-botanical information.

IV.2.3.1 Description of the study areas

The municipality of "*Habana Vieja*" belongs to the province of Havana, Cuba. Located in the north and central part of the province and the west of the bay, it borders up on North with the Florida Strait, at south with "*Cerro*", at the east with "*Centro Habana*", and at west with "*Plaza de la Revolución*" municipalities. Counting with 30.42 km^2's territorial extension and a population of 140 233 inhabitants, being the municipality with the higher population density of Cuba[141].

Camaguey municipality belongs to the province of the same name with a superficial extension of 1 098.58 Km^2 and a population of 326 743 inhabitants. On the north border with "*Esmeralda*", and "*Sierra de Cubitas*" municipalities, while on the south with "*Jimaguayú*", on the east "*Minas*" and "*Sibanicú*", and on the west "*Vertientes*" and "*Florida*" municipalities[141]. At last, "*Santiago de Cuba*" is the main municipality of a province with the same name. Reaches an extension of 1 031.74 km^2 and a population estimated at 510 563 inhabitants. Located on the south side of the island facing the Caribbean Sea, it limits to the north with the municipalities of "*San Luis*" and "*Songo La Maya*". To the west with the municipalities of "*Guama*" and "*Palma*" while to the east with the municipality of "*Songo La Maya*"[141].

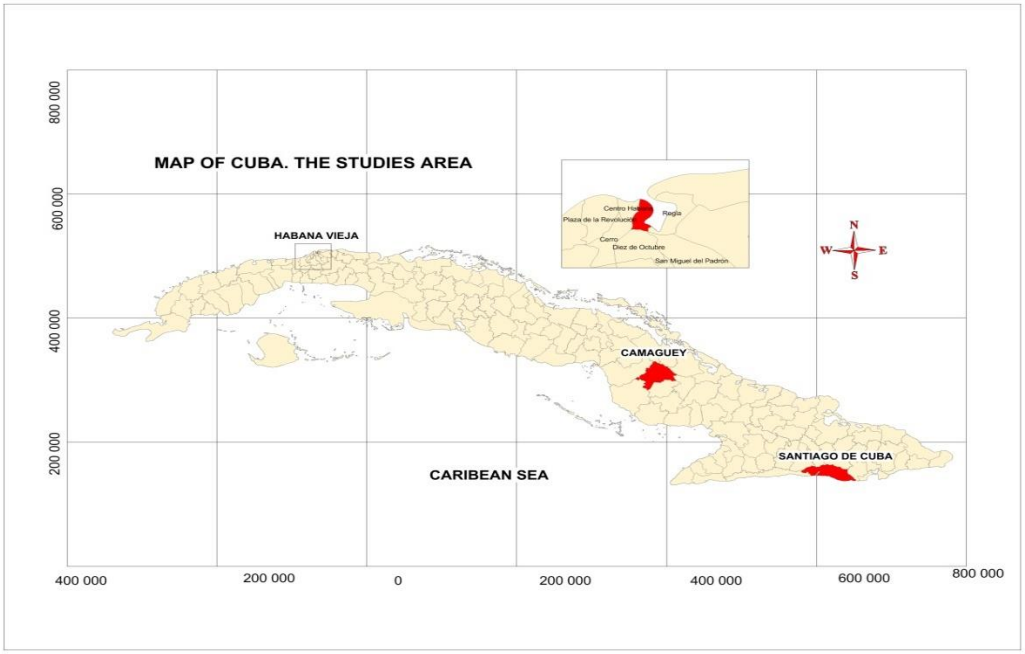


Figure IV.1. Cuban municipalities considered in the ethnobotanic study. *WGS84 projection (EPS 64326) MapInfo Professional software version 12.0.

IV.2.3.2 Study design and test sample

The study design covered all the inhabitants of the communities and neighbourhoods of the municipalities previously selected. In turn, the sample was selected through a consecutive non-probabilistic sampling including people from sexes, different ages, traditional healers, homemakers, retirees, students, and professionals dealing with medicinal plants in the study areas. It included participants who wished to cooperate voluntarily in the research and who knew or had heard of the species under study. Written informed consent for all face-to-face quizzes, especially from key sampled interviewees cohort in the sample was obtained (traditional healers and health professionals who encourage the consumption of medicinal plants); respecting the principle of autonomy and guaranteeing the confidentiality of its participation.

IV.2.3.3 Data collection

Data information was collected following the requirements established in the "Traditional Medicine in the Islands" TRAMIL methodologies[142]. Interviews were based on a designed semi-structured questionnaire elaborated on the basis and criteria of the objectives of this research, collecting socio-demographic and ethnopharmacologic variables (see Annex 1). The socio-demographic variables included sex, scholar level, occupation, and age; whereas, the ethno-pharmacologic variables involved medicinal uses of the specie, part of the plant used, methods and ways of preparation, concentration or plant/solvent ratios, times and consumption frequencies, side effects noted, and concomitant consumes with conventional drugs with actions on the Central Nervous System.

The criteria used to define the medicinal uses indicated by the interviewees were based on the testimonies of the participants and the diseases referred by them, to be later classified and grouped in a homogenous pharmacologic category.

IV.2.3.4 Plant authentication

As first approach, a voucher specimen was collected in each one of the regions selected and taxonomically identified by a taxonomist from other region of Cuba. The plant collected in Santiago de Cuba was identified by a taxonomist from Universidad de Camagüey (the same who informs the introduiction of the species in Cuba). In the same way, the plant collected in Camaguey was identified by a

specialist from the National Institute of Ecology and Systematic in Havana, and the collected in Havana identified by a taxonomist from the Eastern Centre of Ecosystems and Biodiversity in Santiago de Cuba (BIOECO, Spanish acronymun). Samples of all species collected were deposited in the herbarium of each institution: The *National Herbarium of Cuba*in Havana (code 41856), the herbarium "*Julián Acuña Gale*" in Camaguey (code 12056); and the the herbarium "*Jorge Sierra Calzado*" in Santiago de Cuba (code 147).

During the field work, the authenticity of the plant material reported by each informant was verified when the interviewer showed a sample of the plant material. Once confirmed by the interviewee that it is the species consumed or consumed by someone he knows, he indicated (when possible) the harvesting area of the plant for consumption, this time being confirmed by the interviewer.

IV.2.3.5 Qualitative analysis

The ethno-botanic information offered by each interviewee was organized in an excel sheet to be later processed in the statistical package IBM SPSS Statistics 20 version 2.1. Chi-Square test for independent samples was used to compare the parameters with binomial nature (sex and cause of the combination of the plant extract with synthetic drugs) between the three regions. Kruskal Wallis test was used for those parameters that consider multiple response options. Mathematical correlations between those variables that could be correlated (i.e., concentration versus frequency and consumed quantities) were done using the Pearson correlation test. All tests were running using the before mentioned statistical package and considering a 95.0 % of confidence level.

IV.2.3.6 Quantitative analysis

The use of quantitative indices in ethno-botanical research was calculated informing the value of use (IVU) and the level of significant use described by Tramil (UST) as follows:[140].

Level of Significant Use of Tramil (**UST**)

$$UST = \frac{Use}{N_{is}} * 100$$

where: Use: Number of summonses for a specific pharmacologic activity.
Nis: Number of interviewed persons.

Value of use index (**IVU**)

$$IVU = \frac{\sum Uvis}{N_{is}}$$

where: Uvis: Number of uses mentioned by each informer
Nis: Number of interviewed persons.

IV.3 Result and Discussion

IV.3.1 Bibliometric study

After refining the search and classifying them, the first stage/moment of the bibliometric analysis (until December 2018) rendered 51 articles, 45 of them classifying within the inclusion criteria of the study: ethnobotanical (19 hits) or chemical/pharmacological information (26 hits). The six discarded papers were related to agronomy and/or botanic information.

The most frequent plant-collected areas were Brazil, South Africa, and Venezuela, while the most active main authors belong to Brazil, Portugal, and South Africa. The countries (by author´s origin) more focused on the chemical and/or pharmacological profiles research were Brazil and Portugal, while Brazil, Venezuela, and South Africa focused more on the ethnobotanical point of view (in this order). The most frequent ethnobotanical reports refer activity as antimicrobial, cold, gastric, and liver disorders. As an important observation is that none of the pharmacologic nor ethnobotanical papers refers to a sedative and/or hypnotic activity, except for one ethnobotanic study developed in the northeast region of Cuba[19].

The second stage evaluation rendered a surprising result of 47 hits: 13 hits for ethnobotanical studies, and 29 hits for pharmacological and/or phytochemical studies. The remaining five hits were related to their biological aspect and/or taxonomy. Brazil continues to be the country that most uses, consumes, and studies the species, followed by South Africa. In South Africa the plant is mainly used to alleviate respiratory ailments, whereas Brazil has the broadest range of applications, beyond the medicinal use. Also it is used against skin infections, disturbed digestion, hepatic insufficiency, as analgesic, and in the dyspepsia[103,117].

Other reports refers to be used as repellents and vector control[143], as antitumor, antimicrobial, antioxidant and anti-inflammatory[124]. Phytoremediation of water and soil after exposure to 2,4-dichlorophenoxyacetic acid (2,4-D) is one of those non-pharmacological uses of the plant[144,145].

In this second evaluation period, Portugal stands out as the country with more papers related to phytochemical and pharmacological studies[110,146]. For Cuba, the number of hits was doubled, with four new hits: two from ethnobotanical information[147,148] and two excluded because belongs to the author´s scientific production.

A simple inspection of both moments/stages of the study shows a growing interest of the scientific international community in the plant. In Cuba, in spite of doublint of the number of hits, three of those four new papers originate from the same author´s research group. In general, the increased number of hits can be also related to the new Journal and database indexations done by Google academic platform. Also, the search confirms that the plant is mainly appreciated as a medicinal plant in Brazil, South Africa, and Portugal for treating respiratory, skin, digestive, and liver disorders.

IV.3.2 Ethnobotanical study

The cross-blind validation study demonstrated that the species growing and used within the regions explored is the same species and correspond to *P. neochilus*.

The traditional uses of the Cuban population of the species are summarized in Table IV.1. In total, more than 3060 uses were counted, which means an average value of more than two pharmacological uses per person interviewed. The most referred uses were the sedative/hypnotic association, being in correspondence with the common Cuban name assigned to the plant: "Meprobamato", which is the commercial name of the synthetic sedative drug methocarbamol. Curiously, these two activities have not been previously reported in any of the ethnobotanical studies outside Cuba as revealed by the bibliometric study, but which is informed in several ethnobotanical papers from Cuban´s authors[19,147]. This observational use as sedative/hypnotic is unique for the Cuban population and so far, has not been reported by people from other latitudes.

On the other hand, anti-inflammatory activity stands out as the most reported common property for Cuba and the rest of the world. The analgesic and the anti-inflammatory activity were preclinically demonstrated and classified "as efficient" in the control of post-operatory pain in female cats submitted to ovary-salpingo-hysterectomy[149]. On the other hand, anti-inflammatory activity has been reported in many species of this botanical gene as happens for: *P. scutellaroides*[150] and *P. amboinicus*[151]. This effect was correlated to the presence of several chemical compounds such as amyrin[152] and flavonoids[153].

Table IV.1 also denotes that the Central region of Cuba attributes different activities such as anti-catarrhal and digestive affections to the species, which were not reported in the other two regions. These activities match with similar ones informed in other latitudes referred to: treat digestive disturbances, pain, edema, skin infections, and respiratory ailments[154,155]. Besides, Daio et al., reported that macerated leaves of *P. neochilus* act as a tonic agent to the gallbladder, stimulating the secretion of bile, favouring the digestion of fats[156]. Also, an ethno-medicine survey in South Africa revealed usage of this species for treating respiratory infections[12].

At the same time, Table IV.1 show that the inhabitants of the Eastern region give the greatest variety of ethnopharmacological properties to this species, followed by the central and western region respectively. However, there are no statistical differences between regions judging by the results of the Kruskal-Wallis for median comparison at 95% of confidence. This suggests that the use of this medicinal plant in Cuba is statistically the same with independence of the region where it is consumed. In this way, these results confirm the isolated observations that the Cuban population uses *P. neochilus* mainly by its sedative and hypnotic properties.

After completing the interviews in the three areas under consideration, female participants prevailed with the 1001 informers, representing 66.73 % of the studied population (1500). This might be explained associated with the role played

Table IV.1. Ethnobotanic uses of *P. neochilus* within the three regions of Cuba

Uses	West	Centre	East	Frequency	Percent (%)
Sedative	312	323	349	984	65,60
Hypnotic	272	285	295	852	56,80
Analgesic	210	201	223	634	42,27
Anti-inflammatory	108	147	157	412	27,47
Ornamental	52	33	49	134	8,93
Anticatarrhal	0	21	0	21	1,40
Digestive affections	0	23	0	23	1,53
Total	**954**	**1033**	**1073**	**3060**	

Source: Adapted from TRAMIL, 2018

by women in society, specifically in family care, and in the rescue of herbalists' traditional preparations for the treatment of common ailments. Additionally, the double workload (at home and the traditional job) leads many to continued stress, affecting their sleep levels.

Age groups with a better knowledge regarding plant use were group 2 (38-59 years old, 46.33%) and group 3 (> 60 years old, with 38.67%), representing 85% of the all-interviewed population. The knowledge of the species for these groups can be related to to the use of medicinal plants as a therapeutic alternative to treat work related stress. Besides, at those ages clinical appearance of signs and symptoms of diseases correlated to the Central Nervous System and sleep disorders frequently appear. Group 2 encompasses people that generally are the center of the family, offering attention to parents and children at the same time, doubling their familiar tasks increasing by default their stress and in consequence insomnia. Some epidemiological studies placed female sex and ageingas predominant factors in anxiety, depression and insomnia[157,158].

Most of the people interviewed belong to the university level (663 representing 44.20 %), followed by those with technician level (399 for 26.60 %). At the same time, most of the informers were workers in concordance with the age of the interviewees. The high scholar level of the polled people is in conformity with the levels reached by Cuban society, results derived from the social programs implemented after the 1959 revolution. All those demographic results are in agreement with previous studies developed by other ethnobotanical surveys developed in different regions of Cuba in which females with age around 40 to 60 years old and at the university level are the majority[139,140,159]. Also, in other Latin-American regions, women represent the better informers related to the use of medicinal plants, suggesting their higher knowledge connected to their home daily activities[155,160-162].

The last socio-demographic variable considered was the occupation of the participantsat the moment when they fulfil the survey. The 70.80 % of the interviewee were workers, representing 45.33 % of them employees by companies of the Government while the independent labour force (private activities) reached

25.47 %. Minor proportions (438 interviewed, 29.20 %) were housewives, retirees, or students.

The 100 % of the 27 traditional healers interviewed showed a high knowledge about the use of *P. neochilus* with medicinal intentions; in contrast to the percentage (32.07 %) of the 106 health workers surveyed. In this cohort of persons (27 traditional healers and 106 health workers) only nine of the interviewees knew or had read about the demonstrated pharmacologic properties and it existence of references about it toxicity of the plant. That is why the staff decided, once these interviews were carried out, to take actions related to the training of the traditional healers and the health workersto improve theirknowledge about the use of this plant. Likewise, he exhorted them to observe in greater detail and interest the aspects related to the use of this medicinal plant, considering the role they play in providing health to the population.

No statistical differences were found in any of the sociodemographic variables between the three Cuban regions studied, considering the Kruskal-Wallis median comparison test at 95% confidence.

Relative to the part of the plant used, all the informers refer to the use of the aerial parts. The 58.66% use the stem and leaves, while 42.33% use leaves alone. Only five informants refer to the use of the stem alone, mainly those associated with digestive disorders. Once again, and with independence of the numeric differences, no statistical differences were detected.

Figure IV.2 represents the preparation modes in which the population uses the plant for medicinal purposes. Decoction highlights as the most used (77.67% of the interviewed) with independence of the region followed by the infusion at 15.40%. The rest of the preparation modes were irrelevant with very low ratios of use. No statistic differences between regions were found.

Regarding the concentration of the beverages prepared, the informers using leaves and/or the aerial parts of the species (1483) were clustered into two main groups. Most of the informers add quantities equivalent from 10 until 20 leaves with or without stems to prepare one litre extract, representing 44.77%, while others add from five to ten leaves (38.10%). None of the participants add less than five leaves (with or without steams) per litre of brew, while those who use more than 20

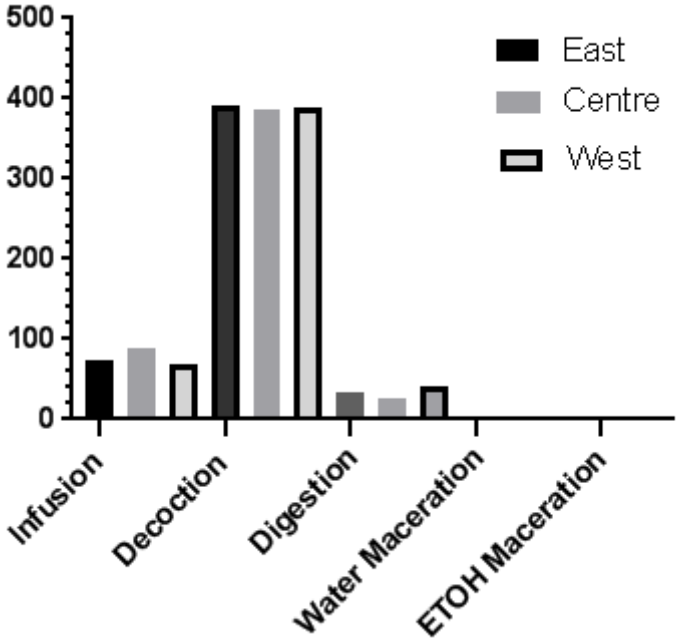


Figure IV.2. Modes of preparation of *P. neochilus* brew with medicinal purposes by the Cuban´s population. Source: Own authorship

leaves (224 representing 15.10%) were mainly concentrated in the western region of Cuba, but without statistic differences.

This extract is consumed preferentially in a teacup with an equivalent volume of around 200 to 250 millilitres, during the night time half an hour before to sleep, (45.27%), while 38.07% (571 interviewees) take it in a coffee cup with an equivalent volume from 50 to 75 millilitres. A strong relationship between the concentration of the brew and the quantity consume was found with a correlation coefficient of r=0.76. In a general sense, 95.53% of the interviewees refer to consuming the medicinal beverage only one time within the day, 45 minutes or one hour before sleep; inferring that the concentration and amount taken are strong enough to elicit the pharmacologic desired effect.

Figure IV.3 shows the main side reactions declared by the *P. neochilus* extract consumers. Even when in general the population, tends to consider natural medicines as non-toxic, the interviewers notified 229 side effect reactions asserted by 141 informers. The common ones were vomiting (5.33%) and (4.87%), followed by headache, morning dry mount, collywobbles, and loss of orientation with 31, 27, 10, and 8 informers respectively. A weak mathematic correlation (r=0.53) between the appearance of these side effects and the concentration of the extract was found. Nevertheless, and supporting this observation, only four of the 141 informers that refer to any kind of side effect, drank the plant extract at the lower concentration (from 5 to 10 leaves per litre of formulation). These side effects referred before, are quite common for those synthetic drugs that act over the Central Nervous System, especially Benzodiazepines[163].

The concomitant consumption of *P. neochilus* extracts with synthetic drugs was another of the variables explored in this investigation. The results presented in Figure IV.4 denote that 35% of the interviewees have combined at least once natural and synthetic medication, the group of benzodiazepines being the one with greater frequency. Curiously, the synthetic drug from which the *P. neochilus* takes their common noun (meprobamato in Spanish) is the one with a smaller incidence (28 reports). Again, no significant differences between the kind of drug used and the studied regions were observed.

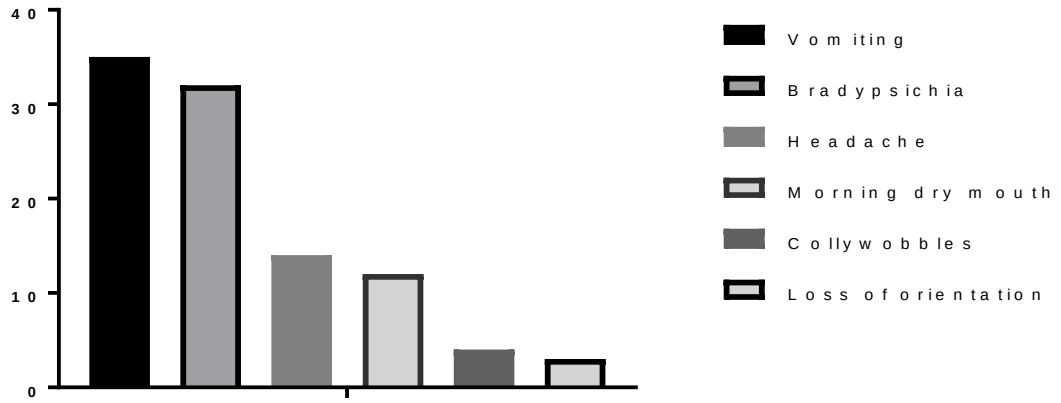


Figure IV.3. Main side effects informed by the Cuban population that consumes extracts of *P. neochilus* with medicinal purposes. Source: Own authorship

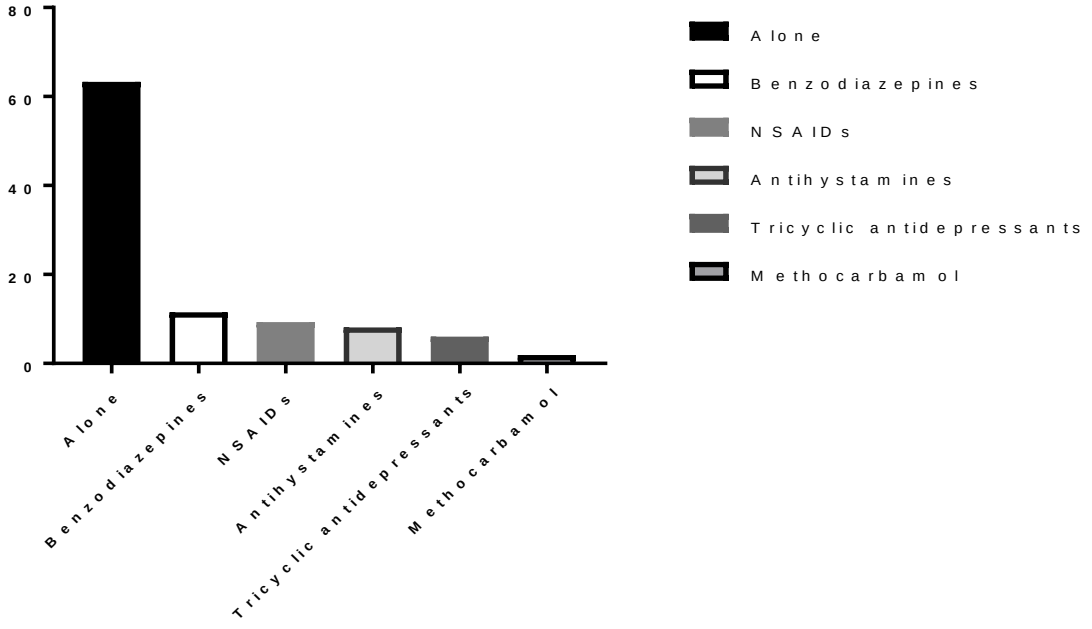


Figure IV.4 Drugs consumed by the Cuban population concomitantly with extracts of *P neochilus*. Source: Own authorship

All of the 551 interviewees that referred to consuming at least oncea combined treatment, were also asked the reason why they combined it. The reply of this question was on 507 occasions the following: "to intensify the activity of the synthetic medication". How the interviewees answer this question reveals statistical differences according to the Chi-Square test for independent samples, pointing out the central region as the one with higher frequency attending this practice of concomitant consumption to achieve a bigger threshold of pharmacologic activity.

Table IV.2 shows the quantitative variables associated with the most significant uses and the total reports informed by the Cuban population. The higher value of IUV in the Eastern region is associated with the higher number of uses reported by the interviewees of this geographical area. It is also this region in which the main activities reported reach the higher values of UST. These results give evidence once again to the high level of use that the Cuban population does of this plant, even when no previous international reports refer to it with its main purpose: The activity on the Central Nervous System. There is only one reference that can be indirectly correlated to this activity and is the *in vitro* inhibition of the acetylcholinesterase (AChE) enzyme referred by Brito et al. [16].

From the chemical point of view, some of the substances isolated from *P. nechillus* extracts refer to the presence of triterpenes as friedelin, α and β-amyrin, fatty acid esters; and flavonids[135], which had been tested against some Central Nervous System disorders[164].

Table IV.2. Traditional uses, total of reports and quantitative ethno-botanic variables informed for *P. neochilus* by the Cuban population.

REGIONS	WESTERN	CENTRAL	EASTERN
IVU	2.78	2.88	3.03
UST (%)	62.4 (Sedative) 54.4 (Hypnotic) 42.0 (Analgesic) 21.6 (Anti-inflammatory)	64.6 (Sedative) 57.0 (Hypnotic) 40.2 (Analgesic) 29.4 (Anti-inflammatory)	69.8 (Sedative) 59.0 (Hypnotic) 44.6 (Analgesic) 31.4 (Anti-inflammatory)

Source: Own authorship

IV.4 Partial conclusions

In light of the results obtained in this research, it was demonstrated through an extensive ethnobotanical study designed specifically for these purposes that this plant is consumed in Cuba alone or together with synthetic drugs (mainly benzodiazepines) to induce or enhance its sedative and hypnotic effects, without detecting statistical differences between the three Cuban regions analysed. Notibly, some data also disclose the plant parts, concentration, preparation mode, and other valuable information. The fact that this use is particularly specific to the inhabitants of Cuba, as revealed in the bibliometric study, let us to design future morphological, chemical and pharmacological experiments that help prove or disprove this widespread use by the Cuban population.

CHAPTER V

CHAPTER V. Determination of quality control parameters of leaves and extracts from *Plectranthus neochilus* Schltr.

Modified version from the published article: Rodriguez-Ferreiro, A.O., Ochoa-Pacheco, A., Méndez-Rodriguez, D., Ortiz-Beatón, E., Font-Salmo, O., Guisado-Bourzac, F., Molina-Bertrán, S., Monzote, L., Cos, P., Foubert, K., Pieters L., Perez-Novo, C., Vanden Berghe, W., Escalona-Arranz, J. C & Setzer, W. N. (2022). LC-MS Characterization and Biological Activities of Cuban Cultivars of *Plectranthus neochilus* Schltr. ***Plants***, 11(1), 134. https://doi.org/10.3390/plants11010134

V.1. Introduction

Throughout evolution, medicinal plants have been used to treat a wide range of diseases, from coughs and colds to microbial infections, inflammatory processes, and nervous disorders. In developing countries, herbal medicines form the basis of primary health care because of limited access to expensive commercial drugs on the international market[165]. In contrast, developed countries advocate the development of alternative therapies based on the natural origin of their active ingredients to treat various chronic, non-communicable and degenerative diseases of the ageing population. This duality favours that many scientific investigations have medicinal plants as potential therapeutic targets.

In this context, natural products and their derivatives continue to lead the way in the development of new active ingredients in drug discovery. Indeed, of the 1,881 new drugs approved during the period 1981-2019, 787 (41.8%) fell into the categories of "Unmodified natural product", "Biological macromolecule", "Naturally derived product" and "Botanical drug (defined mixture)", which are directly attributable to a natural origin. In addition, 489 analogues that mimic natural compounds were synthesised, which together account for two-thirds of the newly developed medicines[166]. The development of herbal medicines should therefore be an alternative for underdeveloped countries to solve their own health problems and a source of income.

The introduction of non-native plant species in different countries has increased as they are used by private/individual or government agencies for medicinal/commercial purposes to increase sources of raw materials as a therapeutic option. However, this intention to cultivate non-original medicinal

plants does not always lead to an exact replication of the bioactive properties that characterise them in their natural habitat. It is well known that secondary metabolism (which is usually associated with the pharmacological activity of the plant) depends on intrinsic and external factors[167]. Intrinsic factors can be better controlled by establishing the identity of the plant as well as its genetic authenticity. However, external factors such as environment, cultivation, harvesting and post-harvest processing/storage practices are more difficult to control, especially if the activity is population-dependent[168].

Therefore, it is necessary to study and characterise the species from a pharmacognostic point of view to determine the morpho-anatomical characteristics of the plant, possible drying methods and the quality parameters of the drug and leaf extracts of the species.

The species *P. neochilus* belongs to the family Lamiaceae and its cultivation has been naturalized mainly in Brazil, although it is native to South Africa and Namibia. In these countries the species is used ethnomedically to alleviate respiratory symptoms, digestive disorders, liver failure and skin ailments[12,103]. Pharmacologically speaking, its potential activity as an antiparasitic and antifungic[112], antibacterial[14,111], anioxidant[79], cytotoxic[169], and and hypoglycemic[17]has been reported.

The occurrence of *P. neochilus* in Cuba was reported in 2016 and its cultivation has expanded throughout the national territory due to its ethnopharmacological and ornamental benefits[18]. Recent ethnobotanical studies have suggested that this species is consumed by the Cuban population for its sedative and hypnotic effects rather than for its traditional antimicrobial, anticatarrhal, and digestive properties[19,147]. This contradictory information was confirmed in the previous chapter IV, when the combination of the bibliometric analysis with a comprehensive and specially designed ethnobotanical survey showed that the Cuban population actually uses this plant alone or together with synthetic drugs (mainly benzodiazepines) to induce or enhance its sedative and hypnotic effects. This scientific concern encouraged us in the present study: to determine the quality parameters of the drug and leaf extracts as well as the chemical composition of the *P. neochilus* species growing in Cuba.

V.2- Materials and Methods

V.2.1-Plant collection

Adult plants were collected in the morning from the experimental plot of the National Centre for Applied Electromagnetism (Lat 20.032316, Lon -75.810585) at the Universidad de Oriente, Santiago de Cuba. The plants were authenticated at the "*Centro Oriental de Ecosistemas y Biodiversidad*" (BIOECO) in Santiago de Cuba with a voucher specimen code deposited at "Jorge Sierra Calzado" Herbarium (see Figure V.1).

V.2.2- Quality control drug parameters determinations

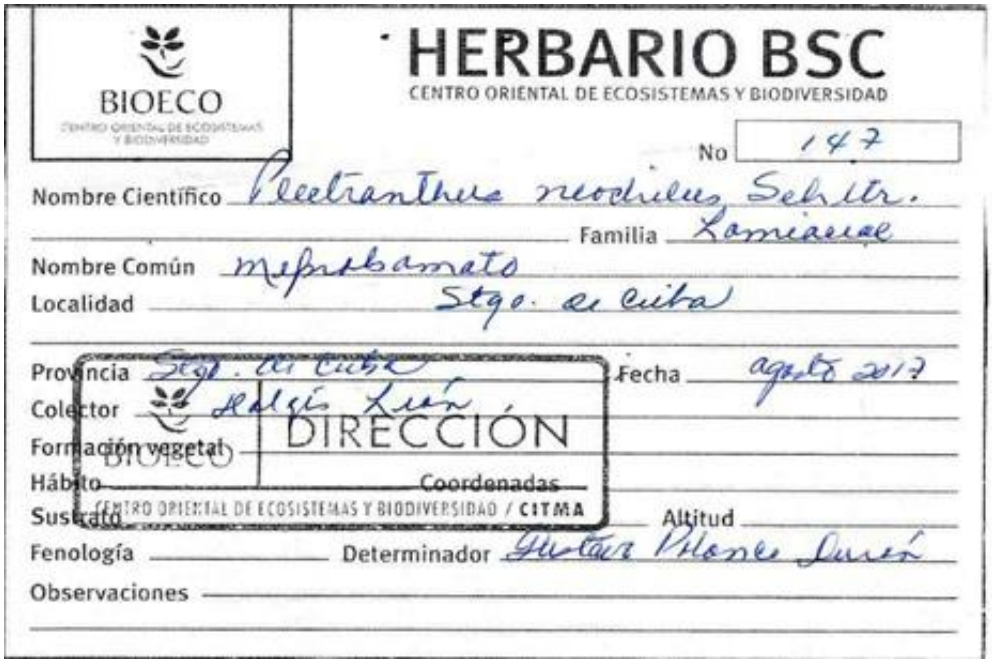
Four batches of plant material (February, May, August and November 2018) were harvested. The collected leaves were divided into two parts: Fresh leaves were used for macroscopic, microscopic and essential oil determinations (EO), while the remaining part was dried using three different drying methods: sun-dried, shade-dried and oven-dried (45 °C). The drying process of the leaves was considered effective if the following four criteria were met: 1) at least the last three weight determinations had a coefficient of variation of no more than 0.1%; 2) the drying period was no more than three weeks (21 days); 3) no evident microbial contamination occurred; and 4) the residual moisture determined by the infrared method (Sartorius MA 35, Göttingen, Germany) was equal or less than 14%[170].

V.2.3- Macroscopic and essential oil yield determinations

The fresh leaves of the four batches were analysed in detail for their anatomical features, size, shape, odour and colour using a stereomicroscope NOVEL NSZ -606 (Nanjing, China) with 40x magnification. In addition, essential oil extraction was carried out for the four batch samples by hydrodistillation (3 hours) using a Clevenger apparatus. The results were expressed as a percentage of the essential oil yield (mean of the two determinations)[171].

V.2.4- Microscopic determination

For the histological studies, four leaves were selected of each collected lot. Cross-leaf sections were dehydrated in alcoholic series of 50, 70, 75, and 90% and stained with safranin diluted in ethanol 90%. After staining the cross sections, they were mounted in glycerinated gelatin and sealed with paraffin. Sections of 8-µm thick were obtained using a microtome Kedee202 A (Zhejiang,


BIOECO
CENTRO ORIENTAL DE ECOSISTEMAS Y BIODIVERSIDAD

HERBARIO BSC
CENTRO ORIENTAL DE ECOSISTEMAS Y BIODIVERSIDAD

No 147

Nombre Científico Plectranthus neochilus Schltr.

Familia Lamiaceae

Nombre Común mejrobamato

Localidad Stgo. de Cuba

Provincia Stgo. de Cuba

Fecha agosto 2017

Colector Adolfo Luis

DIRECCIÓN

BIOECO

Formación vegetal

Hábito

Coordenadas

Sustrato

CENTRO ORIENTAL DE ECOSISTEMAS Y BIODIVERSIDAD / CITMA

Altitud

Fenología

Determinador Gustavo Pilonet Jurén

Observaciones

Figure V.1. Drugs consumed concomitantly with extracts of *P neochilus* by the Cuban population.

China). The sections were stained with safranin and methylene blue, cleared in xylol, and ridded in Canadian Balsam as previously described[172]. The epidermis, cell wall, stomata, and mesophyll were characterized according to the methodology described by Theobald and collaborators[173]. The cuts were observed and photographed in a microscope Novel N-220M (Nanjing, China) coupled with a Canon digital camera 1600×1200 pixels at 400×.

V.2.5- Total ash content and total extractable matter

These parameters were determined according to the protocols proposed by WHO[174]. Accordingly, we calculated the mean of the three replicates for each of the four batches. For the determination of the total ash content, 2 g of the dry plant material was burnt in a glowing silicate crucible at 700 °C for 2 hours (Boeco MF 8/1100 muffler, Hamburg, Germany). This process was repeated until the mass was constant. The cooled residue was weighed and the values expressed in percent.

The soluble substances in water and ethanol were determined by the cold maceration method. Five grams of the dry plant material were placed in a 250-mL Erlenmeyer flask with a frosted lid and 100 mL of the solvent (water or ethanol) was added. After 12 hours of stirred maceration and 6 hours of rest, the contents were filtered and 25 mL of the filtrate was transferred to a flat-bottomed dish to evaporate the solvents at 105 °C in a Carl Roth SC 150 (Karlsruhe, Germany) thermostated bath. The cooled residue was weighed, expressing the values (w/v) in percent.

V.2.6- Plant extracts preparation and quality control determinations

Three different types of extracts were prepared: One followed the indications from the ethnobotanical information and consisted of an aqueous decoction of 10 mg of the fresh, crushed leaves in 100 mL of water (FLD). The other two extracts were obtained in the same proportion but using dried leaves which were subjected to maceration during 48 h with either water (DLW) or commercial ethanol at 94% (DLE) as solvents.

V.2.7- Determination of the physical and physicochemical parameters of the extracts

The determination of organoleptic properties, pH value, and total extractables was carried out according to standard protocols[175]. Organoleptic properties were assessed by simple inspection of appearance (colour, texture and odour). The pH value was measured using a calibrated pH-meter (Hanna Instruments, Spain). Total solids were determined by a gravimetric method after drying 5 mL of the extracts in a porcelain capsule. The results of these last two parameters were given as the mean and standard deviation (SD) of three determinations.

V.2.8- Determination of the phytochemical profile of the extracts

Chemical composition was analysed in an UPLC-DAD-MS/MS system using a Xevo G2-XS QTof spectrometer (Waters, Milford, MA, USA) coupled with an ACQUITY LC system equipped with MassLynx version 4.1 software. Five µL of each extract (FLD, DLW, DLE) at 100 µg/mL were injected on a BEH Shield RP18 column (100 mm × 2.10 mm, 1.7 µm; Waters, Milford, MA, USA). The mobile phase solvents consisted of H2O+0.1% FA (A) and ACN+0.1% FA(B), and the gradient was set as follows (min/B%):0.0/10, 5.0/10, 20.0/15, 30/15, 40.0/25, 45.0/25, 55.0/40, 60.0/40, 65.0/100, 70.0/100, 75.0/10, 85.0/10.

Full scan data were recorded in ESI (−) and ESI (+) mode from m/z 50 to 1500 and the analyser was set in sensitivity mode (approximate resolution: 22,000 FWHM). The spray voltage was set at either +1.5 kV and −1.0 kV; cone gas flow and desolvation gas flow at 50.0 L/h and 1000.0 L/h, respectively; and source temperature and desolvation temperature at 120 °C and 550 °C, respectively. Data were also recorded using MSE in the positive and negative ionization modes (two analyses per mode), and a ramp collision energy from 20 to 30 V was applied to obtain additional structural information. Leucine Encephalin was used as lock mass. DAD spectra were recorded between 190 and 500 nm.

Using the software ReifycsAbf Converter, the UPLC-DAD-MS/MS raw data were converted to *.abf files and processed with MS-DIAL version 4.24[168] for mass signal extraction between 50 and 1500 Da from 0 to 22 min (range in which the main peaks appears). A centroid mode with a tolerance of 0.01 and an optimized detection threshold of 8000 for MS1 and 5000 for MS2 was fixed. The Global

Natural Products Social (GNPS, https://gnps.ucsd.edu) export function of MS-DIAL[176] was used to export the results and created a molecular network with the Feature-Based Molecular Networking (FBMN) workflow[177]. Major peaks were tentatively characterized by means of MS/MS spectra comparing with those found in the literature and the public databases PubChem (https://pubchem.ncbi.nlm.nih.gov/), ChemSpider (https://www.chemspider.com/ MassBank of North America (MoNA) (http://mona.fiehnlab.ucdavis.edu/) and NIST Mass Spectrometry Data Center (http://chemdata.nist.gov/).

V.2.9- Statistical Analysis

Statistical analysis was carried out in GraphPad Prism 8 (GraphPad Software, San Diego, USA). Tukey's test was used as a multiple comparison test for independent samples to define significant differences (p value under 0.05) between the quantitative quality control parameters of dried leaf lots and derived physico-chemical parameters of extracts. Before statistical data analysis, normal distribution was verified using the Kolmogorov-Smirnov test implemented in the same software. Decision limits for upper and lower cut off values of the standard deviations were estimated, when possible, by each quantitative quality control parameter for a 95% confidence.

V.3 Results

The apparent inconsistent ethnomedicinal use of *P. neochilus* extracts motivated us to explore the potential differences in the pharmacognostic and phytochemical profile of Cuban cultivars. For this purpose, four batches of previously identifiedplant material (February, May, August and November 2018) were harvested.

V.3.1- Plant quality control parameters

V.3.1.1- Fresh leaves

Macroscopic determinations did not reveal phenotypical differences throughout the year of study (four batches collected) and are in agreement with the deposited botanical information for this plant in Cuba[18]and Brazil[178]. Leaves with creased edges, perinervic venation, petiole with a wedge-shaped base, membranous texture, greenish-grey colour and a strong and characteristic aromatic odour are

the most representative macroscopic quality control parameters. Other remarkable characteristics are an adaxial and abaxial pubescent leaf surface, with generally short hairs. In spite of the strong and characteristic aromatic odour, none of the eight determined essential oil (EO) yields (two replicates for the four batches) allows us to obtain quantifiable values greater than the sensitivity of the determination method. Accordingly, the EO yield values (EOY) measured did not exceed the detection limit (EOY < 0.01%).

In general, the EO in the species is informed at relative low yields. For the species that grows in Brazil, values around 0.03% have been regularly reported[103,111]; while for the species that grow in Portugal[13] and South Africa yields barely exceed 0.2 %[132].

The micro-morphological analysis revealed that both leaf surfaces are covered with trichomes and abundant orange-coloured glandular cells which are more evident in the adaxial surface when observed under the stereo-microscope 40× (see Figure V.2A). The multicellular and uni-serial nature of non-glandular trichomes (see Figure V.2B) as well as the orange-coloured glandular cells (see Figure V.2C) can be better observed when using higher magnifications of the bright field microscope at 100×. The adaxial surface has a cuticle of approximately 1 μm thick with a single layer epidermis with polygonal cells of 10 μm in size, periclinal and anticline walls, tracing straight or convex lines that are up to 1.25 μm thick (see Figure V. 2D). The abaxial epidermis also is 1.25 μm thick without cuticle, and has diacytic stomata (see Figure V.2E) with a density of 22.5/100 μm^2. The parenchyma is homogeneous, lacunar, with 5 to 6 strata of amorphous cells reaching a thickness of 35 μm.

V.3.1.2- Dry leaves

The drying process was carried out by sun-drying, shade-drying and oven-drying (45 °C). Due to the succulent characteristics, none of the air-exposure methods was effective, since more than three weeks were needed to achieve drying, causing microbiological contamination in the case of shade-dried batches. Under these circumstances, only the oven-dried method was able to eliminate the water from the leaves in 6-8 days, preserving most of the organoleptic characteristics.

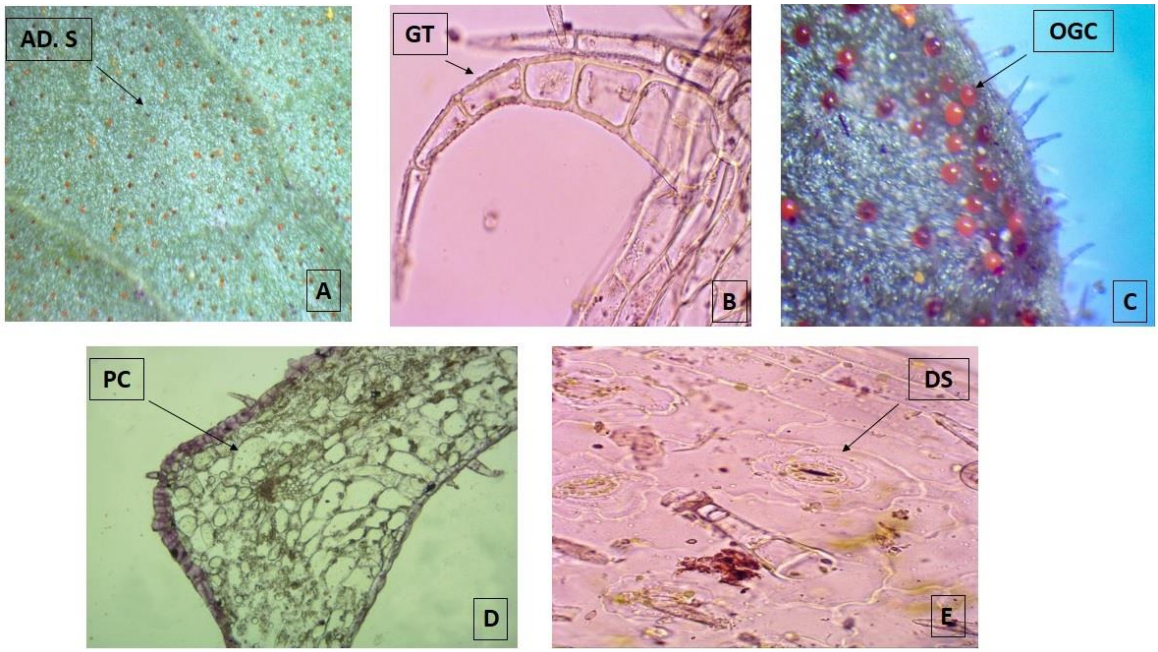


Figure V.2. Photomicrograph of transverse sections of leaves from *P. neochilus*collected in Santiago de Cuba. A) Adaxial surface of the foliar lamina. B) Non-glandular trichomes. C) Orange-colored glandular cells. D) Polygonal cells. E) Diacytic stomata. A x 40X and B, C, D, E x 100X

The residual moisture content was 10.5 ± 1.7% (azeotropic method) (n = 8, two determinations on the four batches).

The other quality control parameters repeatable within batches (see Table V.1) showed no appreciable differences between the different harvesting moments. In all cases it was possible to define an upper and lower decision limit, allowing predicting a range in which the results should be expected. Even when no statistical differences ($p > 0.05$) among batcheswere found for none of the quality control parameters, the batch selected to prepare the extracts from *P. neochilus* leaves for the further experiments was batch 2 (May), considering the maximal number of soluble compounds.

V.3.2- Plant extracts and quality control determinations

V.3.2.1- Physical and physicochemical parameters of plant and extracts

Three extracts were prepared: the first following the ethnobotanical information using water and fresh crushed leaves (FLD). The other two extracts used the dried leaves and water (DLW) or commercial ethanol at 94% (DLE) as solvents. All three extracts were prepared using the leaf material coming from batch number two. Table 2 (see Table V.2) summarizes the physical and physicochemical parameters of the extracts (three replicates).

As can be seem in Table V.2, the quality control parameters are different in all extracts, especially those of a quantitative character. Water stands out as solvent with high capacity to extract constituents of *P. neochilus* leaves. In relation to pH, dried leaves favour the extraction of acidic compounds, especially when ethanol is used as solvent.

V.3.2.2- Determination of the phytochemical profile of the extracts.

The three extracts of *P. neochilus* FLD, DLW, and DLE were analysed by UPLC-DAD-MS/MS. Visual evaluation of the total ion chromatogram (TIC) of all extracts (see Figure V.2) revealed resemblance between the extracts with regard to the position of peaks, but displayed a difference in relation to the relative amount of the compounds present. This was confirmed by the dereplication analysis performed and in-depth exploration of the main peaks in the three chromatograms, which reveals that the major changes observed are related to changes in relative intensity of the peaks.

Table V.1. Quantitative quality control parameters of dried leaves from *P. neochilus* collected in Santiago de Cuba at different times.

Parameter	Batch 1 (February)	Batch 2 (May)	Batch 3 (August)	Batch 4 (November)	LDL (95%)	UDL (95%)
Total ash content (%)	$8.1^{a} \pm 1.4$	$8.4^{a} \pm 2.0$	$9.7^{a} \pm 1.3$	$8.5^{a} \pm 1.7$	4.8	12.5
Ethanol soluble substances (%)	$18.5^{b} \pm 2.0$	$20.2^{b} \pm 2.7$	$17.5^{b} \pm 1.9$	$20.2^{b} \pm 0.4$	14.5	23.7
Water soluble substances (%)	$22.6^{c} \pm 1.3$	$24.1^{c} \pm 1.4$	$22.1^{c} \pm 1.1$	$23.5^{c} \pm 1.6$	19.8	26.3

LDL Lower Decision limit, UDL Upper Decision limit. Equal letters within the same row indicate no statistical differences (LSD Tukey test, α= 0.05).

Table V.2. Physical and physicochemical parameters of the extracts prepared from batch 2 (May-2018) of *P. neochilus* leaves growing in Santiago de Cuba.

Parameter	Fresh leaves decoction (FLD)	Dry leaves water maceration (DLW)	Dry leaves ethanol maceration (DLE)
Organoleptic characteristics	Color: light green Smell: characteristic of the plant Texture: slightly dense	Color: light brown Smell: characteristic of the plant Texture: slightly turbid	Color: dark green Smell: characteristic of the plant Texture: transparent
Total extractable substances (%)	$15.99^{b} \pm 0.01$	$20.19^{c} \pm 0.01$	$10.67^{a} \pm 0.01$
pH	$5.27^{c} \pm 0.01$	$4.85^{b} \pm 0.02$	$4.12^{a} \pm 0.01$

Different letters within the same row indicate statistical differences (LSD Tukey test, α= 0.05).

Table V.3.Assigned compounds, [M-H]- and ESI negative fragment ions of the eighteen peaks detected in *P. neochilus* fresh leaves decoction (FLD) extract.

Compound	Rt (min)	Accurate Mass [M-H]$^-$ (*m/z*)	Error (ppm)	MS/MS ions (Rel. Intensity, %)	Molecular Formula	Tentative identification	AEP
1	6.45	387.1647	-1.3	207(17), 163(8)	$C_{18}H_{28}O_9$	12-Hydroxyjasmonic acid glucoside	DLW
2	7.95	593.1553	1.5	503(12), 473(37), 413(5), 383(11), 353(19)	$C_{27}H_{30}O_{15}$	Vicenin-2	DLW, DLE
3	11.36	491.0858	-1.0	475(51), 315(59), 299(64)	$C_{22}H_{20}O_{13}$	4´-Methoxy-quercetin-3-*O*-glucuronide	DLW, DLE
4	11.61	461.0721	-0.9	285(57), 255(22)	$C_{21}H_{18}O_{12}$	Luteolin-*O*-glucuronide	DLW, DLE
5	11.99	491.0829	0.4	315(69), 299(33)	$C_{22}H_{20}O_{13}$	7-Methoxy-quercetin-3-*O*-glucuronide	DLW, DLE*
6	12.05	437.1805	0.7	377(100), 359(86), 341(22), 331(30), 315(62)	$C_{22}H_{30}O_9$	3,6,7,12,16-Pentahydroxy-2-acetyl-5,8,12-abietatrien-11,14-dione	DLW*, DLE*
7	12.38	437.1816	-0.2	377(38), 359(41), 289(71)	$C_{22}H_{30}O_9$	2,3,7,12,16-Pentahydroxy-6-acetyl-5,8,12-abietatrien-11,14-dion	DLW, DLE
8	12.46	467.2131	0.4	437(18), 421(36), 289(100)	$C_{20}H_{36}O_{12}$	2-(8-(Hydroxymethoxy) oct-1-en-3-yloxy)-hexoside-pentose	None
9	12.79	359.0778	1.6	197(25), 179(23), 161(48), 135(7)	$C_{18}H_{15}O_8$	Rosmarinic acid	DLW, DLE
10	14.66	489.1032	-1.4	313(57), 298(19), 283(18)	$C_{23}H_{22}O_{12}$	3´,4´-Dimethoxy-luteolin-7-glucuronide	DLW, DLE
11	15.71	479.1918	-1.5	419(86), 401(62), 359(41), 341(24), 313(21)	$C_{24}H_{32}O_{10}$	6,11,12,14,16-Pentahydroxy-3,17diacetyl-8,11,13-abietatrien-7-one	DLW*, DLE*
12	16.04	475.0871	-1.3	299(73), 284(31)	$C_{22}H_{20}O_{12}$	Methoxy-kaempferol-7-glucuronide	DLW*, DLE
13	16.12	475.0874	-0.6	299(100), 284(39)	$C_{22}H_{20}O_{12}$	Methoxy-kaempferol-3-glucuronide	DLW*
14	16.28	459.0930	-0.4	283(100), 268(51)	$C_{22}H_{20}O_{11}$	Methoxy-apigenin-5-glucuronide	DLW, DLE
15	16.76	511.2578	1.0	493(27), 467(76), 305(9)	$C_{26}H_{40}O_{10}$	Hexosyl-6β-hydroxicarnosol	DLW
16	18.58	435.1661	1.1	375(42), 357(19),327(9)	$C_{22}H_{28}O_9$	3,6,11,12,14-Pentahydroxy-2-acetyl-5,7,11,13--abietatetraen-7-one	DLW, DLE
17	20.27	477.1798	-1.0	417(100), 387(17), 357(23), 327(11)	$C_{24}H_{30}O_{10}$	6,11,12,14,16-Pentahydroxy-3,17-diacetyl-5,8,11,13-abietatetraen-7-one	DLW
18	21.21	419.1721	-1.4	359(51), 341(6)	C22H28O8	3,6,12-Trihydroxy-2-acetyl-8,12-abietadien-7,11,14-trione	DLW, DLE

Rt → Retention Time, AEP→ Peak presence in alternative extract (DLW and DLE), *→Traces

Dereplication is a strategy that provides a fast identification of known metabolites in complex biological mixtures, speeding up the process to identify natural products[179], while Feature-Based Molecular Networking (FBMN), available on the Global Natural Products Social Molecular Networking (GNPS) web platform supports the analysis of the LC-MS/MS data. This process rendered 22 library hits. The matched compounds were mainly glycosides, glucuronides and methoxy derivates of quercetin, kaempferol, luteolin and apigenin; abietane type diterpenoids, fatty acyl derivates and rosmarinic acid; helping the further process of compound identification.

Considering that the aim of this research is to evaluate why Cuban cultivars are not typically used for their potential antimicrobial, anticatarrhal, and digestive properties, the identification of chemical metabolites was focused on the extract obtained by the method that most resembles the inhabitants' way of preparation (FLD). Peaks with at least 20% of relative intensity were considered and their presence was evaluated in the other two studied extracts (DLW and DLE). Under these conditions 18 peaks were selected and tentatively identified (see Figure V.3, Table V.3). The chemical structure proposed for each one of the 18 compounds is shown in and Figure V.4.

Compound 1 with a retention time of 6.45 min shows a peak with m/z 387.1647 [M-H]- yielding a fragment at m/z 207 [M-H-C6H12O6]-due to the loss of a neutral hexoside residue (180 Da) and another at m/z 163 [M-H-C6H12O6-COO-]- as result of the loss of the carboxyl function. No other secondary peaks were detected; therefore peak 1 was tentatively identified as 12-hydroxyjasmonic acid glucoside considering the role of this kind of compound and their distribution in the plant kingdom. It was detected only in watery extracts (see Table V.3).

Compounds from 2 to 5 as well as 10, 12, 13 and 14 were identified as flavonoids (flavone and flavonol type) derivates in which the nature of the aglycone was inferred with the help of the MS2 ESI positive mode[180]. All those compounds were detected in the three extracts with the exception of compound 13, detected only in water extracts (see Table V.3). Compound 2 with a m/z 593.1553 [M-H]- and fragments at m/z 503, 473, 413, 383, 353 was identified as vicenin-2. Figure V.5 shows the fragmentation pattern proposed assigning those product ions as result

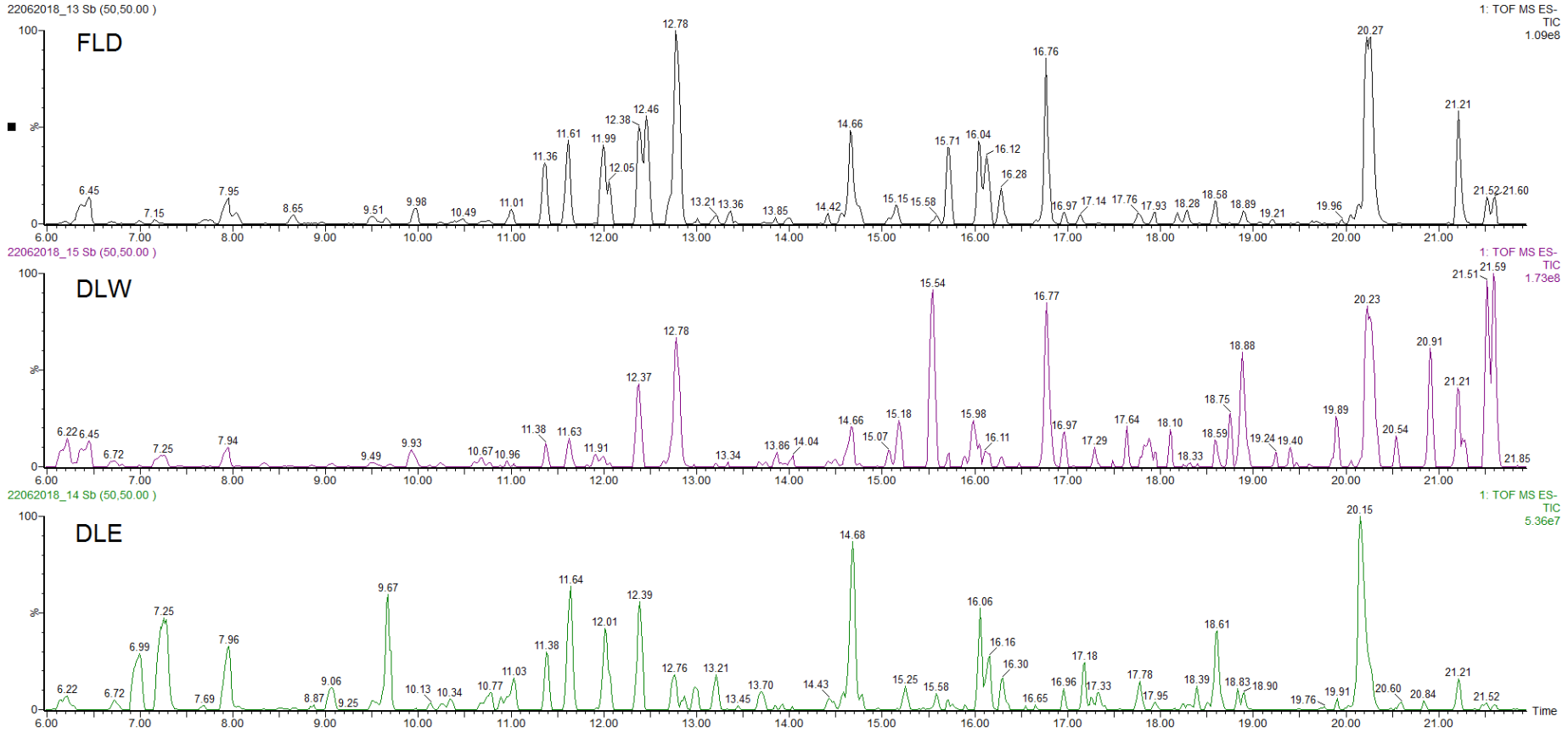


Figure V.3. UPLC-DAD-MS/MS Total Ion Chromatogram (TIC) profiles of *P. neochilus* extracts

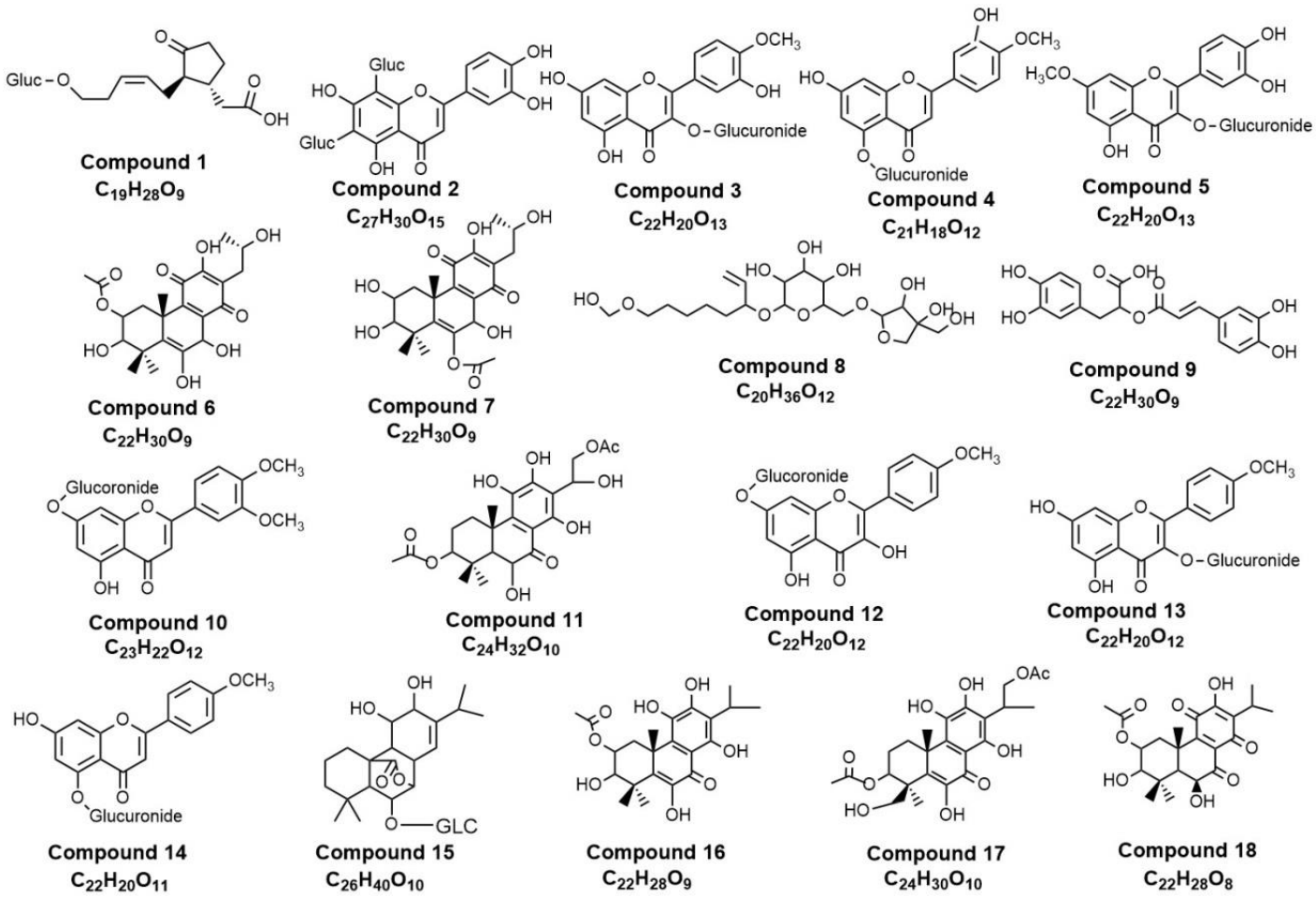


Figure V.4. Chemical structures of the 18 compounds identified in *P. neochilus* leaves growing in Cuba.

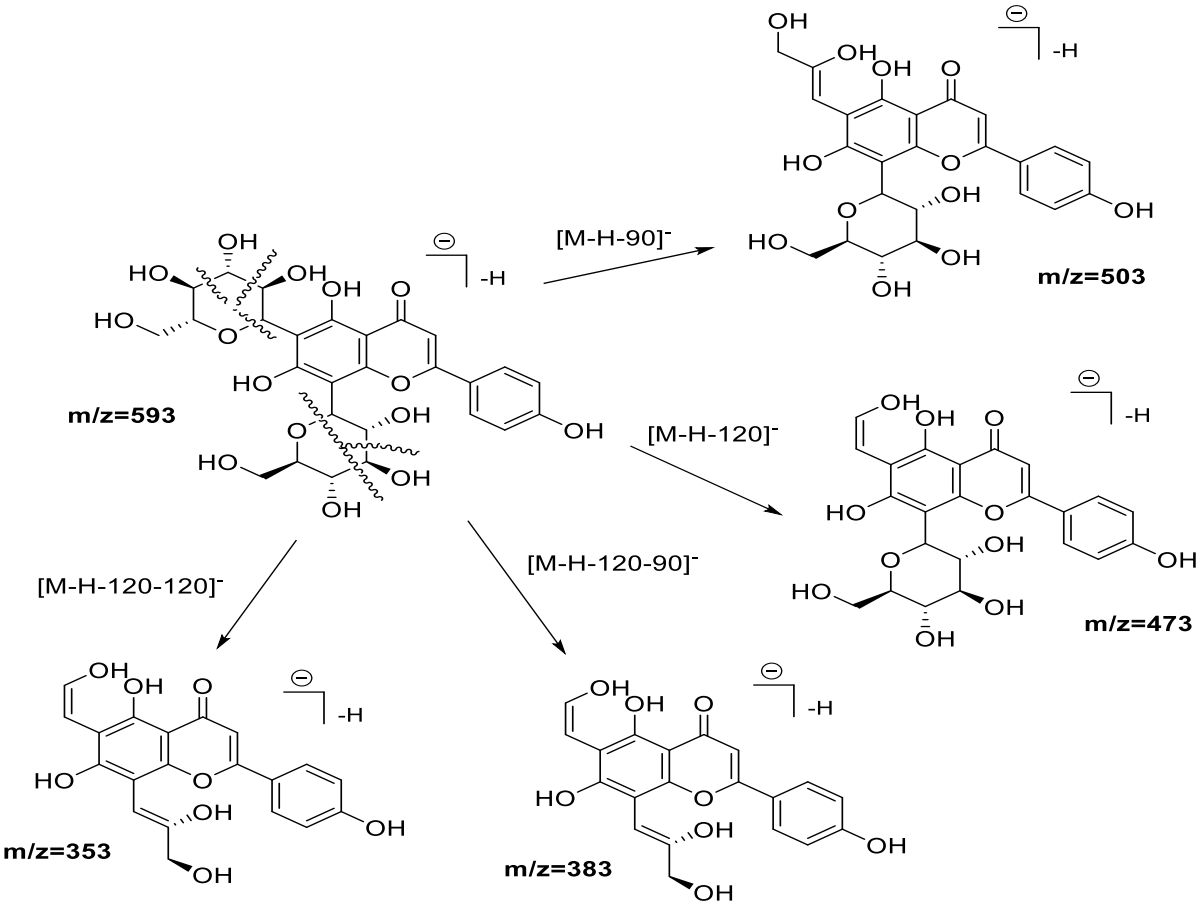


Figure V.5. Fragmentation pathways (ESI negative mode) proposed for Vicenin 2.

of the cross-ring cleavages in di-hexose C-flavonoid glycoside[181,182]. The UV spectra confirm the flavonoid nature of compound 2 with peaks of maxima absorption at 225, 270 and 375 nm.

Compound 3 and 5 were classified as position isomers showing similar molecular ions m/z 491.0858 and 491.0829 [M-H]- as main (100% relative intensity) and fragments m/z 315 and m/z 299 consistent with glucuronide loss (176 Da). As unique difference, peak 3 displays an extra fragment at m/z 476 with relative high intensity (51%), suggesting an easier loss of a methyl group from an ether moiety than peak 5 that on the contrary shows the fragment m/z 299 with higher intensity. For compound 3, a peak at 153.0118 (ESI positive mode) confirms that the -OCH3 moiety is not present in the A-ring. Those observations together with the information derived from the FBMN analysis, and the chemotaxonomic information for the genus *Plectranthus*[183], allows us to assign position 4´ and 7 for the methoxy group of compounds 3 and 5, respectively, while the glucuronide group was placed at position 3.

Compounds 6 and 7 also have almost the same molecular ions (m/z 437.1805 and 437.1816 [M-H]-) and similar initial fragmentation pattern with fragment ions at m/z 377, 359 representing the loss of an acetyl group (-60 Da) and a water molecule (-18 Da). The difference between both compounds is defined by the rest of the fragments and its relative intensity. For peak 6, fragments at m/z 341, 331 and 315 shows medium-low relative abundance while for peak 7 those fragments appear in a very low relative abundance and a new fragment with 71% of intensity at m/z 289 sign the main difference between both compounds. Considering this fragmentation pattern (see Figure V.6), the results of dereplication analysis, the abundance of reports of abietane diterpenoids in the Lamiaceae family and its description for several species of *Plectranthus*[150,183], compound 6 was identified as 3,6,7,12,16-pentahydroxy-2-acetyl-5,8,12-abietatrien-11,14-dione or 2-hydroxy-6-acetyl-scutellarioidol A and compound 7 as 2,3,7,12,16-pentahydroxy-6-acetyl-5,8,12-abietatrien-11,14-dione or 6-hydroxy-2-acetyl- scutellarioidol A. The position of the hydroxyl and acetyl substituents are conditioned to the cleavage of the ring A (-C(CH3)2-CO, m/z = -70 Da), which is only possible in compound 7 after the loss of the acetyl group of position 2.

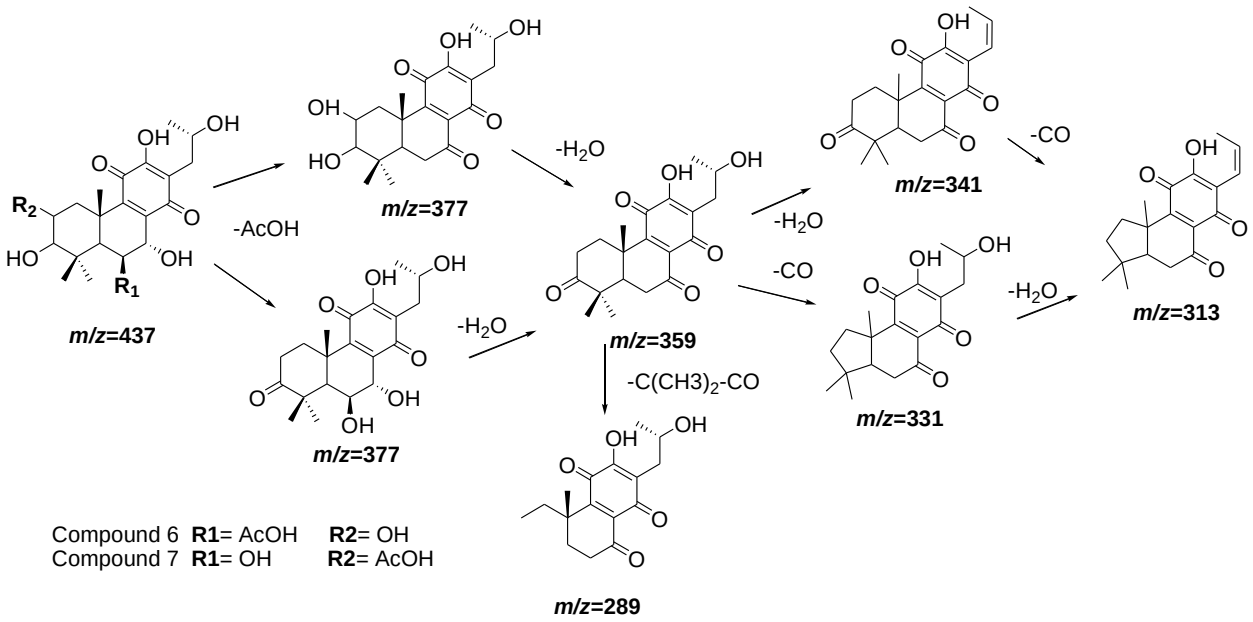


Figure V.6. Fragmentation pathways (ESI negative mode) proposed for compounds 6 and 7 in *P. neochilus* extracts.

Compound 8 was coincident with a fractionation pattern of a fatty acyl glycoside with an m/z value of 467.2131 Da in negative ion mode and tentatively identified as 2-(8-(hydroxymethoxy) oct-1-en-3-yloxy)-hexoside-pentose. Fragment ions at m/z 437 and 421 are in correspondence with the cleavage in the both sides of the ether bond of the aliphatic chain. The base fragment (100% intensity) at m/z 289 is a consequence of the further loss of the pentoside unit.

Compound 9 and its fragmentation pattern allowed us to identify this peak as the well-known compound rosmarinic acid. Both the fragment ions at m/z 197 and 179, correspond to the cleavage of the ester group while fragment m/z 161 corresponds to the loss of two or one water molecules of each one of the previous fragments respectively. Last fragment observed: m/z 135 (even in a very low intensity) confirms the assigned substance. Based on the peak area at UV detection, this compound classifies as the main compound in FLE and DLW extracts, and as second in DLE extract. This result is in concordance with previous information[16].

Compounds 10, 12, 13 and 14 are the last flavonoids identified with [M-H]- at 489.1032, 475.0871, 475.0874, and 459.0930, respectively. All are methoxy-glucuronide-flavonoid derivates. Typically for all compounds is the loss of the glucuronyl group generating the fragments at m/z 313, 299, 299, and 283, respectively (see Table 3). In the case of the first two, this fragment results in the second most abundant, while for compounds 13 and 14, the fragments m/z 299 and 283, respectively, represent the main peak in the spectrum (see Figure V.7). Further fragments are in agreement with the loss of methyl groups from the methyl ether (-15 Da).

For compound 10 the ESI positive mode shows a distinctive fragment at 179 (0,4B+), characteristic for the C-C cleavage at positions 0/4 of luteolin[184]. Considering all those facts, the chemotaxonomic information available and the relative intensity of the [M-H]-, it is suggested that glucuronyl group is attached at position 7, while methoxy groups are placed at 3´ and 4´[184,185], that is why this compound was tentatively identified as 3´,4´-dimethoxy-luteolin-7-glucuronide. ESI positive mode of compound 12 shows fragments at m/z 121 (0,2B+) and 165 (0,2A+), characteristic of the C-C cleavage at positions 0/2 of kaempferol[184], the rest of the factors are similar to compound 10. Having no further information

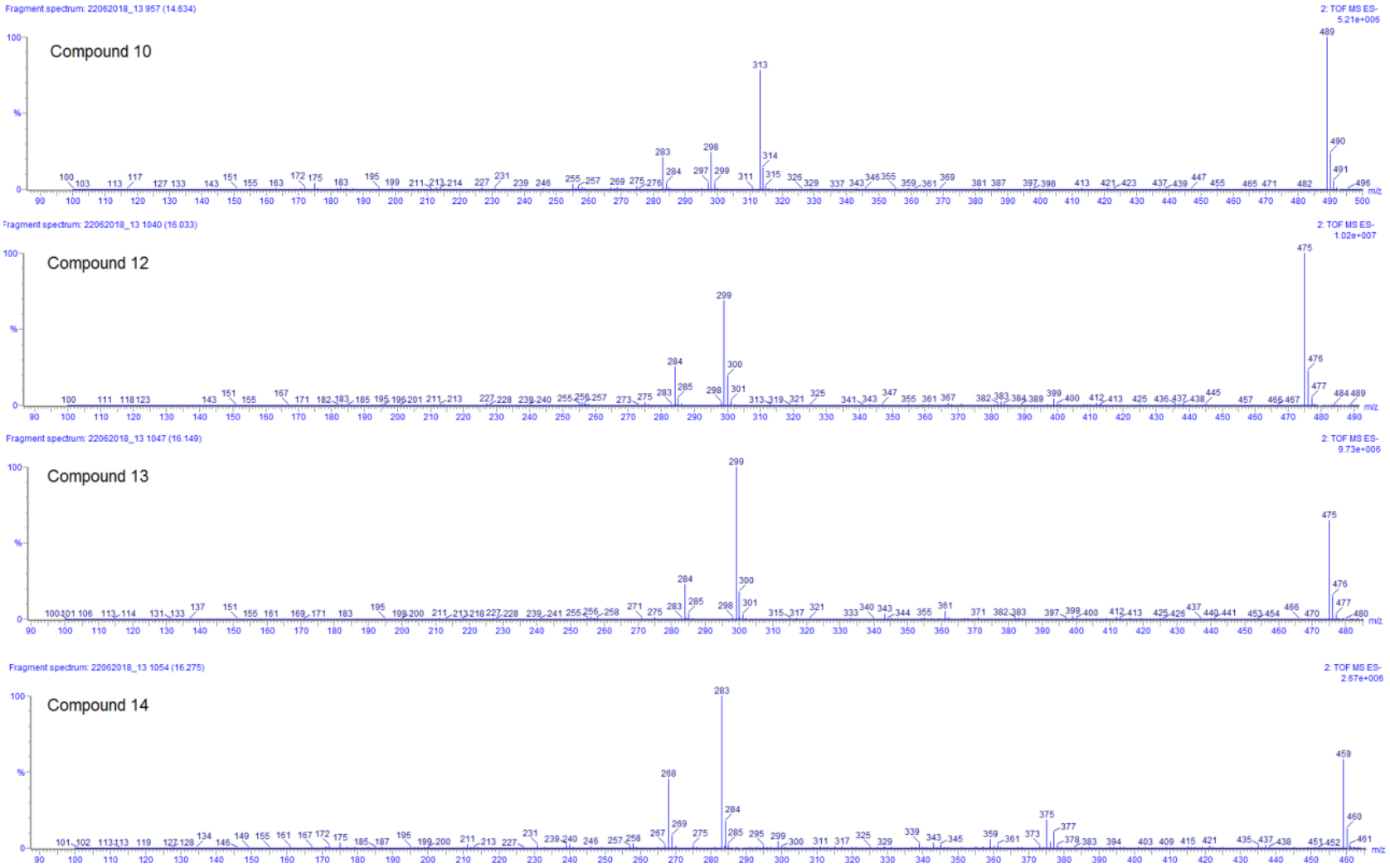


Figure V.7. ESI negative ion mode MS2 spectra of compounds 10, 12, 13 and 14 of *P. neochilus* extracts

about the possible position of the methoxy substituent, compound 12 was tentatively identified as methoxy-kaempferol-7-glucuronide. Compound 13 has almost the same characteristics than compound 12 showing a pseudomolecular ion [M-H]- at m/z 475.0874. Also, the same fragment peaks (m/z 299 and 284) as well as the same behavior in positive ion mode (data not shown) and similar UV maxima at 227, 268 and 335 nm (226, 268 and 341 nm for compound 12, data not show). The only remarkable difference between compounds 13 and 12 is related to the relative intensity of the mass peaks. For compound 13 the main fragment was found at m/z 299, resulting from the loss of the glucuronyl, compared to compound 12 where the pseudomolecular ion [M-H]- was seen as the main ion in the spectrum (see Figure V.8). This allowed us to infer that in flavonoid 13 the glucuronyl substituent is placed in the "non-favored" position 3[185], that is why this compound was tentatively identified as methoxy-kaempferol-3-glucuronide. At last, compound 14 corresponds to an apigenin derivate considering the fragment in ESI positive mode of m/z 163 (0,4B+). The main fragment (m/z 283) does not correspond to the pseudomolecular ion [M-H]-; therefore, the glucuronyl substituent was linked to the "less favored" apigenin hydroxyl substituent position 5[185]. The methoxy group can be arbitrary placed in position 7 or 4´ that is why compound 14 was tentatively identified as methoxy-apigenin-5-glucuronide.

Compound 11 with a pseudomolecular ion at m/z 479.1918 [M-H] - and molecular formula C24H32O10 was inferred as a diacetylditerpenoid. The consecutive loss of both acetyl fragments followed by the loss of a water molecule and the similarity with coleon D and coleon U, compounds isolated from *P. barbatus*, *P. fasciculatus*, *P. forsteri*, *P. grandidentatus*, *P. madagascariensis*, *P. nummularius*, *P. sanguineus, P. argentatus, and P. myrianthus*[171], allows us to identify compound 11 as 6,11,12,14,18-pentahydroxy-3,17diacetyl-8,11,13-triene-7-one (Figure V.9).

Compound 15 presents a pseudomolecular ion [M-H]- at m/z 511.2578 and fragments m/z 493[M-H-18]-and m/z 467 [M-H-44]- as a result of a trans-elimination of a water molecule (18 Da) and by loss of a carboxyl group (44 Da), respectively. Additional loss of a fragment of 162 Da from the fragment ion m/z 467 resulted in a signal with a low relative intensity at m/z 305 indicating the presence of a hexose unit. This fragmentation pattern, the FBMN analysis and

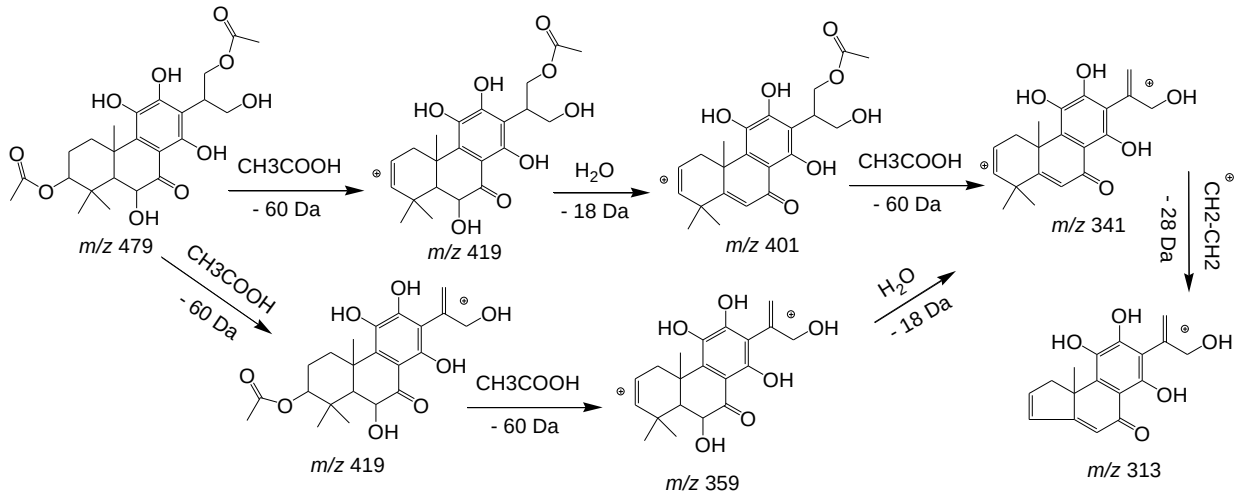


Figure V.8. Fragmentation pathway (ESI negative mode) proposed for compound 16: 6,11,12,14,18-pentahydroxy-3,17diacetyl-8,11,13-triene-7-one.

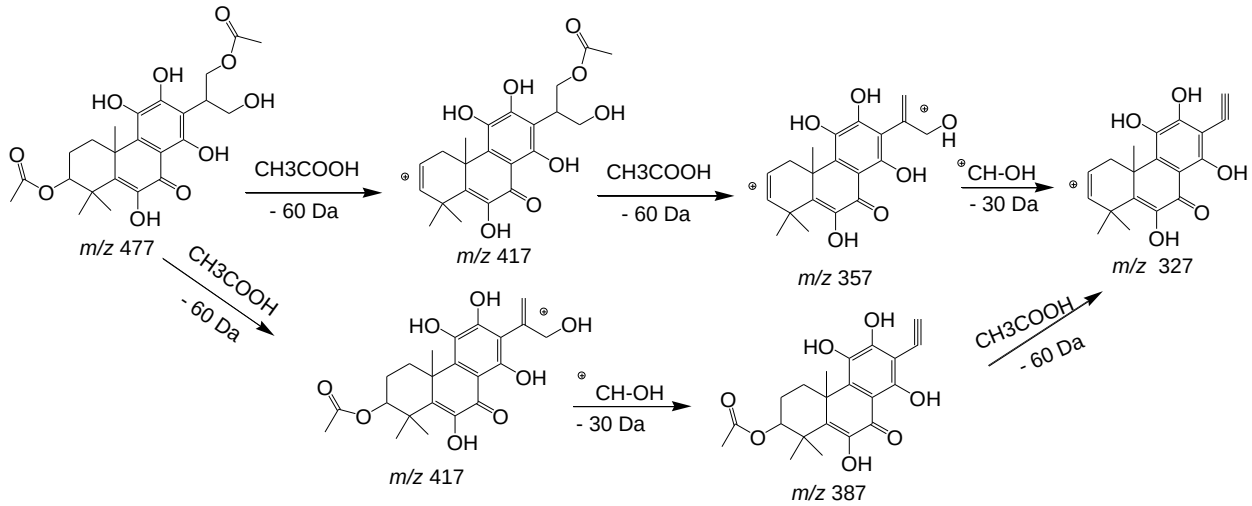


Figure V.9. Fragmentation pathway (ESI negative mode) proposed for compound 17: 6,11,12,14,16-pentahydroxy-3,17diacetyl-5,8,11,13-tetraene-7-one.

chemotaxonomic information available for *Plectranthus* genus allow us to tentatively identify compound 15 as hexosyl-6β-hydroxicarnosol. The aglycone 6β-hydroxicarnosol has been already reported for *P. barbatus*[186]. The unfavored loss of the hexosyl group is associated to the steric hindrance provoked by 7,20-epoxyabieta-20-one moiety, being consistent with β position.

Compound 16 with a retention time of 18.58 min and pseudomolecular ion [M-H]- at m/z 435.1661 seems to have a similar backbone as compounds 6 and 7 but with an additional double bond. The fragments suggest the same sequence of fractionation with the loss of H_2O (-18 Da), CH3COOH (-60 Da), H_2O (-18 Da) and at last CO (-28 Da) generating the fragments at m/z 417, 375 (base peak), 357 and 329 respectively. This information allows us to identify compound 16 as 3,6,11,12,14-pentahydroxy-2-acetyl- 5,7,11,13-abietatetraen-7-one, which appears in all the extracts evaluated (see Table V.3). Similar compounds have been isolated been isolated from *P. madagascariensis*[171] and *P. scutellarioides*[150].

Compound 17 shows a pseudomolecular ion at m/z 477.1798 [M-H]- corresponding to the molecular formula C24H30O10, being similar to compound 11 but with an extra double bond. A fragment at m/z 417 (base peak) represents the loss of an acetyl substituent, followed by two possible fragmentation ways: the successive loss of the second acetyl group (-60 Da) and CH-OH+ (-30 Da) rendering the fragments m/z 357 and 327 or vice versa; first CH-OH+ (-30 Da) followed to the second acetyl group (-60 Da) to render the fragments m/z 387 and 327 (Figure V.9). This information allows us to tentatively identify compound 17 as 6,11,12,14,16-pentahydroxy-3,17-diacetyl-5,8,11,13-abietatetraen-7-one,compound previously isolated from *P. scutellarioides*[150].

The last compound identified at a retention time of 21.21 min and a base peak and pseudomolecular ion at m/z 419.1721[M-H] -, seems to have a similar abietane backbone as compounds 6 and 7 as well as compound 17. The fragment at m/z 359 (second most abundant with 51%) can be explained by the loss of a neutral acetyl group [M-H-60]- with the consequent oxidation of the adjacent hydroxyl group at position 3, as described before with compound 7 (see Figure V.6). The fragment, with a very low relative intensity (6%) at m/z 341 represents the loss of a water molecule. Based on this information, the hydroxyl group should be positioned at

place 6 generating a double bond. The other hydroxyl substituent was placed in a position that does not allow easy water loss, position 12 (as chemotaxonomic pattern in most of the abietane diterpenoids identified in *Plectranthus* genre). All this information and the hits suggested by the FBMN analysis allows us to tentatively identify compound 18 as 3,6,12-trihydroxy-2-acetyl-8,12-abietadien-7,11,14-trione. The coleon U-quinone nucleus suggested for compound 18 has been recurrently reported for *Plectranthus* species such as *P. madagascariensis*, *P. sanguineus, P. forsterii, P. grandidentatus and P. myrianthus*[171].

V.4- Discussion

From a macromorphological and phenotypical point of view, there is no difference between the plants growing in Cuba and those reported in the bibliography. However, the essential oil production did not reach a level that would have allowed quantification in any of the four batches. Also, when analysing the micromorphological aspects, most of the parameters of the plant studied were in agreement with the international references, but no glandular trichomes were found on either the abaxial or adaxial leaf surface[11,110]

Glandular trichomes play a fundamental role in the storage and excretion of the volatile substances that attract or repel natural enemies. Their absence in the samples studied in the present work could be the explanation for such a low production of essential oils, which probably remain stored in the inner vesicles and maintain the characteristic smell of the plant. It is internationally recognised that biotic and abiotic aspects lead to changes, especially in sedentary organisms such as plants. This is even more evident in tropical islands such as Cuba, where climate change is increasing the aridity and salinity of soils through the combined effects of increasing drought, high temperatures and evaporation[187].

P. neochilus, is a plant native to South Africa (a completely different ecosystem from Cuba), but is nevertheless distributed worldwide. There are quantitative and qualitative chemical differences in the production of the essential oil. While in the coldest regions such as South Africa and Portugal the yield of essential oil production reaches at least 0.2% and the main constituent is α-terpenyl acetate, in the plantations of Brazil, citronellol is the main component and the yield barely reaches 0.03%. The reduction or even failure of essential oil biosynthesis due to

drought or high temperatures has already been described in the literature[167]. Based on this assumption, it is possible to hypothesise that under Cuban climatic conditions (high temperatures and evaporation levels) *P. neochilus* "protects" its essential oil production in internal vesicles rather than in glandular trichomes exposed to the sun, making it less accessible for extraction.

Another consequence described by the effects of drought or high temperatures is the reduction of photosynthesis. This leads to an increased production of phenolic and/or polyhydroxylated compounds to counteract the increased production of reactive oxygen species[188]. In the present study, 16 of the 18 identified compounds show a high oxidation pattern. In general, the isolated compounds can be classified into two main types of metabolites: Flavonoids and Abietane diterpenoids. From the eight flavonoids identified, seven have at least six oxygen atoms in their structure and fiveshows a free or methoxylated catechol moiety in ring B, indicating a high oxidation pattern. Reports on flavonoids in the genus *Plectranthus* are not abundant, but most of them are flavones and flavonols[184]. Exactly these types of flavonoids were identified in this study: flavones (compounds 2, 4, 10, and 14) and flavonols (compounds 3, 5, 12, and 13). Recently, the presence of rutin for *P. neochilus* has been informed[110]and although this compound was not isolated in this work, compounds 3 and 5 are structurally close. On the other hand, abietane diterpenoids are frequently reported in the genus *Plectranthus*[171].

In this work, we tentatively identified seven abietane diterpenoids (compounds 6, 7, 11, 15, 16, 17, and 18), and with the exception of compound 15, the degree of oxidation in the other six can be considered high (with an average of eight to ten oxygen atoms within the diterpene backbone). Due to the scarce information available on the non-volatile metabolites of *P. neochilus*, to the best of our knowledge all identified metabolites, with the exception of rosmarinic acid (compound 9), were detected for the first time in this species. Furthermore, the high content of phenolic and/or polyhydroxylated compounds could contribute to the divergent phytochemical compositions and properties of the Cuban cultivars of *P. neochilus* under these particular climatic conditions.

In general, there is a correspondence between the ethnobotanical information reported for Cuba and the pharmacognostic characteristics and phytochemical

composition of the species growing on the island. Regularly, the information reported worldwide on this species relates to the production of essential oils and its pharmacological activity as an antimicrobial and antiparasitic agent[13,14,103,111,113]. The low yield of essential oils that *P. neochilus* cultivars have in Cuba could reduce their antimicrobial activity and thus contribute to the fact that *P. neochilus* extracts for antiseptic use are not very popular among the island's inhabitants. On the other hand, the high oxidation pattern of flavonoid and abietane derivatives could lead to new pharmacological activities.

V.5- Partial conclusions

In the present study, the pharmacognostic and phytochemical profile of Cuban cultivars of *P. neochilus* was investigated for the first time. Macro and micromorphological analysis revealed a low yield in essential oil production and the absence of glandular trichomes, compared to plants growing in other latitudes. Different quality control parameters were evaluated for drying the leaves before the different extraction procedures. The traditional extraction of leaves by the inhabitants of Cuba was compared with the extraction of dried leaves with aqueous solvents or ethanol. The three extracts differ mainly in the proportions of their main constituents but are similar in their chemical composition.

CHAPTER VI

CHAPTER VI. Effects of *Plectranthus neochilus* Schltr. extracts on mice behaviour and brain gene expression.

Modified version of the article that will be submitted to theEtnopharmacology journal: Rodriguez-Ferreiro, A.O., Perez-Novo, C., Molina-Bertrán, S., Llauradó-Maury, G., Guisado-Bourzac, F., Morales-Aguilera, R. A., Rodeiro-Guerra, I., Escalona-Arranz, J. C. Vanden Berghe, W.* (2023). Effects of *Plectranthus neochilus* Schltr. extracts on mice behaviour and brain gene expression.

VI.1. Introduction

Developing countries resort to traditional medicine to make up for the lack of conventional medicines. In this context, the use of medicinal plants emerges as an alternative to alleviate their ailments while maintaining their ethnopharmacological practises. But not always this use is evidence based as claimed by the international community, nor implemented in the same way, differing according to each country´s national health programmes and legal/regulatory framework.

Cuba has been implementing since the end of the '80s of the last century, a structural and systematic program for the development of natural medicines and in particular phytotherapy. The creation of several research institutes, the implementation of National Guidelines coordinated by the Ministry of Public Health or MINSAP (Spanish acronym), and the modification of the legislative/regulatory landscape allowed the creation of what was called the "National Program of Traditional and Natural Medicine" [189] . This creates many opportunities for scientific research of Cuban medicinal plants, which are increasingly popular due to the lack of conventional drugs in the currentunderdeveloped economicalsituation, suffering from a blocked economy due to the US embargo.

In this context, *P. neochilus* considered as naturalised in Cuba in 2016 due to its quick expansion within the island because of its hypnotic, sedative, analgesic, and muscle relaxation effects rather than their worldwide traditional uses[18]. Early efforts of our research group proved that the plant grown in Cuba lacks quantifiable essential oils, does not show significant antimicrobial activity; and produces several flavonoid and abietan diterpenes with high degrees of oxidation that, together with rosmarinic acid as the main compound, could act at the level of the Central Nervous System (CNS). In this sense, the present study aims to scientifically validate the

possible *in vivo* behavioural effects of traditionally prepared extracts of *P. neochilus* based on brain-specific gene expression changes of some of the most common receptors involved in CNS activities.

VI.2. Material and methods

VI.2.1 Material and reagents

Resazurin, L-glutamine, Tamoxifen, Penicillin, and Streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM and RPMI-1640 culture media, heat-inactivated foetal bovine serum (FBS), and heat-inactivated horse serum were all purchased from Gibco® (New York, NY, USA). RNAlater®, QIAzol® Lysis Reagent, RNA and cDNA Kits all purchased from Thermofisher, (Massachusetts, USA). All primers were purchased to Integrated DNA Technologies (IDI, Iowa, USA).

VI.2.2. Plant material and plant extracts preparation

Plant collection followed the same procedure as stated in the previous section **"V.2.1-Plant collection"**, while plant extract preparation follows the methodology described in **"*V.2.6- Plant extracts preparation and quality control determinations*".** In this way, the three extracts (FLD, DLW, and DLE) explored in their quality control parameters and phytochemical profile will trail the biological tests.

VI.2.3. Cell viability assay

Cell viability was fluorometrically determined using the resazurin dye reduction assay. For this purpose, two cell lines derived from CNS tissues were chosen: PC12 (rat adrenal medulla CRL-1721) and BV2 (mouse microglial cells CRL-2467). The cell lines were acquired from the American Type Culture Collection (ATCC). PC12 cell were cultured in RPMI 1640, while BV2 in were cultured in DMEM medium. All media were supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, whereas for PC12 5% horse serum was used. Cultures were always maintained at 37°C in a humidified atmosphere at 5% CO_2.

Six different concentrations of: 512, 256, 128, 128, 64, 32, at 16 µg/mL were evaluated for each cell line. In 96-well plates were seeded 190 µL per well of PC12 (5 x10^4 cell/mL) and BV2 (2.5x10^3 cell/mL) respectively. Then, 10 µL of the extract to be evaluated were added at the desired concentration for a final volume of 200 µL, to be incubated later at 37 °C and 5% CO_2 for 24 hours. Subsequently, 50 µL/well

of diluted resazurin (2.2 μg/mL) was added and re-incubated for another 4 hours under the same conditions stated before. Cell viability was fluorometrically assessed in a microplate reader (TECAN GENios, Germany); setting the λex and λem parameters in 550 and 590 nM respectively.

The 100% of cell growth was settled from values obtained in the untreated-control wells while 0% of cell growth was set from wells where only medium was added. Solvents used for dilution: DMSO and ethanol were evaluated as solvent controls. CC_{50} values informed corresponds to the mean of the two replicates performed, expressing in percent the reduction of cell growth/viability when compared with the control wells. Tamoxifen was used as cell growth control reference drug.

VI.2.4 *In vivo* behavioural and motor activity assays

VI.2.4.1 Animals

Male albino mice of the OF1 line (18-21 g body weight and four weeks old) purchased from the Centre for the Production of Laboratory Animals (CENPALAB, Havana) were used. The animals were maintained in quarantine for 7 days before starting the experiment at room temperature and humidity, with natural light/dark cycles, water, and food *ad libitum*. Animals were feeding with pelletized feed supplied by CENPALAB, Havana. The experiments were performed from 08:00 until 13:00 hours, following the ethical principles established for "The handling use of laboratory animals" [190] recommended by the Cuban and international guidelines, endorsed in the Work Standards Procedures of the Institute of Marine Sciences (ICIMAR acronym in Spanish). Experiments were done in a blinded mode and reproduced by two independent researchers.

VI.2.4.2 Experimental design

The 50 mice were divided into five groups of ten mice each: Group I: Control (untreated), Group II: Diazepam (DZP) 2mg/kg (positive control), Group III-V experimental groups receiving a single dose/application of 500 mg/kg of FLD, DLW, and DLE extracts respectively. The dosage stated in the experimental (FLD, DLW, and DLE extracts) and diazepan® groups were selected according to Archana, 2013[191]. The extracts, the vehicle (un-treated group), and Diazepam were orally administered (dissolved in 0.9 % saline solution), by gastric cannula at a volume equivalent to 0.01 mLg-1 of body weight. The administration of test extracts was

applied from 08:00 while for Diazepam 30 min later. FLD, DLW, and DLE extracts were prepared the same day of administration by simple dilution in the saline solution of the previously freeze-driedextracts. Five hours after extract administration, mice were euthanized with pentobarbital sodium (125 mg/kg, intravenously) followed by decapitation. Whole brains were transferred into a vial with RNAlater® for 24 h at 4°C, and immediately stored at −80°C until the RNA isolation procedure.

VI.2.4.3 Open Field Test

The behaviour and motor activity exploration were tested with the Open Field Test, consisting in a circular white wall of 15 cm high and 30 cm diameter. An acrylic square was used as floor, on which a 10 cm diameter concentric circle was drawn[192]. Behavioural observation starts after 1 hour of administration when placing each animal in the central circle. Variables measured were: rearing (R), line crossing (LR), centre square entries (CSE), centre square duration (CRD), grooming (G), urination (U), and Fecal Pellet number (FPN). Data analysis considered lump values of all variables for each animal. Blind observation using a manual-digital stopwatch to calculate the times was used. The total observation time was 6 minutes.

VI.2.4.4 Rotarod test

Dunham similar equipment was used to evaluate the motor coordination[193], consisting of a 3 cm diameter horizontal roller that rotates at 8 rpm. Before the experiment, animals were previously acclimatized (2 min) on the roller without rotating. Subsequently, the rotation starts counting the time (with a manual-digital stopwatch) when the animals fall from the roller (in seconds). The experiment duration was settled in two minutes when the roller is stopped. The test was twice repeated within 60 min intervals, expressing as average time for statistical comparisons.

VI.2.5 Gene expression analysis

Thirty micrograms of whole brain tissue from each experimental animal were disrupted and homogenized in 700 µL QIAzol® Lysis Reagent in a Tissue Lyser II. Total RNA was then isolated using the RNA kits (Thermofisher, MA, USA), according to the manufacturer's protocol. During the purification process, contaminating

genomic DNA was removed by DNase digestion. The purity and concentration of total RNA samples were assessed using the Qubit (Thermo Scientific, Wilmington, USA).

Reverse Transcription-PCR and Real-Time Quantitative PCR

Total RNA (750 ng), was reverse-transcribed to cDNA by the GoScript™ Reverse Transcriptase kit according to the manufacturer's protocol. Real-time PCRs were carried out on the Applied Biosystems Quantstudio 3 Real-Time PCR System (ThermoFisher, Waltham (Massachusetts), USA). Gene expression was calculated by the qBase plus program[194] using actinB (*ACTB)* and beta-2-microtubulin *(B2M*) as housekeeping genes. The following gene-specific primer sets ordered to "Integrated DNA Technologies (IDT)" were used: *PPARGC1A, SIRT3, SOD1, NFKB1, TLR3, TLR4, S100B, CXCR2, CSF3R, HSPA1B, SELL, TNFA, NRF1, GABRA1, GABRA3, GABRA4, GABRA5, GABRG2, GABBR1, OPRM1, OPRD1, OPRL1, DRD1, DRD3, DRD4, CHRM2, CHRNA1, HTR1A, HTR2A, ATP2A1, CLCN1, BDNF, CREB1, CASP8 and CASP9*. The experiment was carried out in duplicate in three independent biological replicates. The thermal cycling conditions included initial denaturation at 95°C for 3 min and subsequent 40 cycles of two-step protocol: denaturation at 95°C for 15s and annealing/extension at 60°C for 60 s.

VI.2.6 Statistical analysis

Data analysis was performed with GraphPad Prism® software (version 8.0, La Jolla California, United States) and the results are expressed as mean ± standard error of the mean (SEM). Data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene's test) before being processed with the nonparametric Kruskal-Wallis test for the open-field test. For the Rotarod result, a one-way ANOVA followed by multiple comparison analysis (Tukey) was used. For the Kruskal-Wallis non-parametric test was also used to analyze the gene expression data. Relative gene expression data were transformed to their log2 values using the Python-based library Pandas. The z-scores calculation, hierachical clustering and heat maps generation were performed using the Bioinfokit v2.1.0 data analysis and visulatization toolkit (http://doi.org/10.5281/zenodo.3698145). In all cases, a $P \leq 0.05$ was taken as the level of significance.

VI.3.1 Cell viability assay

The cell lines PC12 and BV2 were used to evaluate potential harmful cell damage associated with the different solvent types and the compunds obtenied of *P. neochilus* leave extracts. Results shown in Table VI.1 indicate that when water is used as solvent, no matter the state of the raw material (fresh or dried), *P. neochilus* extracts did not induce cytotoxicity in any of the cell lines at the maximum concentration tested (512 µg/ml). On the contrary, the ethanol extract induces cytotoxicity in the cell lines tested, for PC12 at CC_{50} >64µg/ml and for BV2 the cytotoxicity was increased with CC_{50} >32µg/ml after 24 hours of exposure. Based on those results, it can be inferred that aqueous extracts (as is usually consumed by the Cuban population) are not toxic for the cell lines under study, and in this way, offering the first evidence of their safety profile.

VI.3.2 *In vivo* behavioural and motor activity assays

The results of the observational assessment for a single dose of the experimental extracts are shown in Figure VI.1. No statistical differences were observed in the general exploratory behaviour between the control group and the three experimental extracts in none of the variables explored; even when, for some variables (especially in grooming behaviour and faecal pellet number (FPN), lower values were registered. At the same time, test groups differ from the diazepam group, in which all variables monitored were significantly decreased in relation to the experimental and control groups. Nevertheless, in some of the variables monitored, test extracts showed lower values than those obtained for the control group, particularly in the experimental group III (FLD), which most closely resembles the traditional extract used by Cuban inhabitants. Accordingly, it can be stated that *P. neochilus*extracts barely modified the "anxiety" behaviour in the mice.

The effect obtained in the evaluation of the Rotarod method after acute administration of FLD, DLW, and DLE extracts of *P. neochilus* are shown in Figure VI.2. As can be noticed, FLD experimental extract had a significant effect ($p < 0.0029$) on motor coordination and grip strength on the rotating rod with respect to the control group, compared with the effect obtained in the diazepam group. The other two experimental extracts did not show statistically significant differences from the control group, but lower values in latency of fall and a different behaviour

Table VI.1. Cytotoxicity expressed as CC_{50} values of *P. neochilus* extracts.

Cell lines/Extract	FLD (µg/mL)	DLW (µg/mL)	DLE (µg/mL)
PC12	>512	>512	>64
BV2	>512	>512	>32

FLD- Fresh Leaves Decoction, DLW- Dried Leaves Water, DLE- Dried Leaves Ethanol

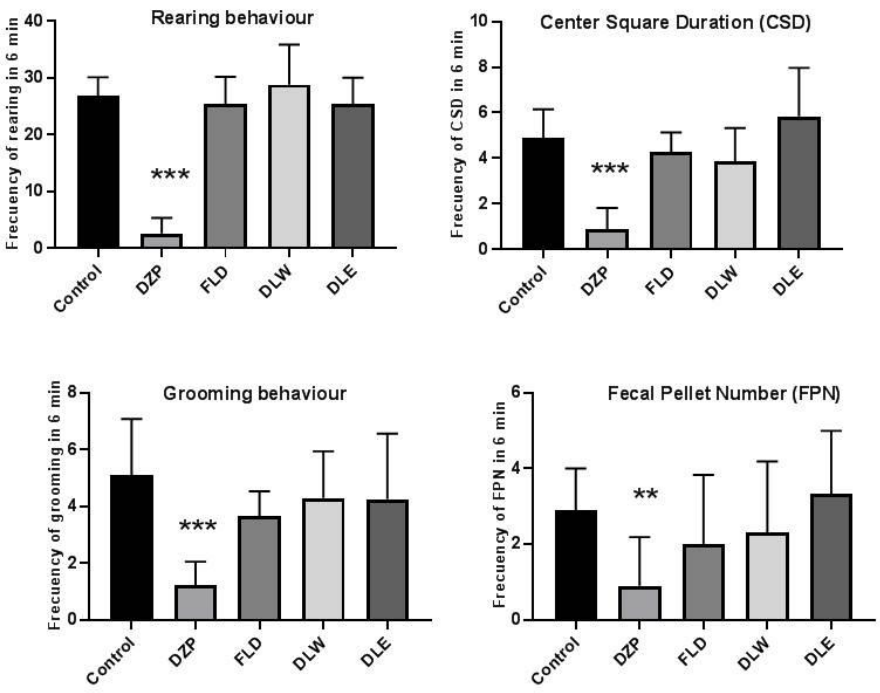


Figure VI.1. Effect observed after acute oral administration of *P. neochilus* extracts (500 mg/kg) on OF1 mice (n=10 per group) in the Open Field Test. All variables expressed the lump values obtained after 1 hour extracts administration and blind observation using a manual-digital stopwatch. Results are expressed as mean ± SEM after a Kruskal Wallis tests used with *P < 0.05, **P <0.01 and ***P <0.005.

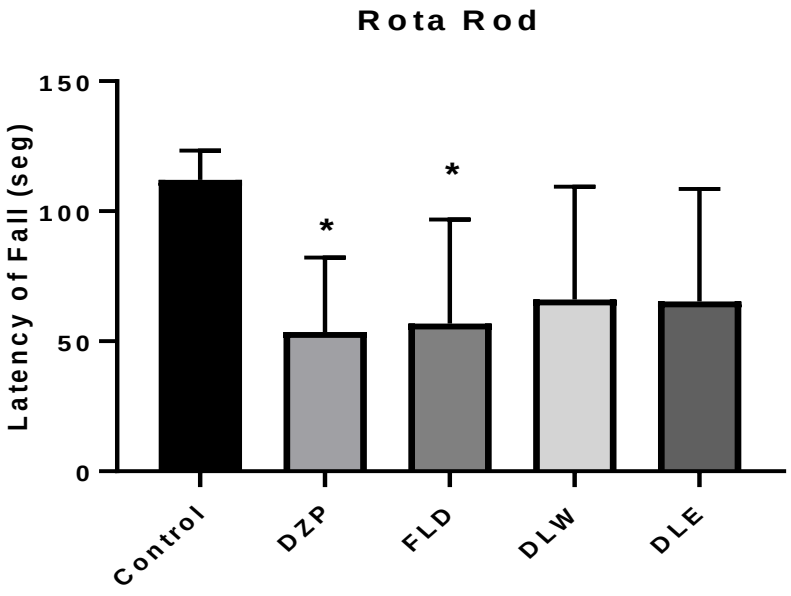


Figure VI.2. Effect observed after acute oral administration of P. *neochilus* extracts (500 mg/kg) on OF1 mice (n=10 per group) in the Rotarod test. Latency of fall expressed the average time in seconds (using a manual-digital stopwatch) that mice remain gripped to the rotor after one hour extracts administration. Results are expressed as mean ± SEM after a Kruskal Wallis tests used with *P < 0.05.

in muscle tone (as contracted) was observed. In general, all *P. neochilus* extracts presented numerical values of the monitored variable closer to the diazepam group than to the control group. These results highlight an appreciable muscle-relaxing property of *P. neochilus* extracts, mainly when fresh leaves are used.

VI.3.3 Gene expression

To further characterize a possible molecular mechanism of action involved in the behavioural *in vivo* effects of *P. neochilus* extracts on mice, whole brain samples from all five experimental groups, were studied to evaluate transcriptional expression changes of 35 genes of interest (Table VI.2). Of these 35 genes, three genes were not expressed and did not exceed the RT-qPCR background noise signal (*CREB1, CASP8* and *CASP9*).

The results on the gene expression show that in general, different types of *P. neochilus* extracts generate significant changes in the level of expression of various selected genes as can be appreciated in the heatmap generated (see Figure VI.3). Various GABA receptors (*GABRA5, GABRG2*) as well as *gapdh* decreased expression when compared to the control and diazepam group, whereas *nrf1* expression was similarly repressed by diazepam and the *P. neochilus* extracts (Figure VI.4). On the contrary, the genes encoding for the dopaminergic receptors: *DRD1, DRD3*, were upregulated compared to the control and Diazepam groups, whereas DLE (and to a less extent FLD) also revealed increased expression of *OPRM1* and *OPRL1* opioid receptors in comparison to the control group (Figure VI.5). To identify the degree of similarity and/or difference in gene expression changes between the treatments, individual cluster analyses (for each experimental group) was performed with the control group (untreated mice), whereas *P. neochilus* extracts FLD group was defined as the reference group because it represents the closest approximation to the type of extract prepared and consumed by Cuban inhabitants. Figure VI.6(a, b, c, d) illustrates gene cluster analyses based on the relative expression changes ($\Delta\Delta$Ct) of all individual genes between FLD, DLW, DLE and DZP treatment versus the control group. Under this premise, those genes clustered at both ends of the cluster dendrogram reveal the most under or overexpressed gene as compared to the control group which may play a critical role in the molecular mechanism associated with the mice behaviour observed in the *in vivo* experiment.

As can be seen on the left side of Figure VI.6a, the gene *NRF1* appears alone in the first cluster, while *GABRA5*, *GAPDH*, *TLR4*, and *GABRG2* appear together in a second cluster asthe most repressed genes with respect to the control group. On the other side of the Figure (right side), the cluster formed by the three opioid receptors explored (*OPRL1*, *OPRM1* and *OPRD1*) stands out; as well as the the *CXCR2* and *DRD1* gene cluster. Those five genes plus *DRD3* (which is part of the next group), are the only ones that are expressed twice or more compared to the control group (see supplementary data Table VI.1).

On the left side of Figures VI.6b (DLW) and VI.6c (DLE) inhibit similar gene clusters as the FLD extract. However, on the right side, significant differences can be appreciated for the upregulated genes. While for FLD experimental group six genes appeared two-fold or more overexpressed, for the experimental groups DLW and DLE only *DRD1* and *DRD1* plus *OPRL1* genes appear twice or more overexpressed (Table VI.2). Another difference detected in the gene cluster pattern by these extracts produced from the dry leaves of the species, is that with exception of DLE, no significant changes are observed for the opioid genes, and the expression of cholinergic genes (see Figure VI.6c).

Finally, Figure VI.6d illustrates the unique cluster dendogram following diazepam treatment, distinct from the *P. neochilus* groups (Figures VI.6a, VI.6b, and VI.6c), with exception of *NRF1* and *TLR4* genes which are similarly under-expressed. At last, Figure VI.6e combines all three *P. neochilus* extracts in one cluster analysis to illustrate that despite the similarities and divergences in the gene expression pattern between experimental groups, the FLD-induced cluster dendogram pattern dominates.

Table VI.2. Gene cluster distribution for *P. neochilus* extracts and diazepam experimental groups. Gene expression values compared to the control group.

Cluster number and gene distribution for FLE extract/ (degree of gene down or up-regulation regarding control group)																	
1	**NRF1** (-53.842)																
2	**GABRA5** (-13.443)	**TLR4** (-6.834)	**GAPDH** (-6.044)	**GABRG2** (-4.618)													
3	**GABBR1** (-2.889)	**GABRA1** (-2.166)	**GABRA4** (-3.243)	**TNF** (-1.617)													
4	**SELL** (-0.955)	**BNDF** (-0.998)	**CSF3R** (-1.176)	**ATP2A1** (-0.727)	**NFKB1** (-0.615)												
5	**HTR2A** (-1.018)	**S100B** (-0.372)	**SOD1** (-0.523)	**SIRT3** (-0.146)	**TLR3** (-0.110)	**CREB1** (0.000)	**CASP8** (0.000)	**CASP9** (0.000)	**CHRNA1** (-0.123)	**HSPA1B** (0.237)	**CHRM1** (0.063)	**HTR1A** (0.022)					
6	**CLCN1** (-0.147)	**PPARGC1A** (-0.383)	**DRD4** (0.670)	**DRD3** (0.618)													
7	**DRD1** (1.238)	**CXCR2** (1.873)															
8	**OPRD1** (2.199)	**OPRM1** (2.844)	**OPRL1** (3.367)														
Cluster number and gene distribution for DLW extract/ (degree of gene down or up-regulation regarding control group)																	
1	**NRF1** (-53.884)																
2	**GABRA5** (-13.451)																
3	**TLR4** (-6.837)	**GAPDH** (-6.006)	**GABRG2** (-4.649)														
4	**GABRA1** (-2.290)	**GABRA4** (-3.298)															
5	**SOD1** (-1.166)	**CSF3R** (-1.379)	**GABBR1** (-1.506)	**TNF** (-1.049)	**BNDF** (-0.959)	**CLCN1** (-1.126)	**ATP2A1** (-0.668)	**S100B** (-0.451)	**OPRD1** (-0.476)	**TLR3** (-0.171)	**SIRT3** (-0.159)	**HTR1A** (0.429)	**DRD4** (-0.201)	**SELL** (0.022)	**CREB1** (0.000)	**CASP8** (0.000)	**CASP9** (0.000)
6	**PPARGC1A** (0.176)	**CHRM1** (0.257)	**NFKB1** (0.185)	**HSPA1B** (0.201)	**OPRM1** (0.329)	**CHRNA1** (0.057)	**HTR2A** (0.897)	**OPRL1** (1.065)									
7	**CXCR2** (1.971)	**DRD3** (2.665)															
8	**DRD1** (8.5987)																

Cluster number and gene distribution for DLE extract/ (degree of gene down or up-regulation regarding control group)

1	**NRF1** (-53.870)																
2	**GABRA5** (-13.492)																
3	**TLR4** (-6.054)	**GAPDH** (-6.717)	**GABRG2** (-4.621)	**GABBR1** (-3.257)													
4	**GABRA4** (-2.923)	**GABRA1** (-2.026)	**CSF3R** (-1.556)	**PPARGC1A** (-2.052)													
5	**CLCN1** (-1.333)	**TNF** (-1.162)	**BNDF** (-0.883)	**HTR1A** (-0.913)	**ATP2A1** (-0.570)	**SOD1** (-0.710)	**S100B** (-0.505)	**HTR2A** (-0.151)	**TLR3** (-0.365)	**SELL** (-0.357)	**NFKB1** (-0.289)	**SIRT3** (-0.245)	**DRD4** (0.229)	**CREB1** (0.000)	**CASP8** (0.000)	**CASP9** (0.000)	
6	**HSPA1B** (0.243)	**OPRM1** (0.528)	**OPRD1** (1.526)														
7	**CHRM1** (1.967)	**CHRNA1** (2.286)	**CXCR2** (1.849)	**DRD3** (2.186)													
8	**OPRL1** (8.978)	**DRD1** (12.158)															

Cluster number and gene distribution for DZP group/ (degree of gene down or up-regulation regarding control group)

1	**NRF1** (-53.417)																
2	**TLR4** (-5.176)																
3	**DRD1** (-0.428)	**CSF3R** (-0.749)	**DRD4** (-0.382)														
4	**OPRD1** (-0.458)	**BNDF** (-0.387)	**HTR2A** (-0.382)	**ATP2A1** (-0.010)	**CXCR2** (-0.024)	**TLR3** (0.387)	**CASP8** (0.002)	**CASP9** (0.001)	**DRD3** (0.025)								
5	**CHRNA1** (0.141)	**TNF** (1.499)	**SIRT3** (0.863)	**PPARGC1A** (0.722)	**GABRA5** (1.522)	**HTR1A** (0.428)	**GABRG2** (2.248)	**CLCN1** (1.090)	**GABRA1** (1.130)								
6	**GABBR1** (1.916)	**HSPA1B** (0.807)	**SOD1** (0.406)	**SELL** (1.192)													
7	**NFKB1** (1.290)	**CHRM1** (0.898)	**S100B** (1.568)	**CREB1** (0.062)	**OPRM1** (1.615)	**GABRA4** (4.297)	**GAPDH** (5.223)										
8	**OPRL1** (2.518)																

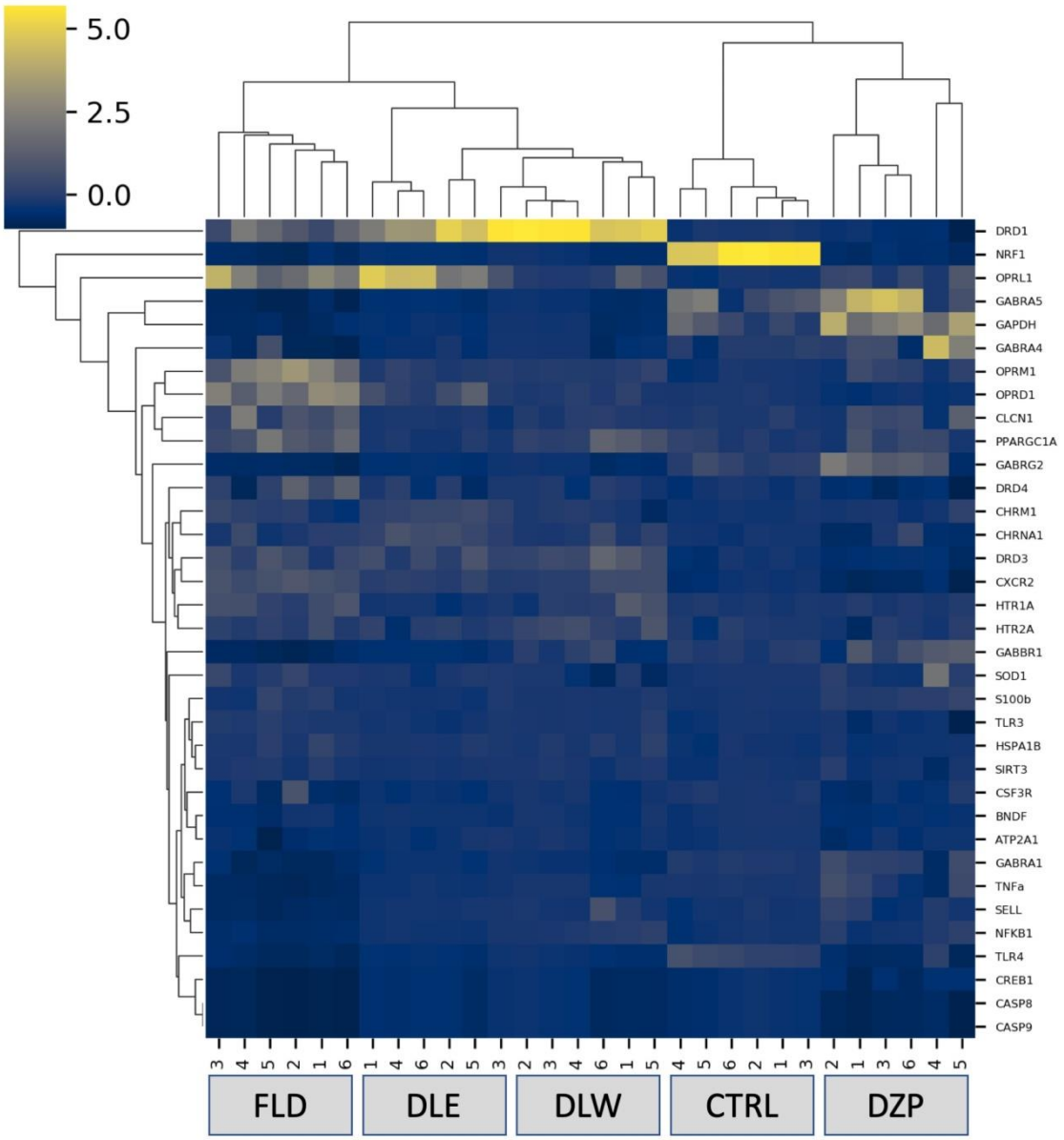


Figure VI.3. Heatmap representation of hierarchical clustering of log2 transformed gene expression values of 35 genes, represented as Z-scores for the different treatment groups.

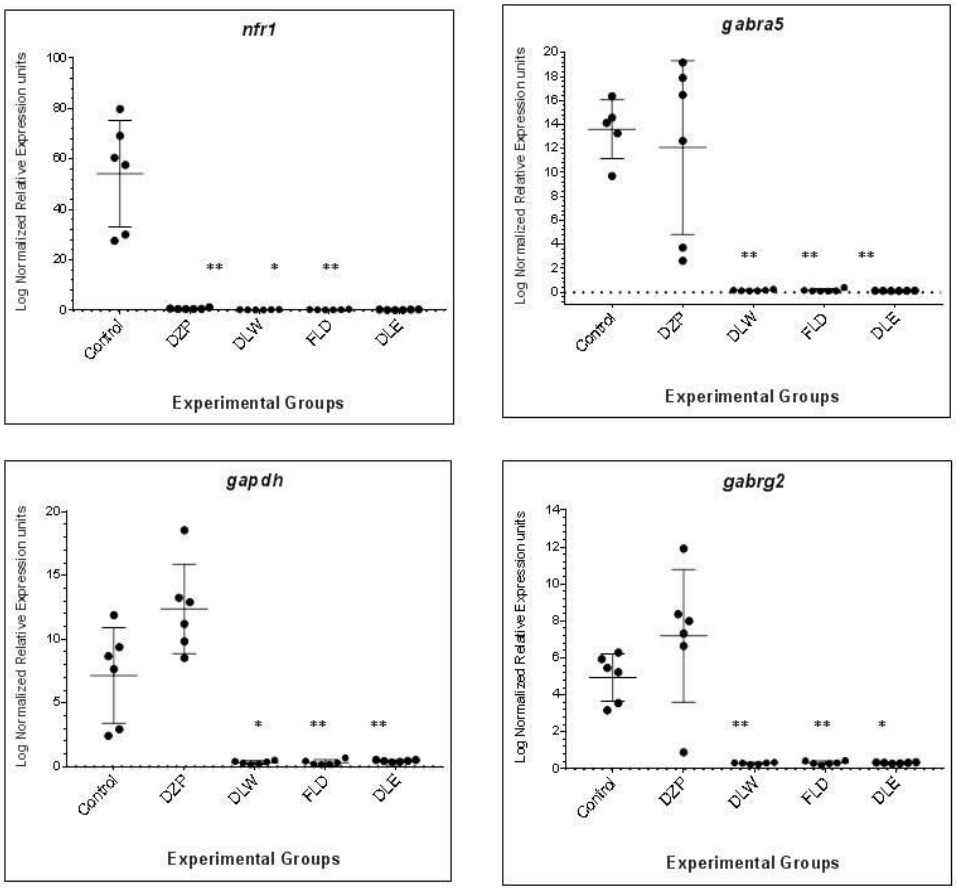


Figure VI.4. Significantly inhibited genes in OF1 mice brain samples after acute oral administration of *P. neochilus* extracts (500 mg/Kg). Statistical significance using Kruskal Wallis tests with *P < 0.05, **P <0.01 and ***P <0.005.

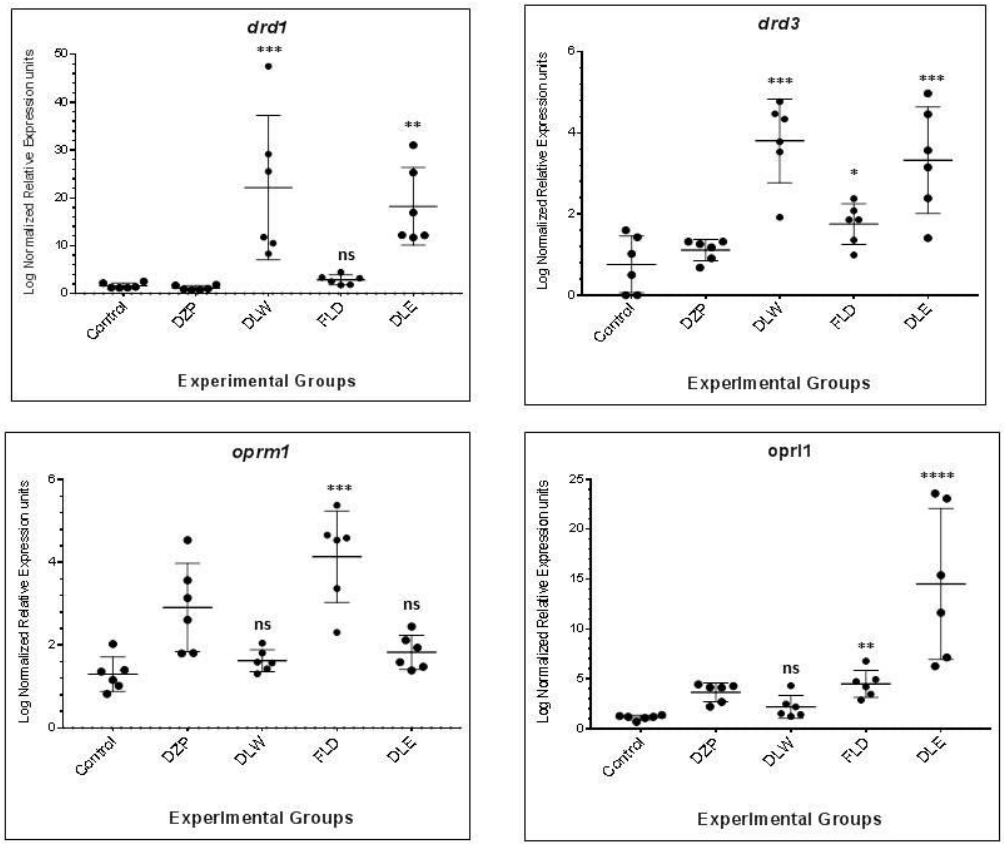


Figure VI.5. Significantly activated genes in OF1 mice brain samples after acute oral administration of *P. neochilus* extracts (500 mg/kg). Statistical significance using Kruskal Wallis tests with *P < 0.05, **P <0.01 and ***P <0.005.

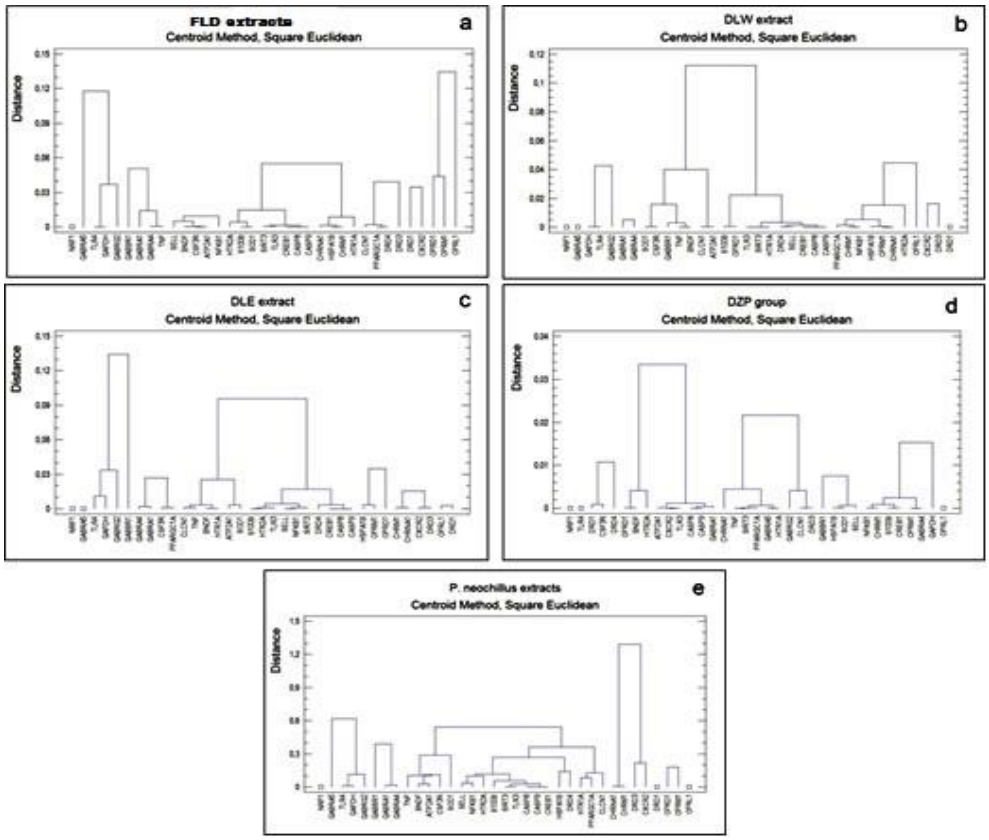


Figure VI.6. Cluster analysis grouping the expression behavior of the 35 genes explored in relation with the untreated group. Figures **a**, **b**, **c,** and **d** represents the difference between the ΔΔCt of each explored gene and the ΔΔCt calculated for the control group in FLE, DLW, DLE and DZP groups respectively. Figure 3**e** represents the same cluster analysis but considering the three *P. neochilus* extracts in a single cluster.

VI.4. Discussion

In Cuba, *P. neochilus* is traditionally more frequently used as sedative, hypnotic, analgesic and muscle relaxant than as anti-microbial agent (most reported use). To further validate scientific evidence for central nervous effects of *P. neochilus* grown in Cuba, this paper aimed to explore the effect of different plant extract formulations on animal behaviour as well as on gene expression of brain function related genes. Firstly, the cytotoxicity of FLD, DLW and DLE extracts in PC12 and BV2 cell lines was evaluated, showing no cytotoxic effect for the aqueous extracts. On the contrary, the ethanol extract (DLE) showed a high cytotoxicity for both cell lines tested. Moreover, upon previous phytochemical characterisation of the three explored extracts (FLD, DLW, DLE), seven abietane diterpenoids were identified at higher concentrations in the ethanol extract (DLE). Those non-polar abietane diterpenoid compounds, extracted with preference in low/medium polar solvents such as ethanol, could be the cause of such differences in the cytotoxic profile. Recent papers have already shown cytotoxicity of various abietane diterpenoids isolated from *Plectranthus* ssp. in several cell lines[146,195]. In a similar approach, the cytotoxic profile of *P. neochilus* was correlated with the solvent polarity in an experimental study using water, methanol, and acetone as solvents, being the acetone extract the most active/toxic[110] .

Watery extracts (as FLD, and DLW), with low quantities of abietane diterpenoids but high levels of polyphenol compounds, might offer a cyto-protection through membrane stabilization and/or by its antioxidant properties[196]. For example rosmarinic acid, the most abundant polyphenol in the tested extracts, has been described as a good neuroprotector by inhibiting ROS formation, lipid peroxidation, and tau protein hyperphosphorylation on PC12 cells[197].

The behavioural and motoric studies showed that mainly the FLD extract induces a moderate decrease in the times of mice fall (expressed in seconds), showing similar behaviour to the positive control group (DZP). To the best of our knowledge, no previous studies refer to this effect of *P. neochilus.* Nevertheless, for other species of the genus, also strong activity on CNS has been informed, such as for *P. amboinicus* leaves extracts in rats[198] and in mice at doses of 500 and 750 mg/kg[191]inducing a remarkable anxiolytic effect and an appreciable decrease in the animal locomotion

activity[199,200]. The fresh leaves essential oil of *P aegyptiacus* also showed a significant central nervous system inhibition activity at doses of 150 mg/kg in the novelty-induced behavioural test (NIB)[201].

Furthermore, gene expression results demonstrated that in general, *P. neochilus* but especially FLD extract generate significant changes in the level of expression of most of the genes explored. All GABA receptors (*GABRA5*, *GABRG2*, *GABBR1*, *GABRA1*, and *GABRA4*) appear downregulated (in this order) when compared to the control group, but especially with the diazepam group. On the contrary, the genes encoding for the dopaminergic receptors, *DRD1*, *DRD3*, and *DRD4*, as well as opioid receptors, appear overexpressed in comparison with the control group. Considering the role of dopamine system in the "fight or flight" behaviour, the overexpression of dopamine genes is surprising in view of the sedative and muscle relaxing profile informed for *P. neochilus* extracts. Dopamine receptors consist of two main families D1 (including *DRD1* and *DRD5* receptors) and D2 (including *DRD2*, *DRD3*, and *DRD4* receptors). Both families are coupled to a G-protein that activate (D1 family) or inhibit (D2 family) the adenylate cyclase. In consequence, one should expect that an increase or decrease level of cAMP generates an antidepressant-like or depressant-like response through a D1 or D2 family receptor activation[202]. Drugs like quinpirole have shown the so-called D-2/D-3 agonist biphasic effect in which at low doses decreases the levels of locomotion, and movement and at high doses increases those behaviour parameters[203].

This biphasic effect has been explained by two ways: the increment in the production of endocannabinoids in the dorsal striatum after D-2/D-3 receptor stimulation, and/or the anatomic location of these expressed D-2/D-3 receptors. Evidence on the first hypothesis sustains that after the endocannabinoid stimulated production by D-2/D-3 receptor activation, a trans-synaptic endocannabinoid inhibitory feedback neutralizes the dopamine-induced facilitation of motor activity[204]. The second hypothesis states that activation of the presynaptic dopamine D2/D3 receptor produces a marked decrease in motor activity by inhibiting dopamine release in the basal ganglia, but the stimulation of post-synaptic dopamine D2/D3 receptors produces enhanced locomotion and characteristic stereotyped behaviours (including jumping, climbing and oral movements[205].

On the other hand, the presence of a D1-D3 receptor heteromer that mediates locomotors activity in rodents, allows a simultaneous interaction between D1R (adenylate cyclase activator) and D3R (adenylate cyclase inhibitor) agonists[206]. In this way, in this heteromer, a mimetic action on D3R increases the affinity of D1R ligands triggering the strongest dopaminergic response mediated by the D1 receptor[207]. This agonists behaviour has been described as "biased signalling" or "functional selectivity" that encompasses the ability of the ligand to selectively activate or block a G protein-dependent or independent signalling, emerging as one of the main challenges in G protein-coupled receptor pharmacology[208]. Nowadays, it is accepted that activation/inactivation of D1 and D2 might reciprocally modulate each other[209].

In this context, all three *P. neochilus* extracts up-regulate *DRD1* and *DRD3* receptor with statistical significance when compared to the control and diazepam groups. As shown in Figure VI.5 and Table VI.2, the *DRD1*/ *DRD3* expression level ratio is higher in the dried leaves extracts (DLW, and DLE, favouring *DRD1* expression). This might explain small variations in behavioural anxiety effects in the Open Field Test (Figure VI.1). A dopaminergic mechanism of action in the reduction of anxiety is not new for plant pharmaceutical derivates, as occur with *Galphimia glauca* Cav., which is a medicinal plant used for treating anxiety and sleep disorders[210].

The interaction between sub-units of the same type of receptor is not the only approach to understand the functionality of the central nervous system moving away from the concepts of the classical pharmacology. At the present times, there is a plethora of evidences proving that receptor-receptor interactions can also altered receptor recognition, pharmacology and signalling[211]. Most of the studies target dopamine heterodimers with GABA and opioid receptors. Evidence has shown that D2-like agonists decrease the activity of the striate pallidal neurons and reduce striatal GABA release, with a resultant non-expression of the GABAergic receptors[212]. This receptor-receptor co-activation has been described also for the three main subclasses of the opioid (μ, δ and κ) receptors. Studies on biomodels, have demonstrated that dopamine modulates nociception in various regions of the central nervous system[213], while immunohistochemistry experiments confirmed a

co-localization in form of hetero-oligomers of μ-receptors with D1-receptors in neurons of the cortex and caudate nucleus[214].

Also, Yoshida et al., stated an increase of dopamine release after fentanyl administration as consequence of the simultaneous activation of μ and δ2 opioid receptors[215]. This multiple association between dopamine/opioid/GABA systems has been described for nociception process in rats[213]. Although the *in vivo* study did not prove the antinociceptive effects of *P. neochilus* extracts, the analgesic effect ranked third among the most common ethnobotanical uses reported in previous studies in Cuba. What has been described above is consistent with the effect induced on gene expression by *P. neochilus* extracts, therefore, it could be considered as a possible mechanism of action.

With respect to the muscle contraction process (main effect observed during the*in vivo* experiments, Figure VI.2), energy metabolism is pivotal whereasinhibition of cell respiration might promote muscle relaxation. In fact, NRF1 gene (nuclear respiratory factor that regulates key metabolic genes, mitochondrial respiration, energy metabolism, cellular growth and DNA replication) appears in all clusters in the last left branch as the gene with the lowest gene expression (Figure VI.6a-c). Along the same way, GAPDH gene expression involved in carbohydrate energy metabolism[216], is also decreased. The depletion of cellular energy resources might contribute to the muscle relaxation process observed *in vivo* and scientifically validate possible muscle relaxing effects of *P. neochilus* extracts reported by the Cuban population.

Finally, inflammatory activity can also modulate stress, sleeping disorders and depression[217]. Reciprocally, the anti-inflammatory effects observed such as inhibition of TLR4, TNFa, NFKB, SOD1, CSF3R and BNDF gene expression may also contribute in alleviation of sleep disorders and inflammatory diseases, in line with the traditional use of the plant in Cuba.

Of particular interest, Rosmarinic acid, one of the main compounds identified in *P. neochilus* extracts, could mediate most of the mentioned molecular mechanisms. Its two catechol groups behave as good antioxidant moieties which may reduce the production of proinflammatory cytokines and microglia activity inhibition[218]. Rosmarinic acid also proves to be an excellent neuroprotectant, modulating the

neuronal apoptosis after an ischemic stroke by an antioxidant defence mechanism[219]. Furthermore, according to Hase et al., pre-treatment with rosmarinic acid restored the function of complex I in the mitochondrial respiratory reaction, and activated the dopamine (DA) signalling pathway by preventing the expression of monoamine oxidase (MAO) B enzyme[220]. Both, mitochondrial respiration and dopamine activation are also plausible mechanisms of action for *P. neochilus* extracts in line with our experimental results.

The presence of other polyphenols in the *P. neochilus* extracts such as flavonol type derivatives could also be linked to CNS activity as reported by the Cuban population, for other medicinal plants. Chamomile flowers (*Matricaria recutica* L.) have been used for centuries for their calming effect attributed to the flavone apigenin. Likewise, linden flowers (*Tilia* sp.) are recognized as tranquilizers, due to the presence of quercetin and kaempferol[221]. It is also well accepted that quercetin (aglycone of some of the flavonoids identified in *P. neochilus* extracts improves impaired mitochondrial biogenesis and mitochondrial function through regulation of the SIRT1/PPARGC1A/NRF1 pathway and inhibition of inflammation through the TLR4/NF-κB pathway[222].

Flavonoid specificinhibition of MAO-A, MAO-B or both, may contribute to protection of neurons against neuroinflammation, activation of synaptic signalling and improvement of cerebrovascular blood flow[223]. Their involvement in Ca^{2+} pathways and phosphorylation/dephosphorylation reactions in 3H-methyl-4-phenylpyridine uptake by human neuronal dopaminergic cells has been suggested as an alternative mechanism of action against depression, bipolar disorder and attention deficit hyperactivity disorder, caused by abnormal dopamine levels. Flavonoids have also demonstrated their ability to act on ion channels and membrane conductance, activating Ca^{2+} release[224].

Abietane diterpenes is another family of compounds we recently identified in *P. neochilus*. Sofar, there are only few reports linking these compounds to anti-inflammatory activity, but rosmarol, a phenolic diterpene isolated from *Rosmarinus officinalis* L., is known for its strong antioxidant and anti-inflammatory activity by down-regulating the gene expression of the inflammatory enzymes iNOS and COX-2 and inhibiting the NF-kappaB activation[225]. In addition, rosmarol and carnosol

(another phenolic diterpene) synergistically alleviate rheumatoid arthritis by inhibiting the *TLR4*/*NF-κB*/HMGB1pathway[226].

Altogether, our study confirms that consumption of fresh leaf extract (FLE) of *P. neochilus*, prepared according to the Cuban ethnobotanic tradition, has significant *in vivo* effect on mice motor coordination behaviour. At the biochemical level, inducible gene expression changes of key genes regulating central nervous system neurotransmitter signaling, as well as energy-metabolism and inflammation may contribute to the reported sedative effects and improvement of sleep quality.

VI.5. Partial conclusions

The obtainedresults show that *P. neochilus* extracts, and FLD in more particular, modify the motor coordination behaviour in the studied mice, which scientifically validates the ethnomedicinal use of this plant for sedative purposes in the Cuban population. Upon further gene expression analysis of the corresponding brain tissue, we identified a preliminar mode of action via activation of the Dopamine/Opioid system and concomitant inhibition of GABAergic, inflammation and energy metabolic signalling pathways. Further studies are needed to evaluate whether recently identified phytochemicals present in *P. neochilus* FLD extract such as rosmarinic acid and some flavone derivatives are the main bioactives responsible for the observed central nervous effects *in vivo.*

CHAPTER VII

CHAPTER VII. General Discussion

The Cuban population has a long tradition in the use of medicinal plants as an alternative therapy to cure and/or alleviate different ailments. This tradition has been inherited from generation to generation in such a way that it is currently part of a "national program" for Primary Health Care in Cuba. The use of phytotherapics opens up a range of opportunities for the economic development of the country and makes up for the deficiencies of conventional medicines.

Lamiaceae, is one of the well-known botanical families and is characterized by having aromatic species that are used as condiments, in the cosmetic industry, and mainly for their medicinal properties. *P. neochilus* species belongs to this family, being native to southern Africa. Its cultivation has become naturalized in South America, mainly in Brazil. It is traditionally used to treat skin conditions, digestive disorders and to relieve the symptoms of respiratory diseases. In 2016 its presence was reported in Cuba and its cultivation has spread throughout the national territory due to the sedative properties[18] attributed by the Cuban population.

Taking into account the different medicinal usage reported for the species by the South African and Brazilian versus the Cuban people respectively, it was decided to setup a systematic bibliographic review and to characterize the ethnobotanical use of the species in Cuba.

The study was divided into two parts, the first related to a bibliometric study and the second to an ethnobotanical study. As a result of the bibliometric study, in total 87 manuscripts were found, 32 of them with an ethnobotanical approach and 55 of a chemical/pharmacological nature. For Cuba, only five hits were found: a first report in 2016 announced the expansion of the species within the island, associated with its ornamental and sedative uses[18], while two other follow up papers ratified the ethnomedicinal use to treat anxiety, depression, and sleep deprivation[19,147]. The most recent 2 hits were published results derived from the current PhD research.

The ethnobotanical study performed confirms that the species *P. neochilus* is popularly known by the common name "Meprobamate" throughout Cuba, attributing mainly sedative, hypnotic, analgesic and anti-inflammatory effects. It is generally consumed as a decoction from the fresh leaves and stems of the plant, and a cup of it is drunk at night approximately 30 minutes before going to bed.

The study also reported that the species was well known by the population over 38 years old, predominantly by women. The study evidenced the prevalence of the university level of education, with the majority of informants being formal workers. Around 500 people who participated in the study declared to have made a concomitant use of medications on at least one occasion, combining natural and synthetic medications. The most frequent practice included the group of benzodiazepines. The participants reported that the reason for the combination of drugs was to achieve a higher threshold of pharmacological activity, highlighting the central region as the one with the highest frequency in attending this practice.

Adverse reactions were reported by consumers of the species, despite the fact that the general population tends to consider natural medicines to be non-toxic. The most common reactions were vomiting, headaches, bradypsychia, dry mouth in the morning, collywobbles and loss of orientation.

Of the 27 traditional healers and 106 health workers who participated in the study, only nine of the interviewees knew or had read about the proven pharmacological properties and the existence of references on the toxicity of the plant.

Taking into account these last two results results (possible side effects and poor knowledge from sanitary personal), and the extensive use of the plant; it is important to suggest training actions from the academia and health authorities. These actions should mainly target doctors specialised in natural and traditional medicine, integrative medicine, family doctors among other categories, so that they can provide validated information on the use of the plant. The design of publicity campaigns through the mass media and the internet, the distribution of printed information material, and interventions in communities and primary schools may be actions to follow in order to disseminate specialized information on the species. These information and education actions for the population should address issues related to ethnomedicinal uses (attributed and proven), the form of preparation, quantity to be consumed, schedules, and adverse reactions, contributing in this way to the rational use of the species. There is also a need to establish phytovigilance strategies between health authorities, family doctors' offices and academia.

Despite the important information obtained, the ethnobotanical study of *P. neochilus* should be deepened, extending it to other communities throughout the country, in

order to increase the size of the sample and to be able to compile as much traditional information as possible on this plant species in the country.

Chapter V was addressed into the determination of the quality parameters of dry and fresh plant material, as well as its derived extracts, which due to its recent presence on the island, lack (to the best of our knowledge) to previous scientific information for the species that grows in Cuba.

The macroscopic parameters determined revealed that there were no phenotypic differences throughout the year of study for the four lots collected. The cultivated species was characterized by having a grey-green colour, with succulent leaves, and many pubescent hairs on both foliar surfaces. The leaves have wrinkled edges, perinervical venation, wedge-shaped base petiole, and a strong and characteristic aromatic odour. These distinctive characteristics of its leaves are in coincidence with those reported for the species growing in Brazil[103,111] and South Africa[11,12,109]

Micromorphological determinations showed that both leaf surfaces are pubescent, but no glandular trichomes were observed. Despite the strong and characteristic aromatic odor, it was not possible to obtain quantifiable values of essential oils by the hydrodistillation method. This condition could be related to the absence of glandular trichomes, substructures that play an important role in the production and storage of essential oils[227. The absence of glandular trichomes could be a consequence of the naturalization/adaptative process of the species in Cuba, since this species is typical of temperate climates and grows on the island in high temperature conditions.

Recent studies have documented that the structure and chemical constituents of trichomes can change due to the particular light regime, water stress, and salinity. Therefore, trichomes represent dynamic protective structures that can be affected due to plant-environment interactions[228]. This hypothesis has also been put forward by several authors proposing that the changes in trichome types and/or their absence are a micromorphological expression conditioned by natural selection pressures[227,229].

Non-glandular trichomes protect plant organs against multiple biotic and abiotic stresses. The protective and defensive functions of these epidermal appendages are crucial for developing organs and can be attributed to the excellent combination of

appropriate structural features and chemical reinforcement in the form of phenolic compounds, mainly flavonoids. Both trichome formation and the accumulation of phenolic compounds are interrelated at the molecular level[228,230].

On the other hand, an indirect defence mechanism against environmental adversities is highly evolved in the Lamiaceae family, related to the presence of jasmonic acid[231,232], which stimulates the expression of defence genes as well as the accumulation of other secondary metabolites such as diterpenoids[233]. Interestingly, UPLC-DAD-MS/MS analysis revealed the presence of: phenolic compounds such as rosmarinic acid, the presence of flavonoids, phytohormones (jasmonic acid) and several abietane diterpenes.

Another indirect measure of its possible adaptation to the dry and arid climate of the island can be seen in the results obtained in the drying process. As a species with succulent leaves and stems, natural drying methods (sun and shade) had to be discarded, since when applied, the samples of *P. neochilus* took more than three weeks to dry. Thus, the risk of microbial contamination was increased, as occurred in the shade dried batches. This resistance to natural drying may be due to the cuticles of the epidermis, which, as described in the micromorphological study, had wax incrustations, which delayed desiccation.

The physical, physical-chemical and chemical quality parameters of the drug determined for the four batches studied showed slightly high total ash values. Total ash values are a measure of the inorganic substances present in the plant, serving as a reference to estimate the amount of salts, sands and metals, affecting their quality if they are very high. The determination of soluble substances pointed to water as the solvent with the highest extractive capacity for the total metabolites of the species. This result could be related to the high polarity of the constituents present in the leaf of the species. No statistically significant differences were observed in any of the parameters between the four batches collected.

Quality control parameters of three extracts derived from the leaves of the species that grow in Cuba were evaluated. One of them was a decoction from fresh leaves in analogy to the traditional methods mentioned in the the surveys collected from the Cuban population and as described in chapter IV. The other two extracts were prepared by maceration from the dry drug using water and ethanol as solvents.

All extracts showed a characteristic odour, while the colour varied according to the state of the drug and the solvent used, further related to the nature of the extracted compounds. The aqueous extract derived from fresh leaves of the species showed a light green colour, the aqueous extract obtained from dried leaves showed a brown colour and the hydroalcoholic extract showed a dark green colour, which may be related to the concentration of photosynthetic pigments after extraction with ethanol. All extracts had the same texture (slightly dense) and their pH values were slightly acidic, in agreement to the weak acidic characteristics of the extracted compounds, such as rosmarinic acid, abietane diterpenes, and flavonoids identified.

Although phytochemical UPLC-DAD-MS/MS profiling of the three extracts revealed qualitative overlap, significant quantitative differences could be observed for each compound in the different extracts. In addition, 18 compounds were identified, of which 15 were reported for the first time in this species. Next, the decoction consumed by the Cuban population was more thoroughly characterized which identified rosmarinic acid as the main compound, a distinctive characteristic for the genus *Plectranthus* and the family Lamiaceae[234].

Remarkably, 15 of the identified compounds showed a high oxidation pattern and classified into two main types of metabolites: flavonoids and abietane diterpenoids. Of the 8 identified flavonoids, 7 have at least six oxygen atoms in their structure and five shows a free or methoxylated catechol function in B-ring, indicating a high oxidation pattern, probably a metabolic reflection of plantwater (draught) stress, heat stress or a combination thereof for species that grow in Cuba[187].

Similarly, for 6 out of 7 abietan diterpenoids a high degree of oxidation can be detected. Once again, it can be hypothesized that the presence of these compounds with a high degree of oxidation could be related to the stress adaptive mechanisms of the new ecosystem in which the species develops during naturalization of the species in Cuba.

It has been described that plants subjected to water stress or the presence of high temperatures reduce their levels of photosynthesis[235,236]. The water stress inhibits plant growth and photosynthesis, it increases reactive oxygen species (ROS), plasma membrane permeability, enzymatic antioxidants, and non-enzymatic antioxidants[237]. These adaptive changes usually manifest themselves in an increase

in the production of phenolic and/or polyhydroxylated compounds to counteract the increased production of reactive oxygen species. With these changes in the chemical structure of the metabolites, a change in the pharmacological profile of the plant can also be expected.

Structural analogues of the chemical compounds reported for the first time for the species growing in Cuba are active on the CNS. The phenolic compound rosmarinic acid identified in the extracts and characteristic for species of the Lamiaceae family has been shown in preclinical studies to be active against a variety of neuropsychiatric disorders such as anxiety[238,239], depression and sleep disorders. Some authors suggest that this effect may be related to its potent antioxidant and anti-inflammatory properties[240].

On the other hand, different authors have reported the presence of this polyphenol for other species of the genus such as *Plectrhanthus*, which have been shown to have sedative-hypnotic effects. Kurman et al., reported the ethnomedicinal use of *P. amboinicus* species to treat different symptoms related to anxiety disorders[241].

The anxiolytic activity of the hydroalcoholic extract of leaves of *P. scutellarioides* was also reported and evaluated in albino rats in preclinical assays. The “ladder model” and the "light and dark chamber model" were the methods used, showing that at the doses of 100 (for the first) and 200 mg/kg (for the second), similar effects to diazepam 2 mg/kg (positive control drug) were achieved [242]. This effect has been also reported for other species of the Lamiaceae family. A recent study on different *Salvia limbata* leaf plantations showed a correlation between the levels rosmarinic acid produced by its leaves and the sedative-hypnotic and anxiolytic effects of its extracts on mice through the “pentobarbital-induced loss of righting reflex test” and the “open-field test” preclinical models[243].

Other compounds identified in extracts derived from *P. neochilus* leaves were flavonoids. These secondary metabolites showed sedative-hypnotic, anxiolytic and antidepressant effects, as well as an improvement in memory capacity in mice. As mechanism of action it was suggested the prevention of antioxidant enzyme impairment and the regulation of serotonergic and cholinergic neurotransmitter pathways[234,244],. In open-field tests, flavonoids such as quercetin exhibited an anxiolytic effect in experimental animals, which may or may not be related to motor

coordination or neuromuscular blockade. This effect may be related to central depressant activity[245].

Recent studies have revealed that flavonoids play a key role as a therapeutic agent in several central nervous system disorders. The most prominent hypotheses on the pathogenesis of anxiety and depression point out quercetin in the context of the search for new therapeutic targets to mediate altered neurotransmitter levels. Depletion of serotonin (5-HT), noradrenaline and dopamine, as systems involved in the dysregulation of physiological arousal and negative emotions, emerge as the focuses. Furthermore, quercetin may be one of the main factors regulating anxiety in the hypothalamic-pituitary-adrenal (HPA) axis, whose function is to respond to chronic stress and the suppression of neurogenesis in the dentate gyrus of the hippocampus[244].

Several pharmacological activities of species belonging to the family Lamiaceae are mainly attributable to their diterpene content. Structural homologues to those identified in extracts derived from *P. neochilus* leaves, but in species of the genus *Salvia*, were shown to be molecular targets of bioactive diterpenes responsible for the treatment of diseases related to the nervous system. Effects on different pathways, including apoptosis signalling, oxidative stress phenomena, beta-amyloid plaque accumulation and tau phosphorylation, have been considered as mechanisms of the anti-Alzheimer's properties of diterpenes. On the other hand, their sedative activity, due to their effect on benzodiazepine and kappa opioid receptors, has been informed. The neuroprotective activity of several Salvia species has been also reported as one of its neuropharmacological properties due to the presence of diterpenes[246].

Another example of the sedative and muscle relaxation activity associated with the presence of abietane diterpenes regards to carnosic acid and seco-abietane derivatives reported for *Salvia leriifolia* Benth. In a preclinal study on mice, these compounds showed a sedative and muscle relaxant activity similar to bromazepam (reference drug)[246]. These compounds are positional isomers to those identified in *P. neochilus* extracts, and may be related to the sedative-hypnotic activity reported by the Cuban population.

In general, the phytochemical characterisation of the extracts derived from the leaves of *P. neochilus* allowed us to suggest which secondary metabolites could be related to the new ethnopharmacological uses reported (sedative and hypnotic). It also allows us to visualise the possible mechanism of pharmacological action, providing a variety of phytochemical markers necessary in the design of further stability and quality parameters studies as a prelude to obtaining a future pharmaceutical form as a final product.

Nevertheless, in order to improve the results showed in this research, quality parameters of the plant drug and extracts derived from *P. neochilus* should be determined for a larger number of batches collected at different geographical areas of Cuba and at different times of the year, with a view to standardisation. The use of other analytical techniques such as NMR to identify the pure compounds isolated from the plant are also welcome. Altogether, efforts should be made to ensure a sustainable source of cultivation of the plant species, which will underpin future research for the development of phytomedicines and their introduction into the Cuban National Health System.

To assess whether the extracts were toxic to the cells under study at the working concentrations, cell viability tests were performed on two cell lines related to the central nervous systems; classifying the aqueous extracts as "non-toxic" in the two cell lines tested. A completely different behaviour was observed in the ethanolic extract, which revealed significant cytotoxicity against all cell lines tested in this study (PC12 >64 μg/mL and BV2 >32 μg/mL).

As reported in chapter IV, the Cuban population uses *P. neochilus* species mainly for its ethnopharmacological benefits, i.e., sedative, hypnotic, analgesic or anti-inflammatory effects following consumption of the aqueous extract obtained from the fresh leaves as a decoction. According to the metabolites detected in chapter V, this effect could be related to the flavonoids and abietan diterpenes identified for the first time for the species although the possible influence of other metabolites that may be present cannot be ruled out. These compounds showed a relative high oxidation pattern which may contribute to a different bioactivity profile and alternative traditional usage by the Cuban population as compared to the generally reported ethnomedicinal use elsewhere in the world.

After analysing the results obtained in chapters IV and V, the neuropharmacological profile of the three extracts was studied to evaluate the effect of the extracts in experimental *in vivo* mouse models, with the aim of verifying the real effect of the extracts without mediating psychological or suggestive factors. In this neuropharmacological study, exploratory behaviour and motor coordination were evaluated in experimental *in vivo* models. The experimental design consisted of 5 groups of 10 male OF1 mice (n=50), using Diazepam as a positive control. Benzodiazepines are reference drugs used to evaluate neuropharmacological effects. It is known that its mechanism of action occurs through the GABAergic pathway, acting as an inhibitory neurotransmitter of the CNS.

As a result of this study, it was shown that the aqueous extract made from the fresh leaves and consumed as a decoction by the Cuban population induces a moderate decrease in the grasping times of the mice's (expressed in seconds), showing a similar behaviour to that of the positive control group treated with diazepam.

As far as we know, there is no documented experimental scientific evidence prior to this research that refers to the experimental evaluation of *P. neochilus* as species as a potential muscle relaxant and sleep inducer. However, this effect has been reported in two other species of the genus *Plectranthus*.

For the species *Plectranthus amboinicus*, the anxiolytic effect and a considerable decrease in its motor activity have previously been reported. This effect was verified using preclinical observational methods to assess anxious behaviour in the elevated maze model, the light-dark model, and the hole-board test in rats at doses equivalent to 350 mg/kg[198] and in mice at doses of 500 and 750 mg/kg[191]. These concentrations are equivalent/similar to those used in this investigation. Besides the shared neuropharmacological aspects by *P. amboinicus* and *P. neochilus* similar antimicrobial, antiinflammatory, antitumor, antioxidant and analgesic effects have also been reported. The species (*P. amboinicus*) grows well under severe drought, as it has a lot of water stored in its succulent tissues. It also survives well in severe heat and burning sun, as well as perennial shade. However, these conditions modify the chemical profile and the pattern of accumulation of bioactive components in different parts of the plant. It has been recognized that its essential oil content varies according to various growth parameters, such as geographical features,

climate and different stages of collection of plant material[247]. Both the quality and the quantity of chemical compounds found in the essential oil are directly related to the collection area. It is rich in monoterpenes and oxygenated sesquiterpenes[104], and its yield varies from an abundant 6.52% to a minimum of 0.55%. Of the non-volatile compounds, rosmarinic acid and several flavonoids of the flavone and flavonol type stand out, all with multiple oxygen atoms that exceed an average of six per compound, although detailed studies of their oxidation pattern depending on the area and collection conditions have not been analyzed, as has previously been done for volatile compounds[247]. Such detailed analysis might contribute in finding a scientific explanation for "new" pharmacological properties attributed to *P. neochilus* that grows in Cuba.

The species *Plectranthus aegyptiacus* also exhibited significant activity on the CNS at doses of 150 mg/kg in the novelty-induced behaviour test (NIB)[201]. The activity on the CNS of the extracts obtained from the leaves of these species could be related to the presence of essential oils, but also to rosmarinic acid and the presence of some polyhydroxylated flavones[248].

In general, the results obtained from the observational methods to evaluate the possible anxiolytic effect of the extracts tend to be inconclusive, and sometimes it is very difficult to discriminate mild differences between the behaviors of the experimental animals by extract groups. This could be mainly due to the fact that the intraspecific differences of the animals sometimes exceed the differences between the treatments, generating high variability of the results when experimenting with crude natural extracts, due to the presence of a mixture of compounds[249]. In these cases, the compounds present in the extracts can act synergistically but also antagonistically, which sometimes masks the individual effect of its components. In contrast, standardized extracts which are chemically well characterized, allow a more robust analysis, as in the present investigation.

In this study, three extracts with different proportions of two classes of compounds were considered: flavonoids (flavonol and flavone type) and abietan diterpenes, which together with rosmarinic acid (as the main compound) make up the complex matrix of *P. neochilus* extracts, as described in chapter V.

In order to elucidate the possible pharmacological mechanism of action of the extracts derived from the leaves of the *P. neochilus* species, a gene expression study of 35 genes was carried out, using whole brains samples of the mice collected at the end of the *in vivo* study. These 35 genes were intentionally selected on the basis of possible underlying mechanisms associated with the neuropharmacological sedative, hypnotic, analgesic and anti-inflammatory activities attributed by the Cuban population.

The results of the gene expression analysis showed no expression for GABA receptors and a differentiated expression of the pathway for dopamine, opioids and a set of genes associated with muscle relaxation, metabolism and the immune/anti-inflammatory response.

Two different families characterize dopamine receptors: the D1-type (including D1 and D5 receptors) and the D2-type (including D2, D3, and D4 receptors). Both receptors are coupled to a G protein, but with different adenylate cyclase stimulation profile. The D1 family mediates activation signals, while the D2 family mediates inhibition signals of such enzyme. For this reason, it is expected that the increase or decrease in cAMP levels may cause a similar response to that is caused by antidepressant or depressants drugs of the CNS, depending on which family of dopamine receptors is stimulated: type D1 or type D2[202]. However, despite these contrary effects; many commercial drugs such as apomorphine or clozapine are not completely selective for any specific type of the five dopaminergic receptors; since all of them share similar structural characteristics and, consequently, they present different affinity constants depending on the type of receptor to which they bind[203].

For more than 30 years, the biphasic effect of the so-called D-2/D-3 agonist, quinpirole, has been recognized: at low doses it decreases locomotion and movement levels and at high doses it increases these behavioural parameters[204].

This biphasic effect has been explained in two ways: increased endocannabinoid production in the dorsal striatum after stimulation of D-2/D-3 receptors, and/or the anatomical location of these D-2/D-3 receptors. Evidence for the first hypothesis supports that following stimulated production of endocannabinoids by activation of D-2/D-3 receptors, a transsynaptic inhibitory feedback of endocannabinoids neutralizes dopamine-induced facilitation of motor activity[205]. The second holds

that activation of the presynaptic dopamine D2/D3 receptor produces a marked decrease in motor activity by inhibiting dopamine release in the basal ganglia; but stimulation of postsynaptic dopamine D2/D3 receptors results in increased locomotion and characteristic stereotyped behaviours (including jumping, climbing, and oral movements)[206].

On the other hand, the presence of a heteromeric D1-D3 receptor that mediates locomotor activity in rodents is well established. The existence of this heteromer causes a simultaneous synergistic interaction between agonists D1R (activator of adenylate cyclase) and D3R (inhibitor of adenylate cyclase), which has been emonstrated by numerous methods and molecular intermediates[207]. Thus, in this heteromer, a mimetic action on the D3R increases the affinity of the D1R ligands, triggering a response in which that mediated by the D1 receptor prevails[208]. This agonist behavior known as "biased signaling" or "functional selectivity" responds to the ability of the ligand to selectively activate or block G protein-dependent or independent signaling. In consequence, the reciprocal modulation of D1/D2 receptors is currently recognized, either in favor of activation (prevalence of the effect mimicked by D1 receptors) or of deactivation (prevalence of the effect mimicked by D2 receptors) [209]. Such a characteristic has been pointed out as one of the main challenges of the modern pharmacology relating to G protein-coupled receptors[250].

In this context, the experimental results in mouse brains show that *P. neochilus* extracts upregulate the D1 and D3 receptors with high statistical significance when compared with the control and diazepam groups. According to the results expressed in the color scale of the heatmap (see Figure VI.3) and/or in the cluster dendrogram constructed in Figure VI.6, the D-1, and D-3 activation levels are more evident in the extracts of dry leaves (DLW, and DLE) than in the fresh leaf extract (FLD) in both types of receptors. This is in full agreement with the results in the Open Field Test (see Figure VI.1), in which the stereotyped behaviors rearing behaviour, central square duration and grooming behavior are increased in the experimental groups of dry leaves in relation to the diazepam and FLD groups. A dopaminergic mechanism of action in reducing anxiety has also been described for *Galphimia glauca*, which is a medicinal plant used to treat anxiety and sleep disorders[210].

Current concepts of modern pharmacology recognize, based on the evidence that receptor-receptor interactions can alter the recognition, pharmacology, and signalling of both or other receptors, that the concepts of classical pharmacology are not sufficient to explain the complex functioning of the CNS[211]. Most studies focus on dopamine heterodimers with GABA and opioid receptors. Experimental evidence has shown, within the framework of what is called receptor-receptor interactions, that the reduction in the striatal release of GABA due to its consequent underexpression of its receptors may be the result of the interaction of D2-type agonists, decreasing the activity of neurons in the striatum pallidus[212]. The striatum is a structure in which most of the cortical information directed to the basal ganglia converges. Furthermore, it is innervated by a very high density of the five types of dopamine receptors, which makes it an important component in a sensorimotor and cognitive region, therefore; in the regulation of locomotor activity and stereotypic movements[251].

Recent results show that a stimulation of central dopamine D2 receptors could produce different discriminative effects on the structure of the nucleus accumbens. Thus, D1 receptor antagonists and/or GABAB receptor agonists may elicit a dopamine D2 receptor-like effect by regulating identical neurons in the brain. The authors of the study conclude that the activation/inactivation of D1 and D2 in the nucleus accumbens can be reciprocally modulated to regulate the brain circuit[209].

This receptor-receptor coactivation has also been described for dopaminergic-opioid receptors. The three major subclasses of opioid receptors (μ, δ, and κ) are involved in the regulation of striatal dopaminergic function. Like μ receptors, dopaminergic signaling plays an important role in pain modulation. Biomodel studies have shown that dopamine modulates nociception in various regions of the CNS, including various regions of the cortex, periaqueductal gray, and basal ganglia[226]. Immunohistochemical experiments demonstrated co-localization in the form of hetero-oligomers of μ receptors with D1 receptors in neurons of the cortex and caudate nucleus[214].

Animal studies suggest a role for dopamine and the opioid system in reward-related processes. Yoshida et al., demonstrated that the simultaneous activation of the μ and δ2 opioid receptors after the administration of fentanyl leads to an increase in the

release of dopamine[215]. In contrast to increased extracellular dopamine release in the nucleus accumbens and dorsal caudate stimulated by μ receptors and δ-opioid agonists in rats, activation of κ receptors down regulates D2 receptors with impact on locomotor activity. Recent evidence supports, at the neuronal level that the inhibition of dopaminergic neurons in the somatodendritic region and in terminal release sites by κ receptor agonists can occur through several signaling pathways and ion channels, these being specific effects at each of the different synaptic sites[252].

Understanding the activity of a plant extract within the complex system of CNS interactions by moving to the binary relationships described above maybe an over simplification. However, this evidence gives some first clues of a possible mechanism that supports the neuropharmacological usage of extracts of *P. neochilus* by the inhabitants of Cuba. In general, our *in vitro/in vivo* results reveal an overexpression of D1/D3, μ, and δ receptors, accompanied by an underexpression of GABA receptors, consistent with the altered locomotor activity and the stereotypic movements observed in mice under study. Similarly, multiple reciprocal associations between dopamine/opioid/GABA signaling systems have been described for the nociception process in rats[252]. Even when the *in vivo* study did not evaluate the antinociceptive effects of *P. neochilus* extracts, the analgesic effect was ranked third among the most common ethnobotanical uses, as stated in chapter IV, and the overexpression of D1/D3, μ, and δ receptors can be interpreted as a possible molecular mechanismof action for such activity.

In addition to the neurotransmitters studied, other genes explored are directly related to the process of muscle contraction. In this context, atpa2a1 receptors (alpha 2 subunit of the Na+/K+ transporter), CLCN1 (voltage-gated chloride channels) and, to a lesser extent, muscarinic (CHRM1) and nicotinic (CHRNA1) receptors show a lower level of expression with respect to the control group, coinciding with the results of the *in vivo* experiment for the muscle contraction process. Energy is essential and the downregulation of genes associated with cellular respiration and energy production is a common finding in experimental groups, but especially in FLD. Therefore, this is another hypothesis to be considered

and, consequently, it also offers experimental evidence of the possible relaxing effect expressed by the Cuban population.

Genes such as NRF1 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) show a seven and five times log fold downregulation respectively, occupying the first and third positions among the 35 genes explored. Other genes associated with energy and/or cellular metabolism, such as ATPA2A1, SOD1, PPARGC1A (peroxisome proliferative-activated receptor, gamma, co-activator 1 alpha) are also expressed at lower levels than the control group. The blocking or inhibition of all these mechanisms for obtaining energy by the cell may be related to the muscle relaxation process observed in the experimental biomodels and, consequently, it also offers experimental evidence of the possible relaxing effect expressed by the Cuban population.

Finally, the results also suggest involvement of an immunomodulatory mechanism of action. Sleep and appetite disorders, anxious mood and difficulty concentrating are some of the characteristics of GAD, associated with psychosocial and functional impairment. Studies have shown that pro-inflammatory cytokines induce microglial activation with consequent neuro-inflammation. Currently, there is solid scientific evidence correlating the level of pro-inflammatory cytokines and microglial activation with behaviour and emotions, while experimental studies link stress and GAD with increased immune system activity[217].

In the brain, there are cellular actors that support immunity, within which microglial cells play an essential role, acting as the resident innate immune system by eliminating damaged brain tissue and pathogens[218]. Activated microglia can be associated with depression and appear increased in the hippocampus and amygdala, brain structures related to this disorder. In this context, our experimental results show that *P. neochilus* extracts moderately decrease expression of most genes associated with inflammatory and/or immune system intermediates such as TNFa, *NFKB*, *SOD1*, *SELL*, *CSF3R*, *BDNF*, *TLR3*, *TLR4*, *SIRT3* AND *S100B*, in this respect, the immunomodulatory effects of the plant may also support the use of the plant by the inhabitants of Cuba to treat sleep disorders.

The only exception within this group of downregulated genes is the *CXCR2* gene, which revealed a 3 logfold increase in expression. Despite its role in the

inflammatory process, this chemokine receptor (activated indistinctly by seven different chemokines) is associated with different signaling pathways at different stages of development, as to enhance the survival of hippocampus neurons. Furthermore, *CXCL1,* CXCL6 and CXCL8 emerge as the most important activators of the CXCR2 gene, being involved in different aspects of dopaminergic neuron development, including proliferation, neurogenesis and differentiation[253]. This versatility means that the effector functions of *CXCR2*, depending on the circumstances, are constantly balancing between the border of protection and destruction[254]. Therefore, the upregulation of this gene may be more associated with its role in the development of dopaminergic neurons than with its anti-inflammatory function, considering that all dopaminergic genes were also overexpressed after the intake of *P. neochilus* in mice.

In Chapter IV, several side effects were reported by the Cuban population after the consumption of *P. neochilus* leaves decoction. These effects could now be associated with the molecular mechanism suggested in the present work, being closely related to the most common adverse reactions to dopaminergic and/or opioid drugs. Thus, nausea and vomiting could be associated with actions on opioid and DR1 receptors, loss of orientation, bradypsychia, and colibacillosis with interaction with opioid and DR2/DR3 receptors, while morning dry mouth is a common side effect of the opioid drugs.[92,255].

Thoroughly, the changes induced in the *in vivo* behavior of mice and gene expression after an acute dose of *P. neochilus* extracts offer scientific evidence that supports the ethnobotanical use that the Cuban population makes of this plant. All this evidence can also be correlated with the chemical composition of the species that grows in Cuba, as was written in chapter V.

Considering the chemical composition of the extracts, rosmarinic acid, as the main compound, could be involved in the aforementioned mechanisms. Its two catechol groups allow it to show good antioxidant activity, consequently reducing the production of proinflammatory cytokines, and inhibiting the microglia activation[240]. Lev et al. reported that this inflammatory response is mediated by the suppression of the *HMGB*1/*TLR4*/*NF-κB* signaling pathway[256]]. Furthermore, and according to Hase et al., pretreatment with rosmarinic acid restores the function of complex I in

the mitochondrial respiratory reaction, and activates the dopamine (DA) signaling pathway by preventing the expression of the enzyme monoamine oxidase (MAO) B[220]. Both mitochondrial respiration and dopamine activation were also potential mechanisms addressed for *P. neochilus* extracts based on experimental results. In addition, rosmarinic acid proves to be an excellent neuroprotector after an ischemic stroke, due to its antioxidant defense mechanism that decreases the apoptotic cascade[219].

However, rosmarinic acid is not the only bioactive to which the various pharmacological activities detected can be attributed. Other polyphenols such as flavones and flavonol-like derivatives detected in *P. neochilus* extracts may also contribute to the anti-inflammatory activity. Chamomile flowers could serve as an example, having been used for centuries for their calming effect, which has been scientifically attributed to the flavonoid apigenin[90]. Likewise, linden flowers (*Tilia* sp.) are recognized as tranquilizers, due to the presence of quercetin and kaempferol, which are responsible for the sedative effect observed[221].

A plethora of experimental results support the activity attributable to flavonoids, such as the case of quercetin (aglycone of some of the flavonoids identified in *P. neochilus* extracts) in Chapter V. This compound proved that ameliorates the impairment of mitochondrial biogenesis and mitochondrial function by regulating the pathway *SIRT1/PPARGC1A/NRF1* and the inhibition of inflammation through the *TLR4/NF-κB* pathway[222]. All these genes appear repressed in the present work, so the quercetin derivatives present in the extracts of *P. neochilus* may also contribute to the pharmacological activities.

Flavonoids are also inhibitors of MAO-A, MAO-B, or both, while protecting neurons against neuroinflammation, activating synaptic signaling, and improving cerebrovascular blood flow[223]. Its involvement in Ca^{2+} pathways and phosphorylation/dephosphorylation reactions in 3H-methyl-4-phenylpyridine uptake by human neuronal dopaminergic cells has been suggested as a possible mechanism supporting its use in depression, bipolar disorder and attention deficit hyperactivity disorder, which are diseases associated with abnormal levels of dopamine. Flavonoids have also demonstrated their ability to act on ion channels and membrane conductivity, activating Ca^{2+} release[224]. Taking into account their

ability to cross the blood-brain barrier (BBB), these compounds stand out as effective modulators of the dopaminergic system and, consequently, could further contribute to the activity observed in *P. neochilus* extracts.

Although bibliographic evidenceis more limited for presence of abietan diterpenes in *P. neochilus* extracts, their activity may also contribute in the observed pharmacological effects. Rosmarol, a phenolic diterpene isolated from *Rosmarinus officinalis* L., has been shown to have antioxidant activity as well as strong anti-inflammatory activity by downregulating the gene expression of the inflammatory enzymes iNOS and COX-2 by inhibiting the activation of NF-kappaB[225]. Likewise, rosmarol and carnosol (another phenolic diterpene) synergistically alleviate rheumatoid arthritis by inhibiting the TLR4/NF-κB/MAPK pathway[226].

In silico studies can also gives light to the molecular mechanisms involved in *P. neochilus* mode of action. Fenoldopam, a selective dopaminergic D1 receptor agonist it's a benzazepine with a catechol group like dopamine. Recent in silico studies demonstrated that Fenoldopam can bind DR1 receptors in the orthosteric binding pocket (OBP) similarly to dopamine. Ligands are very dynamic in the receptor binding pocket and different ligand binding modes could lead to conformational heterogeneity of the intracellular receptor pocket, which may contribute to multiple active state conformations and recruitment of various transducers. Nevertheless, the extracellular loop residue L190ECL2 plays an important role in the stabilisation of catechol agonists[257].

Considering the possible ligand-receptor binding site of this selective DR1 receptor agonist drug and its similarity in terms of the presence of catechol groups to some of the chemical compounds identified in the *P. neochilus* extracts, an analogous behaviour could be suggested. Of the compounds identified for the extracts: rosmarinic acid (compound 9), some of the flavones (compounds 2 and 5) and the abietane diterpenes (compounds 16, 11 and 17) have a catechol group in their structure. In addition, compounds 11 and 17 have an acetyl group in their structure, which behaves as an electron donor isostere of the dopamine amino group (NH2), so these compounds could behave as dopamine bioisosteres. Therefore, it could be possible that these compounds identified in extracts of the plant species under study

could activate the dopaminergic receptor families DR1 (DR1 and DR5) and DR2 (DR2, DR3 and DR4).

Future preclinical pharmacological and toxicological studies must be carried out to complement and/or deepen the mechanism of action of the extracts obtained, and in particular, the one that reproduces the popular consumption of the leaves of the plant in Cuba as a sedative. Preclinical studies at repeated doses to observe the behaviour of the extracts over time; *in vitro* pharmacological studies with selective dopaminergic (D1R and D2R) and opioid (μ, κ and δ) receptor agonists are some of those who could provide the most information.

CHAPTER VIII

CHAPTER VIII. Conclusions

- The species that grows and use the Cuban population corresponds to *P. neochilus*, confirming and detailing the use of the plant by Cuban population as hypnotic and sedative throughout the island, being different with what is worldwide informed.
- Quality control parameters of Cuban *P. neochilus* cultivars revealed the absent of glandular trichomes, essential oil production, as well as 15 new compounds for the species, highlighting 8 flavone derivatives, 7 abietane diterpenoids and rosmarinic acid as main compound.
- Plant extracts caused sedative effect as well as a significant muscle relaxing effect in mice biomodels. Additionally modify on brain samples the expression of dopaminergic and opioid genes; while muscle contraction related genes and inflammation intermediaries were negatively modulated.

CHAPTER IX

CHAPTER IX. Future Perspectives

- The ethnobotanical study of *P. neochilus* should be deepened, extending it to other communities throughout the country, in order to increase the size of the sample and to be able to compile the greatest amount of traditional information existing in the country on this plant species.
- To replicate the tests for the determination of pharmacognostic quality parameters with more diverse cultivars (other regions of the country) for future standardisation of the results; and to repeat the tests for the determination of pharmacognostic parameters of extracts derived from leaves of cultivated species of *P. neochilus* (other regions of Cuba) in order to standardise their physical, physical-chemical characteristics
- Carry out preclinical studies at repeated doses to observe the behaviour of the extracts over time. Conduct *in vitro* pharmacological studies with selective dopaminergic (D1R and D2R) and opioid (μ, κ and δ) receptor agonists. Toxicological testing for product safety.

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ANNEX

ANNEX 1. Modelo de encuesta etnobotanica sobre la planta conocida popularmente como meprobamato

Localización de la entrevista: _____

I.- Datos socio-demográficos del informante:

- **Sexo**: Masculino _____ Femenino _____
- **Nivel de Escolaridad terminado:**

  Primaria ___ Secundaria __ Preuniversitario ____ Universitario ___

  Técnico ___ Obrero calificado ____ Sin nivel escolar terminado___

- **Edad:** 18- 27 ___ 28- 37___ 38-47___ 48-57___ 58-67___ 68 y más___
- **Ocupación:**

Estudiante __ Ama de casa __ Trabajador __ Jubilado __ Sin ocupación ___

II.- Datos de la planta.

1. ¿Conoce Ud. y ha utilizado la planta nombrada popularmente como "meprobamato"?

Sí __ No__

2. ¿Conoce Ud. acerca de algún uso que se le da a esta planta? Sí___ No___

Medicinal ____ Ornamental ____ Religioso ____ Otros ___

3. ¿Conoce cuál (es) usos medicinales se le atribuyen a esta planta?

Sedante___ Para tratar trastornos digestivos___ Relajante muscular___
Somnífero___ Otros (especifique)___

4. ¿La ha empleado alguna vez con fines medicinales? Sí ___ No ___ **¿Para qué?**

5. ¿Cuándo la consume, experimenta algún efecto en el organismo? Sí ___ No ___ **¿Cuál(es)?**

6. ¿Cómo clasificaría Ud. el efecto causado por ella en el organismo?

Muy beneficioso ___ Beneficioso ___ Perjudicial ___

De ser no beneficioso o perjudicial el efecto causado por la planta, especifique cuál o cuáles fueron estos.

7. ¿Cómo conoció las propiedades medicinales de esta planta?

Familiares __ Vecinos__ Amigos __ Religiosos __

Yerberos__ Otros (cuál) __

8. ¿Qué parte de la planta emplea para su uso medicinal?

Hojas __ Raíz __ Tallo ___ Ramas __ Planta completa __

9. ¿Cómo la utiliza?

Fresca____ Seca ___ Fresca y seca ___ No sé___

10. ¿Cómo obtiene la planta que utiliza?

De yerberos___ La sembrada en su casa___ De espacios abiertos___

De un jardín ___ No sé ___ Otros (especifique)___

11. ¿En qué forma usted la consume?

Decocción __ Infusión __ No sé__ Otras (Especifique)_____

12. Cuantas hojas usted utiliza para la elaboración de preparación vegetal. Marque el rango donde se encuentra esta.

1-5 hojas___ 6-10 hojas___ 11-15 hojas___ Más de 15 hojas__

Otras _____

13. ¿Qué cantidad de agua usted utiliza para elaborar la preparación anterior?

Menos de un vaso__ Un vaso __ Más de un vaso __ Un litro __

Otra cantidad (especifique) _____

14. ¿Qué cantidad de la preparación usted consume diariamente?

Menos de una taza__ Un taza__ Dos taza (un vaso) ___

Un litro __ Otra cantidad ____

15. ¿Cuáles son las vías de administración que emplea?

Oral __ Nasal __ Dérmica (tópica) __ Rectal __ Vaginal __ Otras __

16. ¿En qué horarios consume Ud. la preparación de la planta?

Mañana ___ Tarde ___ Noche ___ Sin distinción de horarios____ A toda hora ____

Mañana y noche ___ Tarde y noche ____

17. ¿Por cuánto tiempo Ud. consume la preparación?

1- 5 días ____ 6 -10 días ____ 11 - 15 días ____ Por tiempo indefinido___

Todos los días __Otro (especifique) _____

18. Marque en cuál de estos períodos, ud. utiliza la planta:

Antes de la enfermedad (Prevenirla)___ Durante la enfermedad (sin la crisis)___

Durante la crisis de la enfermedad___ Durante toda la enfermedad___

19. ¿Consume la preparación conjuntamente con alimentos? Sí __ No__.

20. ¿Cuándo la consume con alimentos experimenta algún efecto adverso?

Sí__ No__ .De ser Sí su respuesta, especifique cuál o cuáles fueron estos:

21. ¿Utiliza la planta de forma conjunta con otros medicamentos? Sí___No____

De ser Sí, su respuesta. Especifique cuál (es) medicamentos son:

a) ¿Ha manifestado algún efecto en el organismo? Sí ____ No _____. De ser Sí, su respuesta. Especifique cuál (es) son :

b) Especifique si estos efectos, fueron negativos: Sí__ No__

22. ¿La ha utilizado en niños? : Sí__ No__

23. ¿Recomendaría Ud. su uso? Sí___ No___ ¿Por qué?

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