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Antidepressant-like activity of green kiwifruit (*Actinidia deliciosa*) on mouse models of depression

S.S.D BIO/01 BOTANICA

Coordinatore: Prof. Matteo Ballottari

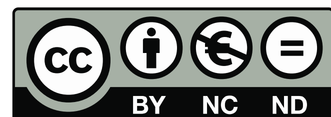
Firma ___________________________

Tutor: Prof.ssa Flavia Guzzo

Firma ___________________________

Dottorando: Dott. Claudio Marcello Marzo

Firma ____________________



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True science teaches us to doubt and, in ignorance, to refrain.

Claude Bernard

Io penso che un uomo senza utopia, senza sogno, senza ideali, vale a dire senza passioni e senza slanci sarebbe un mostruoso animale fatto semplicemente di istinto e di raziocinio, una specie di cinghiale laureato in matematica pura.

Fabrizio De André

To my love Stefania...

Abstract

Depression is a common and heterogeneous mental health disorder that affects more than 322 million people across the world (4.4% of the world's population). Antidepressant pharmacotherapies have a slow onset of clinical efficacy which is critical for therapeutic outcome and patient's compliance. In recent years, an increasing number of studies suggests that phytochemical compounds activity on mood might be a clinically relevant co-adjuvant to current medications.

Many authors suggested a positive association between fruits and vegetables-rich diet and prevention of depression, anxiety and cognitive decline; therefore, the consumption of a whole phytocomplex could increase bioavailability of plant-based secondary specialized metabolites both at central nervous system and systemic levels.

To date, no data are available concerning the administration of whole fruits to assess antidepressant activity *in vivo*: only single compounds have been tested. The aim of this study was to evaluate the activity of kiwifruit (*Actinidia deliciosa* cv. Hayward) extracts on mood in murine models of depressive behavior. Moreover, kiwifruit is rich in polyphenols (e.g. ferulic acid, kaempferol, catechin, etc.) with a known inhibitory effect on both monoamine oxidases A and B.

To reach this goal independent groups of naïve male C57BL/6J mice were used and fed with three increasing dilutions of kiwifruit extracts under chronic regime. Immobility time was then assessed in tail suspension test (TST) and forced swimming test (FST); additionally, the efficacy of kiwifruit with respect to vehicles and the selective serotonin reuptake inhibitor fluoxetine (20 mg/kg, i.p.) were investigated. The highest kiwifruit concentration produced a reduction of 45% and 40% in immobility time in TST and FST respectively, without causing hyperlocomotion in the open-field test (OFT). To find the molecules possibly responsible of these findings, pharmacokinetic profiles and metabolomic analyses

were assessed and allow us to discover two kiwifruit molecules probably responsible to this effect, quinic acid and caffeic acid 3-β-D-glucoside.

The two molecules were evaluated in immobility time in the above-mentioned behavioral test alone or in combination. Quinic acid and the combination of the two molecules produced a reduction of 42% and 54% in immobility time in TST and 29% and 27% in FST respectively, without causing hyperlocomotion in the OFT. Caffeic acid had proven to be ineffective when administered alone.

Interestingly, when administered alone, the two molecules have lower serum and brain levels after UPLC-ESI-MS analysis indicating that, probably other molecules present in the fresh fruit are necessary for the correct adsorption and/or stability of the two metabolites.

Moreover, evaluation of inhibitory activity on both MAOs isozymes were evaluated through an *in vitro* assay. Kiwifruit and its metabolites strongly inhibit both isoforms of the enzyme with quinic acid that is selective only against MAO-B.

Taken together, these data suggest that kiwifruit may have a mood-improving effect through inhibition of the MAOs isozymes and could be used in clinical state such as depression.

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List of abbreviations

5-HT – 5-Hydroxytryptamine (Serotonin)

ACN – Acetonitrile

ANOVA – Analysis of Variance

APA – American Psychiatric Association

BSA – Bovine Serum Albumin

CA – Caffeic Acid

CBT – Cognitive-Behavioral Therapy

CGA – Chlorogenic acid

DAT – Dopamine Transporter

DHEA – Dehydroepiandrosterone

DMSO – Dimethyl Sulfoxide

DSM-V – Diagnostic and Statistical Manual of Mental Disorder

ECT – Electroconvulsive Therapy

EPM – Elevated Plus Maze

ESC – Escitalopram

FLX – Fluoxetine

FST – Forced Swim Test

FW – Fresh Weight

GABA – γ-Aminobutyric acid

GPCR – G protein-coupled receptor

HBV – Hepatitis B virus

HCOOH – Formic Acid

HPLC – High Performance Liquid Chromatography

I.G. – Intragastric

I.P. – Intraperitoneal

IC_{50} – Half maximal inhibitory concentration

ICD-11 – International Classification of Diseases

ICSS – Intracranial Self-Stimulation

IP – Interpersonal Therapy

MAO – Monoamine Oxidase

MAOIs – Monoamine Oxidase Inhibitors

MDD – Major Depressive Disorder

MeOH – Methanol

MS – Mass Spectrometry

MWCO – Molecular Weight Cut-Off

NE – Norepinephrine

NET – Norepinephrine Transporter

NMDA – *N*-Methyl-d-aspartic acid

O2PLS-DA – Orthogonal 2-block projection to latent structures-discriminant analysis

OFT – Open Field Test

PK – Pharmacokinetics

QA – Quinic Acid

RLU – Relative Light Unit

SERT – Serotonin Transporter

SNRI – Serotonin/Norepinephrine Reuptake Inhibitor

SPT – Sucrose Preference Test

SSRI – Serotonin Reuptake Inhibitor

TCA – Tricyclic Antidepressant

TMS – Transcranial Magnetic Stimulation

TNF-α – Tumor Necrosis Factor-α

TST – Tail Suspension Test

UPLC – Ultra Performance Liquid Chromatography

WHO – World Health Organization

Aim and outline of the PhD thesis

In recent years, diets rich in fruits and vegetables have been correlated with an improvement of psychological well-being and cognitive functions. The fundamental goal of the work described in this thesis was to investigate the possible antidepressant-like effects of plant-based food, specifically green kiwifruit (*Actinidia deliciosa* cv. Hayward) to provide a better understanding of the role played by this fruit and its specialized secondary metabolites.

The experimental methods have concentrated on behavioral, neurochemical and analytical techniques. This methodological approach was used to provide information in two main areas:

- First, to investigate the effect of kiwifruit in two animal models of depression and to unveil, through UPLC-ESI-MS analysis, possible kiwifruit compounds to be studied in further details.
- Second, to provide, through *in vitro* assay, some insight into neurochemical mechanisms of action.

The work presented in this PhD thesis is organized in the following chapters:

- ***Chapter 1*** *– General introduction*

  a preface on depression and mental health, current antidepressant pharmacotherapies focusing also on nutrition and nutraceuticals products. In this chapter the attention was also focused on the tools of modern *omics* science for the study of food properties.

- ***Chapter 2*** *– In vivo experiments on well-validated behavioral models combined with metabolomics approaches reveal antidepressant-like activity of kiwifruit*

In this chapter I evaluated the effect of green kiwifruit in two animal models of depression after chronic treatment regime. Pharmacokinetic experiments together with analytical investigation were also assessed in this chapter.

- **Chapter 3** – *Quinic acid is able to mimic kiwifruit juice effects on murine models of depression*

  The cornerstone of this chapter was the *in vivo* evaluation of the two specialized metabolites of kiwifruit identified as possible candidates for activity in chapter 2. The activity of the two molecules were assessed after chronic treatment in the above-mentioned animal models together with PK and mass spectrometry investigations.

- ***Chapter 4*** – *Kiwifruit extract and some of its metabolites, quinic acid included, are able to inhibit human Monoamine Oxidase enzyme*

  The focus of this chapter was to address a potential inhibitory activity of kiwifruit and its metabolites found in the previous chapter on monoamine oxidase enzymes.

- **General discussion & concluding remarks**

  A final resume of the work and the results achieved in this PhD thesis is presented together with the discussion of the possible directions and objectives that this research might address in the future.

Chapter 1 – General introduction

1.1 – Depression, history and mental health

Prior to the coinage of the term depression, other terms such as spleen, nerves, bile, hypochondriasis, fits, hysteria and being “down in the dumps” have been used to describe symptoms which we may today associate with depression (Rousseau 2000). Some historical figures, such as Vincent Van Gogh (Blumer 2002) and John Keats, have suffered with this condition. The historical diagnoses which most closely relate to the modern diagnosis of depression are *melancholia* and *acedia*. Melancholia is considered by Rousseau (2000) and most authors in the field (Jackson 2008) as the primary precursor to modern depression. A combination of the words melas, meaning black and kholé, meaning bile, the word melancholia came about when the Four Humours (blood, phlegm, yellow and black bile) theory of medicine rose in popularity around 400 B.C. The term depression was derived from the Latin verb *deprimere*, “to press down”. It was used, for the first time, in 1665 in English author Richard Baker's Chronicle to refer to someone having “a great depression of spirit”, and by English author Samuel Johnson in a similar sense in 1753 (Berrios 1988).

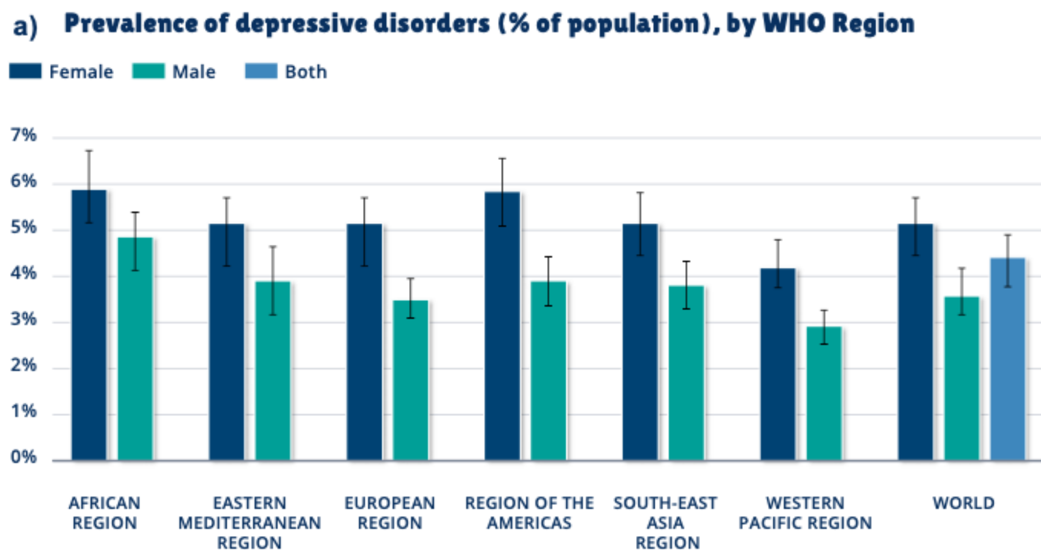
Mental health is regarded as “an integral part of health and well-being” where an individual's health status is defined as complete well-being, encompassing physical, mental and social functioning (WHO 2017). Mental health can be impacted by various factors in life such as individual physical disease (Prince et al. 2007), socio-economical, cultural, believe, political and environmental factors as well as individual psychological and biological immune systems (Hungerford et al. 2018).

Depression is a common and heterogeneous mental health disorder, a significant contributor to the global burden of disease and affects people in all communities across the world. Today, depression is estimated to affect 322

millions people (4.4% of the world's population), nearly half of these people live in the South-East Asia Region and Western Pacific Region, reflecting the relatively larger populations of those two Regions (which include India and China, for example) (WHO 2017).

Depression disorders are the most common mental illnesses and the primary source of disability in the world affecting in excess of 10-15% of the population at some time in their lives. The World Mental Health Survey conducted in 17 countries found that on average about 1 in 20 people reported having an episode of depression in the previous year. Depressive disorders often start at a young age; they reduce people's functioning and often are recurring. For these reasons, depression is the leading cause of disability worldwide in terms of total years lost due to disability and is characterized by a high risk of suicide (the second most common cause of death in those aged between 15 and 29) (WHO 2017; Lim et al. 2018).

Figure 1.1 shows prevalence of depressive disorders among WHO regions and across the lifespan.



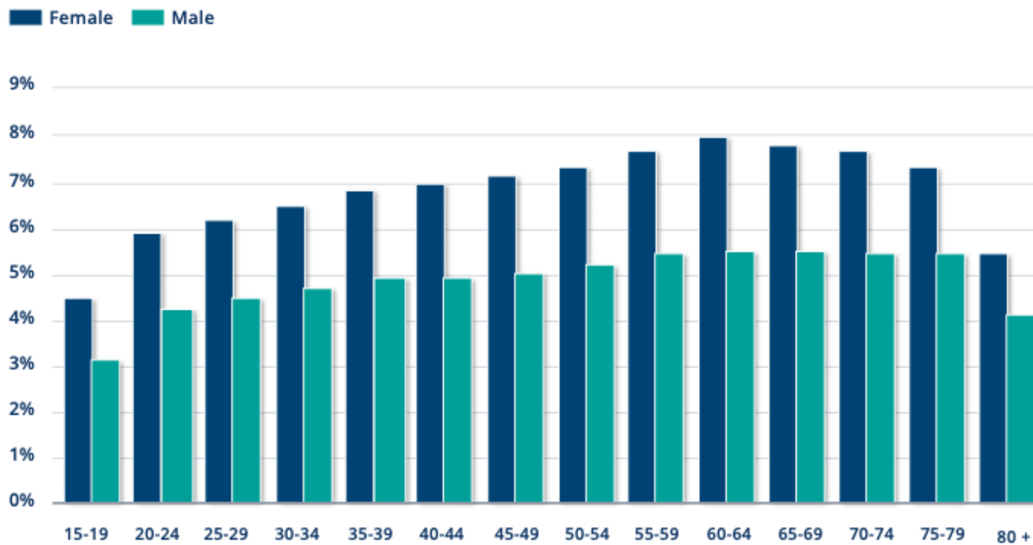


Figure 1.1. **Prevalence of depressive disorders across the world**. The proportion of the global population with depression in 2015 is estimated to be 4.4%. **a)** Upper and lower uncertainty intervals are represented in the bar graph by the error bars. Depression is more common among females (5.1%) than males (3.6%). **b)** Prevalence rates vary by age, peaking in older adulthood (above 7.5% among females aged 55-74 years, and above 5.5% among males). Depression also occurs in children and adolescents below the age of 15 years, but at a lower level than older age groups. Regional data shown are age-standardized estimates.
Source: Global Burden of Disease Study 2015 (http://ghdx.healthdata.org/gbd-results-tool) and modified form Who 2017.

1.1.1 – Theories of depression

Across history, there have been many different explanatory theories regarding the onset and the mechanism of action of depression including biological (e.g. the monoamine deficiency hypothesis) and psychological theories (Bernaras et al. 2019). Biological theories have, from a variety of different perspectives, postulated that depression may occur due to noradrenalin deficits (Schildkraut 1965); endocrine disorders (Birmaher et al. 1996), sleep-related disorders (Sivertsen et al. 2014; Pariante 2017), alterations in brain structure (Whittle et al. 2014), or the influence of genetics, around 40% (Scourfield et al. 2003). Otherwise psychological theories have attempted to explain depression on the basis of psychoanalysis and, more specifically, in terms of attachment theories (Bowlby 1969; Ainsworth et al. 1978; Bigelow et al. 2018), behavioral models

(Skinner 1953; Ferster 1966; Lewinsohn 1975), cognitive models (Seligman 1975; Abramson et al. 1978; Beck 1987), the self-control model (Rehm 1977; Rehm et al. 1979), interpersonal theory (Markowitz and Weissman 1995; Milrod et al. 2014), stressful life events (Reinherz et al. 1993; Frank et al. 1994) and sociocultural models (Lorenzo-Blanco et al. 2012; Chang et al. 2013; Reeves et al. 2014).

The monoamine deficiency hypothesis is one of the well-known and credited theories of depression. Since the monoamine system originates in the deep archaic brain (*raphe nuclei*, *locus coeruleus* and *substantia nigra* for serotonin, noradrenaline and dopamine respectively, and branches almost to the entire brain, it modulates a plethora of cognitive functions (Belmaker and Agam 2008). Some of the first antidepressant drugs were responsible for the blockade of the reuptake of serotonin and norepinephrine at presynaptic level. The main effect of these pharmacological actions is to increase the bioavailability of these compounds at synaptic level rising the postsynaptic stimulation. For that reason, also inhibitors of the monoamine oxidase, the enzyme that catalyze the oxidation of monoamines, achieve the same effect. All these discoveries led to the formulation of the above-mentioned theory. One of the key points of this theory is its predictive power since every compound created with the purpose of inhibiting MAO has been proved to act as antidepressant drug.

Table 1.1 summarizes all theories taken into account regarding depression. Since the components of the brain are highly interconnected, it is not difficult to find possible integrative frameworks between two or more of the various theories. For that reason, clinical data could not be categorized into such neat theories and every situation must be evaluated subjectively.

Table 1.1. Additional Biologic Theories of the Pathophysiology of Depression

Theory	Supporting Evidence	Contradictory Evidence
Altered glutamatergic neurotransmission	- Glutamate and glutamine levels in the prefrontal cortex are reduced Intravenous ketamine, an NMDA antagonist, induces rapid, sustained antidepressant effect; - Cortical messenger RNA levels of glutamate transporters and of the enzyme that converts glutamate to glutamine are reduced	- Glutamate levels in the occipital cortex are increased - Ketamine binds to high-affinity-state D2 dopamine receptors - It is not clear whether antidepressants affect AMPA receptors in the brain
Reduced GABAergic neurotransmission	- Levels of GABA in plasma, cerebrospinal fluid, the dorsolateral prefrontal cortex, and the occipital cortex are reduced - GABA-modulating agents have effects in animal models of depression - Antidepressants affect GABAergic function - GABA neuron immunoreactivity is reduced in the prefrontal cortex	- GABA occurs in more than 30% of brain synapses, suggesting nonspecificity - There is a lack of difference in prefrontal cortex GABA levels on MRS in depression - GABA neurotransmission may be related to symptoms of anxiety in depression
Abnormal circadian rhythms	- Sleep deprivation and light therapy have antidepressant effects - Some patients with depression have circadian abnormalities of mood, sleep, temperature, and neuroendocrine secretion - Rodents active during the day become depressed when daylight is shortened	- The association between clock-related genes and depression is inconsistent
Deficient neurosteroid synthesis	- Cholesterol levels are low in plasma and the brain during depression - DHEA has antidepressant effects in patients with depression	- The findings in schizophrenia are similar - Neurosteroids (neuroactive steroids in the brain that modulate neurotransmitter recepetors) mostly affect memory and sleep
Impaired endogenous opioid function	- δ-Opioid–receptor agonists have antidepressant-like effects in rodents and up-regulate levels of BDNF in the brain - Capacity for cortical μ-opioid–receptor binding is decreased in response to sustained sadness	- Although early reports suggested that opiates may be effective in treating depression, data from large, controlled, randomized trials are lacking
Monoamine–acetylcholine imbalance	- Depressed mood can be induced in humans by administration of physostigmine, an acetylcholinesterase inhibitor - Nicotinic acetylcholine receptor antagonists potentiate antidepressants	- Mecamylamine, a nicotinic acetylcholine receptor antagonist, reduced symptoms of depression - Many antidepressants are not anticholinergic
Cytokine-mediated crosstalk between the immune system and the brain	- Depression is common in infectious and autoimmune diseases - Exposure to cytokines induces depressive symptoms, and cytokine secretion is increased in major depression - Antidepressants have antiinflammatory effects - Cytokines affect the hypothalamic–pituitary–adrenal axis and monoamines	- Most studies are correlative - Cytokine-induced depressive symptoms are temporary and not replicated in all studies - Substance P antagonists are not therapeutic in depression
Thyroxine abnormalities	- Levels of transthyretin are reduced in the cerebrospinal fluid in patients with depression - Thyroid hormones modulate the serotonergic system in the brain - Brain neurogenesis is decreased after the administration of thyroxine in adult rats with hypothyroidism - Rate of response to triiodothyronine is increased during depression	- Thyroxine monotherapy is ineffective - Hypothyroidism is not manifested in most patients with depression
Dysfunction of specific brain structures and circuits	- Transcranial magnetic stimulation of the prefrontal cortex and deep-brain stimulation of the anterior cingulate affect mood - Glucose use is reduced in the prefrontal cortex and subgenual prefrontal cortex - Circuit dynamics in the hippocampus are altered in a rat model of depression	- Implicated brain areas differ from study to study - Inconsistent findings with respect to blood flow, volumetric, glucose utilization, and postmortem methodologies

1.1.2 – Classification, symptoms and diagnosis

Only in the past four decades have precise criteria to diagnose depression. Mood disorders are now classified on the basis of symptoms, natural history (including the age of onset, course and outcome) patterns of familial transmission and response to treatment (Kandel et al. 2012). Nowadays, medical conditions and therefore also depression are classified under two diagnostic system: the Diagnostic and Statistical Manual of Mental Disorder (**DSM-V**), from the American Psychiatric Association (APA) and the International Classification of Diseases (**ICD-11**) from the World Health Organization (WHO) even if the first cover mental disorders in deep details (Gaebel 2015). In the DSM-V depressive disorders have been separated from Bipolar and related Disorders, making the classification of Unipolar and Bipolar depressive disorders (people affected by both depression and maniac symptoms) obsolete.

According to the DSM-V (APA 2013) depressive disorders include:

- **Disruptive mood dysregulation disorder**. The core feature is chronic, severe persistent irritability that occur between the age of 6 and 10.
- **Major depressive disorder** (MDD; otherwise known as unipolar depression). It is characterized by discrete episodes of at least 2 weeks duration (although most episodes last considerably longer) involving clear-cut changes in affect, cognition, and neurovegetative functions and inter-episode remissions (**Table 1.2**).
- **Persistent depressive disorder (dysthymia)**. More chronic form of depression that can be diagnosed when the mood disturbance continues for at least 2 years in adults or 1 year in children.
- **Premenstrual dysphoric disorder**. Its essential features is the expression of mood lability, irritability, dysphoria, and anxiety symptoms.
- **Substance/medication-induced depressive disorder**. The diagnostic features include the symptoms of a depressive disorder, such as major depressive disorder; however, the depressive symptoms are associated

with the ingestion, injection, or inhalation of a substance (e.g., drug of abuse, toxin, psychotropic medication, other medication).

- **Depressive disorder due to another medical condition**. Prominent and persistent period of depressed mood or markedly diminished interest or pleasure in all, or almost all, activities that predominates in the clinical picture.
- **Other specified/unspecified depressive disorder**. This category applies to presentations in which symptoms characteristic of a depressive disorder that cause clinically significant distress or impairment in social, occupational, or other important areas of functioning predominate but do not meet the full criteria for any of the other disorders in the diagnostic class.

The two more frequent depressive disorders are MDD and dysthymia. Dysthymia is a chronic form of mild depression, whereas MDD is the most severe form of depression (APA 2013; Amos et al. 2018). The symptoms vary from one type of mental illness to another, but they are mainly characterized by problems relating to emotions, ways of thinking, and how people behave and relate to each other in society. Some mental health problems are also comorbid with one or more mental health conditions such as depression and anxiety. Mood disorders and anxiety are in general discussed together because both involve negative emotional state. There is also evidence for overlapping risk factors between these pathologies since nearly 60% of patients with major depressive disorders also suffer from anxiety disorder. Anxiety most commonly precedes the onset of depression. According to DSM-V, to be classified as MDD, one has to have a minimum of five out of nine of the symptoms in the **Table 1.2** and **Figure 1.2** during the same 2-week period with the symptoms representing a change from previous functioning and with at least one of the symptoms being either depressed mood or loss of interest or pleasure in activities of daily life (WHO 2017). Depending on the severity of the symptoms, the episode can be classified further into mild, moderate or severe:

- **Mild depression**: Few, if any, symptoms in excess of those required to make the diagnosis are present, the intensity of the symptoms is distressing but manageable, and the symptoms result in minor impairment in social or occupational functioning.
- **Moderate depression**: The number of symptoms, intensity of symptoms, functional impairment, or all of these variables are between those specified for “mild” and “severe.”
- **Severe depression**: The number of symptoms is substantially in excess of that required to make the diagnosis, the intensity of the symptoms is seriously distressing and unmanageable, and the symptoms markedly interfere with social and occupational functioning.

Table 1.2. DSM-V Criteria for Major Depressive Disorder*

1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, or hopeless) or observation made by others (e.g., appears tearful). (In children and adolescents, a depressed mood can be an irritable mood)
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation).
3. Significant weight loss when not dieting or weight gain (e.g., a change of >5% of body weight in a month) or decrease or increase in appetite nearly every day. (In children, failure to gain expected weight should be considered.)
4. Insomnia or hypersomnia nearly every day.
5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).
6. Fatigue or loss of energy nearly every day.
7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

*A comprehensive assessment of depression should not rely simply on a symptom count but should take into account the degree of functional impairment, disability, or both
Modified from (Park and Zarate 2019)

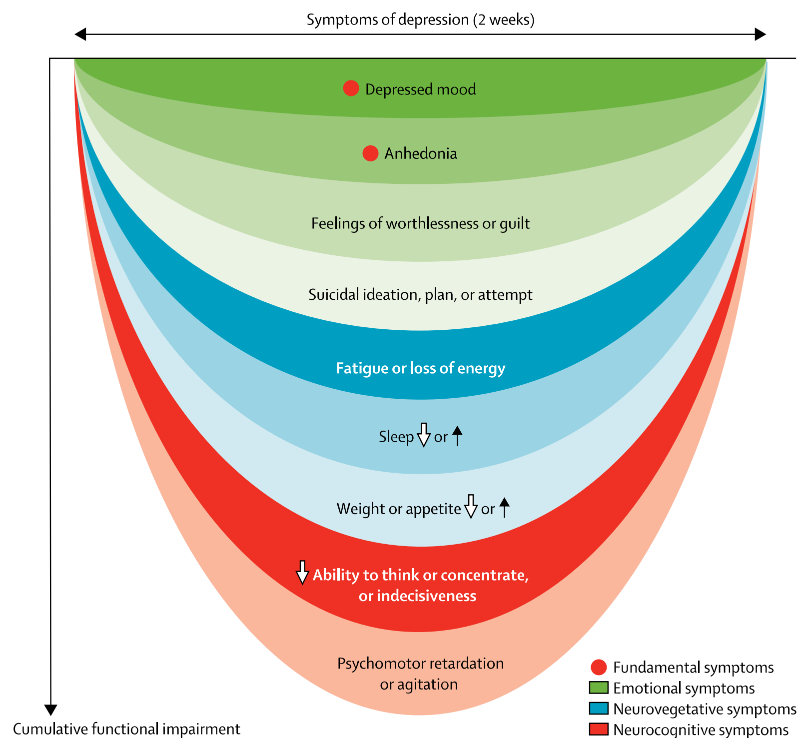


Figure 1.2. Key symptoms of MDD according to DSM-V classified on the basis of sector involved. From Mahli and Mann 2018.

1.1.3 – Treatment of depression

Treatments for depression can be divided into three; those involving using medication (referred to as pharmacotherapy), those using alternative biological treatments, and those using psychotheraphy. Treatments categorized as part of pharmacotherapy include antidepressant, mood-stabilizing, and antipsychotic drugs. Alternative biological treatments can be Electroconvulsive therapy (ECT), Transcranial magnetic stimulation (TMS), deep brain stimulation, bright light therapy, whereas psychotherapy includes Cognitive-Behavioral Therapy (CBT), Behavioral Activation treatment, Interpersonal Therapy (IP), and family and marital therapy (Hooley et al. 2017).

Many different antidepressants have established efficacy in treating depression (Millan 2003). However, antidepressant drugs have slow onset of clinical efficacy, which is critical for therapeutic outcome and patient compliance, indeed current pharmacotherapies take weeks to months to achieve their full effects with

increased risk of suicidal behavior (Trivedi et al. 2006; Machado-Vieira et al. 2010). During this “therapeutic lag-time”, 15% of patients who suffer from MDD commit suicide (Nock et al. 2008; WHO 2017). This is the reason that electroconvulsive therapy may be the treatment of choice for agitated depressed patients with a high risk of suicide. Additional issues come from the limitation in efficacy of all antidepressant drugs, since at least 20% of all depressed patients are refractory to multiple different antidepressants at adequate doses. In general, if a patient does not respond to a given antidepressant after an 8-week trial on an adequate dose, then switching to another antidepressant with a different mechanism of action is a reasonable next step (e.g., SSRI to SNRI) (Rush et al. 2006). All these issues rise the urgent need to find more active drugs with lower side effects.

The most commonly used medications, often referred to as second-generation antidepressants, are the selective serotonin reuptake inhibitors (SSRIs) and the serotonin-norepinephrine reuptake inhibitors (SNRIs), which have greater efficacy and safety compared to older drugs (i.e., first-generation antidepressants). Relatively selective norepinephrine reuptake inhibitors also have been developed as antidepressants (e.g., maprotiline, reboxetine) (Ferguson 2001).

In monoamine systems, reuptake of the transmitter is the main mechanism by which neurotransmission is terminated; thus, inhibition of reuptake can enhance neurotransmission, presumably by slowing clearance of the transmitter from the synapse and prolonging the dwell-time of the transmitter in the synapse. Reuptake inhibitors inhibit either SERT, NET, the neuronal norepinephrine (NE) transporter; or both (**Figure 1.3**). Similarly, the first-generation drugs, which include monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCAs), also enhance monoaminergic neurotransmission: the MAOIs by inhibiting monoamine metabolism and thereby enhancing neurotransmitter storage in secretory granules, the TCAs by inhibiting serotonin or 5-hydroxytryptamine (5-HT) and norepinephrine reuptake. While efficacious, these first-generation agents exhibit side effects and drug and food interactions that limit their use relative to the newer drugs (Brunton et al. 2011).

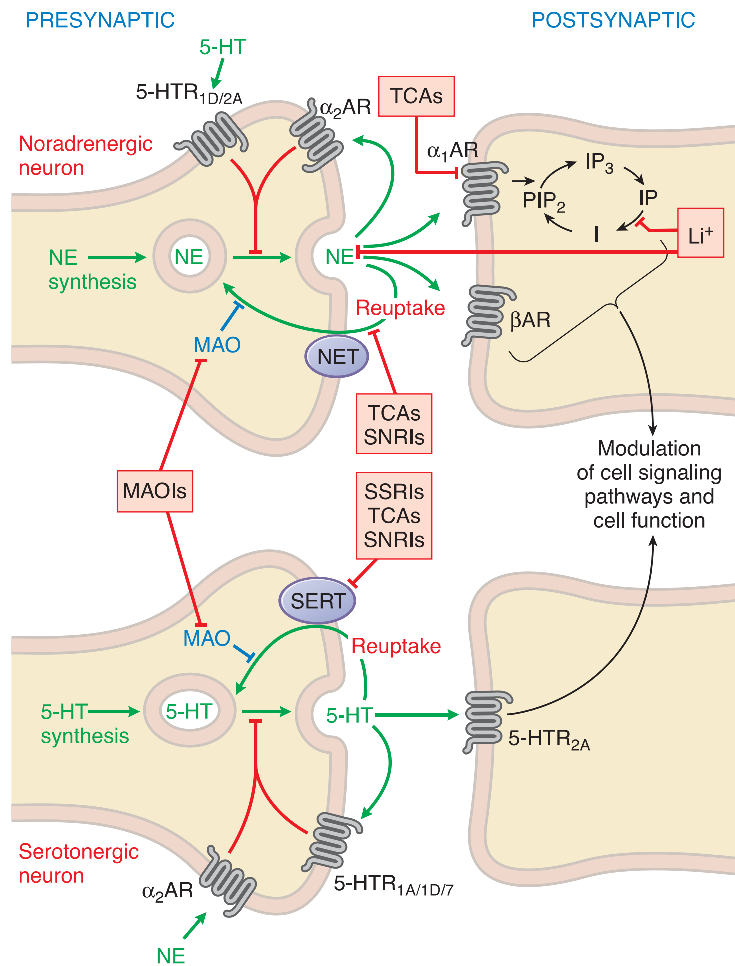


Figure 1.3. **Sites of action of antidepressants**. Schematics representing noradrenergic (top) and serotonergic (bottom) nerve terminals. SSRIs, SNRIs, and TCAs increase noradrenergic or serotonergic neurotransmission by blocking the norepinephrine or serotonergic transporter at presynaptic terminals (NET, SERT). MAOIs inhibit the catabolism of norepinephrine and serotonin. Some antidepressants such as trazodone and related drugs have direct effects on serotonergic receptors that contribute to their clinical effects.
Chronic treatment with a number of antidepressants desensitizes presynaptic autoreceptors and heteroreceptors, producing long-lasting changes in monoaminergic neurotransmission. Post-receptor effects of antidepressant treatment, including modulation of GPCR signaling and activation of protein kinases and ion channels, are involved in the mediation of the long- term effects of antidepressant drugs. Note that NE and 5-HT also affect each other's neurons. From Brunton et al. 2011.

SSRIs have been developed from 1984-1997 show a clear improvement in safety margin compared to the TCAs and are much safer in overdose, and in clinical practice have affected a broad range of psychiatric, behavioral, and medical

conditions, for which they are used, on and off label. SSRI treatment causes stimulation of 5-HT_{1A} and 5-HT_7 autoreceptors on cell bodies in the *raphe nucleus* and of 5-HT_3 autoreceptors on serotonergic terminals, and this reduces serotonin synthesis and release toward pre-drug levels. With repeated treatment with SSRIs, there is a gradual down-regulation and desensitization of these autoreceptor mechanisms. In addition, down-regulation of postsynaptic 5-HT_{2A} receptors may contribute to antidepressant efficacy directly or by influencing the function of noradrenergic and other neurons via serotonergic heteroreceptors. Other postsynaptic 5-HT receptors likely remain responsive to increased synaptic concentrations of 5-HT and contribute to the therapeutic effects of the SSRIs. Further, repeated treatment with SSRIs reduces the expression of SERT, resulting in reduced clearance of released 5-HT and increased serotonergic neurotransmission.

SNRI block both SERT and NET with lesser side effect compared to TCAs. Several antagonists of the 5-HT_2 family of receptors are effective antidepressants, although most agents of this class affect other receptor classes as well.

The SSRIs, unlike the TCAs, do not cause major cardiovascular side effects. The SSRIs are generally free of antimuscarinic side effects (dry mouth, urinary retention, confusion), do not block histamine or α adrenergic receptors, and are not sedating. The favorable side effect profile of the SSRIs may lead to better patient compliance compared to that for the TCAs. However, SSRIs are not free from side effects. Excessive stimulation of brain 5-HT_2 receptors may result in insomnia, increased anxiety, irritability, and decreased libido, effectively worsening prominent depressive symptoms. Excess activity at spinal 5-HT_2 receptors causes sexual side effects including erectile dysfunction, anorgasmia, and ejaculatory delay; these effects may be more prominent with paroxetine. Stimulation of 5-HT_3 receptors in the CNS and periphery contributes to GI effects, which are usually limited to nausea but may include diarrhea and emesis (Vaswani et al. 2003).

The SNRIs have desirable safety advantages over the TCAs. SNRIs have a side effect profile similar to that of the SSRIs, including nausea, constipation, insomnia, headaches, and sexual dysfunction.

TCAs are potent antagonists at histamine H_1 receptors; H_1 receptor antagonism contributes to the sedative effects of TCAs. Antagonism of muscarinic acetylcholine receptors contributes to cognitive dulling as well as a range of adverse effects mediated by the parasympathetic nervous system (blurred vision, dry mouth, tachycardia, constipation, difficulty urinating). Weight gain is another side effect of this class of antidepressants.

Hypertensive crisis resulting from food or drug interactions is one of the life-threatening toxicities associated with use of the MAOIs. Foods containing tyramine are a contributing factor. MAO-A within the intestinal wall and MAO-A and MAO-B in the liver normally degrade dietary tyramine. However, when MAO-A is inhibited, the ingestion of certain aged cheeses, red wines, sauerkraut, fava beans, and a variety of other tyramine-containing foods leads to accumulation of tyramine in adrenergic nerve endings and neurotransmitter vesicles and induces norepinephrine and epinephrine release. The released catecholamines stimulate postsynaptic receptors in the periphery, increasing blood pressure to dangerous levels (cheese-reaction). These episodes can be reversed by antihypertensive medications. Even when the patient is highly vigilant, dietary indiscretions or use of prescription or over-the-counter medications that contain sympathomimetic compounds may occur, resulting in a potentially life-threatening elevation of blood pressure. In comparison to tranylcypromine and isocarboxazid, the selegiline transdermal patch is better tolerated and safer. Another serious, life-threatening issue with chronic administration of MAOIs is hepatotoxicity.

A comprehensive list of antidepressant drugs, with the selectivity against SERT, NET and DAT is presented in **Table 1.3**.

Table 1.3. Potencies of Antidepressants at the Human Transporters for Norepinephrine (NET), Serotonin (SERT), and Dopamine (DAT)

DRUG	NET	SERT	DAT	Selectivity
NE Selective				**NET *vs* SERT**
Oxaprotiline	5	4000	4350	800
Maprotiline	11.1	5900	1000	532
Viloxazine	156	17,000	100,000	109
Nomifensine	15.6	1000	55.6	64
Desipramine	0.8	17.5	3200	22
Protriptyline	1.4	19.6	2130	14
Atomoxetine	3.5	43	1270	12
Reboxetine	7.1	58.8	11,500	8.3
Nortriptyline	4.4	18.5	1140	4.2
Amoxapine	16.1	58.5	4350	3.6
Doxepin	29.4	66.7	12,200	2.3
5-HT Selective				**SERT *vs* NET**
S-Citalopram	7840	1.1	>10,000	7127
R,S-Citalopram	5100	1.4	28,000	3643
Sertraline	417	0.3	25	1390
Fluvoxamine	1300	2.2	9100	591
Paroxetine	40	0.1	500	400
Fluoxetine	244	0.8	3600	305
Clomipramine	37	0.3	2200	123
Venlafaxine	1060	9.1	9100	116
Nor_1-citalopram	780	7.4	—	105
Nor_2-citalopram	1500	24	—	63
Zimelidine	9100	152	12,000	60
Trazodone	8300	160	7140	52
Imipramine	37	1.4	8300	26
Norfluoxetine	410	25	1100	16
Amitriptyline	34.5	4.3	3200	8.0
Duloxetine	11.2	1.6	—	7.0
Dothiepin	45.5	8.3	5300	5.5
Norsertraline	420	76	440	5.5
Milnacipran	200	123	—	1.6
DA Selective				**DAT vs NET**
Bupropion	52,600	9100	526	1000

Values shown are experimentally determined constants (Ki values, nM) for inhibiting the function of human NET, SERT, and DAT expressed in cell lines. Drugs shown include clinically used antidepressants, important metabolites, and experimental drugs not used clinically. Selectivity is defined as ratio of the relevant Ki values (SERT/NET, NET/SERT, NET/DAT). Bupropion is selective for the DAT relative to the NET and SERT. Source: Data are adapted from Brunton et al. 2011.

In recent years, there has been a particular focus from the pharmaceutical industry on the reduction of side effects associated with earlier medications, particularly with the creation of the second (SSRIs) and third (non-SSRIs) generation antidepressants (Olver et al. 2001). In general, their safety profile is good, with a very low chance of fatality (Hawton et al. 2010). In sum, standard antidepressants appear to have some efficacy in relieving depressive symptoms, particularly for those in a severe depressive episode and when administered in combination with other antidepressants medications or psychotherapy. However, a number of issues have been raised regarding both the time taken to induce meaningful treatment impact and also the side effects associated with their administration. Most importantly however, standard antidepressants provide no meaningful improvement for a substantial proportion of MDD patients.

1.1.4 – Healthcare cost of treating depression

The treatment of depression is another aspect that should be taken into account. In 2016, the WHO and the World Bank announced that investing in the treatment of depression and anxiety leads to four-fold returns, since these pathologies cost the global economy one trillion US $ each year (Bernaras et al. 2019). The cost of treating depression is similar to the cost of other degenerative diseases, and its magnitude is similar to that of cancer, AIDS, and coronary heart disease. Due to relapse rates of 50-70% (Zajecka 2000), the global cost of depression in terms of functional impairments such as lost productivity, absenteeism and reduced productivity at work, as well as greater health care costs, is one of the higher compared to other mental diseases (Hamre et al. 2010; Chiu et al. 2017; Amos et al. 2018).

1.2 – Nutrition and mental health

The beneficial effects of a diet rich in fruits, vegetables, nuts and vegetable-based ingredients (whole grain, olive oil) especially on cardiovascular disease

(CVD) prevention, are well known and generally recognized (Oyebode et al. 2014; Wang et al. 2014). In addition, poor nutrition correlates with high blood cholesterol levels, obesity, heart disease, diabetes, and some types of cancer (Bremner et al. 2020).

In recent years, however, evidence of possible effects of diet rich of fruits, vegetables, nuts, whole grain and olive oil on various aspects of mental health are emerging.

Over the past decade, nutritional psychiatry has developed into a promising research area in a field with great unmet need. Nutrition and mental health are interconnected and affected by each other. It has been found that healthy nutrition plays an important role in the onset and severity of a various mental health disorders, indeed people with mental health disorders often have nutritional deficiencies (Lakhan and Vieira 2008). This link between nutrition and mental health has been noticed primarily with depression (Berk and Jacka 2019; Marx et al. 2020).

Earlier observational and small intervention studies pointed mainly on possible effects of diet rich in the above dietary items or of the entire dietetic patterns containing them. An example is the so-called "Mediterranean diet", plant-based diet typical of the olive growing areas of the regions around the Mediterranean Sea, as described by Kromhout et al. (1989) and, more recently, by Vitiello et al. (2016).

These reports pointed mainly on cognitive performances (Kang et al. 2005; Nooyens et al. 2011), prevention of depression (Sanchez-Villegas et al. 2009; Tsai et al. 2012; McMartin et al. 2013; Skarupski et al. 2013; Sanchez-Villegas et al. 2015) and, generally, on psychological well-being (Beezhold et al. 2010; Piqueras et al. 2011; Blanchflower 2012; White et al. 2013; Conner et al. 2015; Huang et al. 2019). The available evidence suggest that increased consumption of fruit and vegetables is associated with both cognitive benefits and psychological well-being. In the meantime, in the interventional study front, randomized controlled registered trials begin to appear (Conner et al. 2017). The observational and

interventional investigation of the effect of the whole dietetic patterns on brain health, while being powerful tools, cannot elucidate which specific fruits and vegetables and which molecules are responsible for the observed effects. Interventional studies with single fruit or vegetables are necessary to assess the possible efficacy of individual fruit/vegetable types on brain health. Up to now, various studies *in vitro* and *in vivo* (the latter on animal models and on human) suggested various possible brain effects of various fruits and vegetables fresh and processed, such as gold kiwifruit (*Actinidia chinensis*, Planch.) (Carr et al. 2013), blackcurrant, blueberry, cherry, cranberry, grape, apple (Bell et al. 2015), orange juice (Kean et al. 2015; Alharbi et al. 2016) and onion (Sakakibara et al. 2008). The *in vivo* investigation has the potential to test fruit/vegetable or their molecule (single or in combination) within the complex contest of a mammalian or even on human, but, on the other side, are generally non able to address the molecular targets of the treatment. *In vitro* investigation can furnish precise information on specific molecular targets but gives not information about the real efficacy in the whole organism.

Around 25% of patients with neuropsychiatric conditions, including mood disorders and schizophrenia, exhibit increased levels of inflammation (Bauer and Teixeira 2019) and oxidative stress (Moylan et al. 2014). It is well reported that healthy diet has anti-inflammatory properties and could ameliorate oxidative stress supplying compound with a well-known anti-inflammatory and antioxidant properties (Giugliano et al. 2006; Cocate et al. 2014; Liao et al. 2019). For example, Vitamins such as ascorbic acid (vitamin C) and alpha tocopherol (vitamin E) have direct free radical scavenging properties (Traber and Stevens 2011). Nutrients such as selenium, zinc and cysteine are cofactors for antioxidant systems such as glutathione peroxidase and superoxide dismutase. Thus, the fruits/vegetables could act on more general processes that are responsible also for the neuropsychiatric disorders. With regards to the possible molecules responsible for the observed activities, very few reports unravel the precise molecular components responsible of the fruit/vegetable activity (e.g. the identification of

two phytochemicals in Concorde grape juice preparation responsible for the reduction of stress and peripheral inflammation (Wang et al. 2018)).

The identification of the precise active molecules of fruit/vegetable is complicated by the fact that some biological effects of fruits and vegetables could depend from additive, synergic and antagonist effects between phyto-complex components rather than on the individual metabolites, and this make it difficult (Liu 2003).

1.2.1 – Kiwifruit composition and properties

The earliest record of kiwifruit (*Actinidia deliciosa*, Chevall. Liang & Ferguson) in the world were the result of adventurous European botanists traveling through China that brought samples of the kiwifruit vine to Europe. Kiwifruit was originally known as “Chinese Gooseberries”, however was considered difficult to market the fruit under that name outside the mainland China. The idea to rename the fruit “kiwifruit” is credited to Frieda Caplan. With its brown furry skin, which resembled New Zealand’s iconic native bird, Frieda suggested to rename the fruit “kiwifruit” to get a better marketing response. “Kiwifruit” is therefore analogous to “*mihoutao*” (monkey peach) widely used in China for the genus *Actinidia*. All *Actinidia* species are climbing vines. Botanically, *Actinidia* fruit can be described as berries with many seeds embedded in a juicy pericarp. Fruit of *A. deliciosa* can contain up to 1400 seeds and fruit size depends on the number of seed set (Boland and Moughan 2013). The most recent revision of the genus recognizes 55 species and 20 varieties. Traditionally, the composition of fruit has been considered primarily in terms of the nutritive components (protein, lipids, carbohydrates, vitamins, and minerals). Kiwifruit is often considered as a high fiber fruit; however, when compared to other fruit, their dietary fiber content is not especially high, at 2–3% of fresh weight (FW), similar to apples, oranges, and bananas.

The fruit is also rich in secondary specialized metabolite compounds belonging to the chemical classes of carotenoids, phenolics, flavonoids, vitamins, chlorophylls, and organic acids. Secondary metabolites play a role in the

interactions of a cell with its environment, represented by other cells in the organism or external organisms or abiotic factors e.g., in case of plants to attract pollinators, or to defense itself against pests and diseases (Verpoorte et al. 2007).

As main secondary metabolites, the fruit of *Actinidia deliciosa* contains phenolic compounds and indolalkaloids (tryptamine and serotonin). Phenolics are a large group of compounds that are characterized by a chemical structure that contains at least one phenolic group. Phenolic compounds have been associated with antioxidant capacity and have been intensely studied because they are believed to provide health benefits for consumers. In plant foods, phenolics are subdivided into two large groups known as phenolic acids and flavonoids. Tryptamine and 5-HT are secondary metabolites belonging to the class of indole-alkaloids and, more specifically, they are indolamines formed by an indole backbone with an ethylamine side chain, both deriving from the amino acid tryptophan. These compounds have been found to be widespread among all the living kingdoms, from bacteria to higher eukaryotes, with very different distributions and functions (Ramakrishna et al. 2011).

It was observed a significative suppression of the growth of insect pests feeding on tryptamine hyperaccumulating leaves (Gill et al. 2003). These evidences, together with the earlier observation that tryptamine administration through artificial assay was able to negatively affect the reproduction rate in *Drosophila melanogaster* (Thomas et al. 1998) by likely depression-induced anti-oviposition effects, strongly suggest that tryptamine might act as an anti-attractant or antifeedant in plants.

Instead, 5-HT has several fundamental physiological functions in plants such as response to biotic and abiotic stresses (Ishihara et al. 2008; Hayashi et al. 2016), plant growth regulation of the development (Erland et al. 2015) and many others actually under debate such as its involvement in the photosynthesis process, regulation of plant rhythms and signaling (Erland et al. 2019).

Kiwifruit is also recognized as being a huge dietary source of vitamin C, potassium and folate, indeed it contains relatively high levels of vitamin E that are

associated with anti-inflammatory effects. For many years, it was assumed that the vitamin E of kiwifruit was contained predominantly in the seeds in association with the oil content, and not bioavailable as the seeds typically resist digestion. However, more recent analysis has shown that the main α-tocopherol form of vitamin E is in the flesh, possibly associated with cell membranes, and therefore potentially bioavailable (McGhie 2013). Proteins are a minor but significant component of kiwifruit. Crude proteins are typically measured at about 1% of fresh weight; however, soluble proteins are much less, around 0.3%. For green kiwifruit, actinidin is the predominant soluble protein comprising up to 40% of soluble protein. Despite its components that are beneficial to health and nutrition, kiwifruit contains a number of elements that can be potentially detrimental to health, in particular, allergens and oxalate. When considering the total benefit to health and nutrition of kiwifruit, it is important to balance both the potential beneficial and deleterious effects (Boland and Moughan 2013).

Kiwifruit also delivers less available energy relative to other foods than is assumed based on traditional measures of food energy content (Drummond 2013). This, together with the key nutritional elements of kiwifruit, supports its position as a highly nutritious, low-calorie fruit with the potential to deliver a range of health benefits. For a review see data from USDA Database (Release 24): http://www.ars.usda.gov/ Services/docs.htm?docid1⁄48964.

1.3 – Omics Era

Nowadays, as with other branches of biological sciences, the study of metabolism (plant and animal) has significantly improved since the quick advent of powerful technological tools for the functional characterization of genes and metabolites in all living systems. The mining and exploitation of the data obtained from classical (e.g. genomics, transcriptomics, proteomics and metabolomics) as well as new-born omics sciences restricted to specific topics (e.g. secretomics,

regulomics, epigenomics, glycomics, phenomics) brought us into a new era of understanding of biological systems (Oksman-Caldentey et al. 2004; Rai et al. 2017; Dhungana et al. 2020). The burst in the development of high-resolution metabolomics techniques based on mass-spectrometry (MS), allowed to establish high accurate and sensitive untargeted methods for the separation and detection of compounds present in trace amounts, e.g at the femtograms scale, in complex mixtures (Breitling et al. 2013). Since the spread of ultra-performance liquid chromatography-MS (UPLC-MS) platforms, which can produce about 20% more metabolite detection than that of high-performance liquid chromatography-MS (HPLC-MS) (Nordstrom et al. 2006) several technological innovations triggered metabolites research.

1.3.1 – Metabolomics approach to unveil food properties

Goodacre defines "metabolomics" or "metabonomics" as the study of global metabolite profiles in a system (cell, tissue or organism) (Goodacre et al. 2004). The metabolome includes metabolites such as carbohydrates, peptides, amino acids, nucleic acids, minerals, organic acids, vitamins, alkaloids and polyphenols. Simplified, as stated by Dettmer *et al*. (2007) the genome describes what can happen, the transcriptome what appears to be happening, the proteome what makes it happen and the metabolome describes what has happened and is currently happening. The metabolome of an organism is influenced by a number of factors, diet included (Zivkovic and German 2009).

While the field of disease-related metabolomics is relatively developed, the area of nutritional metabolomics, and especially how this relates to food intake, is still in its infancy. The food metabolome has been defined as "the part of the human metabolome directly derived from the digestion and biotransformation of foods and their constituents" (Scalbert et al. 2014). Traditional methods for assessing dietary exposure include dietary records, food diaries, 24-hour dietary recalls, food frequency questionnaires and diet history records. These methods can be unreliable since depend on subjects' own reports with under reporting as

one of the main problems (Coulston et al. 2013). Instead, using analytical tool such as metabolomics, allow the simultaneous characterization of a large number of metabolites in biological samples, providing the possibility of mapping the complex metabolism of food consumption and the biological consequences of different diets (Wishart 2008). Recently nutritional metabolomics is rapidly evolving to integrate the multitude of nutritional patterns that arise from the ingestion of foods or their bioactive food components with complex data to discover new biomarkers of nutritional exposure and can help disentangle the molecular mechanisms by which diet affects health and disease (Trujillo et al. 2006). Moreover, applying chemometric tools, i.e., multivariate data analysis, global metabolic profiles/fingerprints can be characterized together with the identification of candidate food biomarkers. Among all the *-omics*, metabolomics plays a crucial role in the field of nutrition because it is more time sensitive than other *-omics* and can reflect the current biological status of an individual. Metabolomics can provide a comprehensive picture of overall dietary intake by measuring the full profile of small molecule metabolites in biological samples such as saliva, blood, and urine. Thus, it could help deepen our knowledge of metabolic pathways relevant to human nutrition (Gibney et al. 2005).

Diet can have effects on two different components of the metabolome: the endogenous metabolome, referring to all metabolites present in a biological sample of the host, and the food metabolome, which includes metabolites that are derived from food consumption and their subsequent metabolism in the human body (e.g., carotene for fruits and vegetables and proline-betaine for citrus fruits) (Heinzmann et al. 2010; Scalbert et al. 2014). Studies of nutritional metabolomics need to account for intersubject metabolic variation and should be able to deal with measurements of subtle metabolic modulations against relatively low doses of bioactive food nutrients or supplements (Rezzi et al. 2007).

In recent years two groups have independently identified proline betaine as a marker of citrus fruit consumption (Mennen et al. 2006; Heinzmann et al. 2010). Other candidate biomarkers for fruit and vegetable intake include antioxidant

vitamins such as vitamin C (Padayatty and Levine 2008) and flavonoids (Mennen et al. 2006), including quercetin (McAnlis et al. 1999). Tea consumption is a major dietary source of polyphenolic compounds, including phenolic acids and flavanoids. Several potential biomarkers of exposure to tea derived polyphenols have been identified (Hodgson et al. 2004). Although biomarkers cannot replace traditional dietary assessment methods, the use of metabolomics in identifying novel and robust biomarkers of dietary exposure and intake can enhance and validate such methods (O'Gorman et al. 2013).

It is important to note that many of the foods consumed are highly correlated, and there is a risk of identifying biomarkers that are not specific to the particular food of interest (Scalbert et al. 2014). For example, vitamin C, several carotenoids, and flavonoids are common to many fruits and vegetables; therefore, they can be used as generic biomarkers of total fruit and vegetable intake but not specific to individual fruits or vegetables. Most studies focused on the identification of dietary biomarkers of specific food and food groups, whereas fewer identified biomarkers of single nutrients (Scalbert et al. 2014).

In this thesis, high resolution UPLC-ESI-MS is used to investigate specific components responsible for kiwifruit activity on brain health.

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Chapter 2 – *In vivo* experiments on well-validated behavioral models combined with metabolomics approaches reveal antidepressant-like activity of kiwifruit

2.1 – Introduction

Animal research has been an integral component of scientific discovery and therapeutic drug development for decades also because many responses to stimuli are well conserved across mammalian species. For that reason, animal models, especially rodents, are potentially relevant to human disorders. The findings that have been facilitated through animal modeling of disease are invaluable, demonstrating their necessity in furthering understanding of human pathophysiology and treatment (Alcantara et al. 2017).

To test the neurobiological theories of common human diseases and to develop novel targets for drug discovery, valid animal models are required. There have been only a few proposed criteria for what constitutes an ideal animal model, and minor adaptations have evolved these criteria to encompass more complex assessments and interpretations, indeed animal models are expected to hold face, construct, and predictive validity. The question of validity criteria was raised by McKinney & Bunney (1969), the authors proposed that an animal model should recapitulate most core symptoms of the disorder being modeled (**face validity**); that symptoms induced by the model should be malleable to pharmacological intervention (**predictive validity**); and should parallel, based on what is known about the human disorder, the modeled condition, the disease etiology (**construct validity**).

Many models meet more than one validity criterion, and as understanding of the underlying biology of disorders advances, one can extend criteria to include "**biological validity**." Recently there has been a push for more biologically relevant

animal models whose main requirement is to mimic the neurobiological correlates of diseases (**pathological validity**); such models require complex tools, and this is a rapidly growing area of research (Krishnan and Nestler 2011). Achieving these criteria could be a challenging task for a number of reasons. The difficulty in achieving the face validity stems primarily from the obvious interspecies differences between mice and men, indeed some symptoms are uniquely human (e.g., depression symptoms like depressed mood and suicidal thoughts). Also, heterogeneity of symptoms in human disease could further complicate the question. Indeed, a large variability of symptoms means that the clinical picture of the disease might vary significantly from patient to patient, and therefore it is not immediately clear which behavioral pattern needs to be reproduced in a model (Nestler and Hyman 2010).

The difficulty in achieving the construct validity is based on the fact that neurobiological etiology of some diseases is not yet completely understood and hence cannot be fully replicated. Therefore, an alternative approach has been suggested, to expose animals to a known environmental risk factor of the disease. As stress has been identified as a primary risk factor for depression (Kendler et al. 1999), this strategy became widely utilized in animal depression studies using different types of stress exposure to induce depression-like behavior.

The following paragraph will discuss some of the animal models currently used in depression research and how they meet current expectations of the field.

2.1.1 – Animal models of depression

To date many different types of stress have been found to induce various depression-like behaviors in rodents. One of the first forms of stress-based models was application of electric foot shock which resulted in behavioral response named *learned helplessness* (Seligman and Maier 1967). This behavioral phenomenon signifies acquisition of a passive approach to coping with stress, which occurs after several exposures in unescapable conditions and manifests itself as a failure to flee the shock when a means of escape is provided.

Passive coping has become a focus of attention in another model, Porsolt's swim test (Porsolt et al. 1977), commonly referred to as a *forced swim test* (FST). Porsolt found that rats placed in an unescapable cylinder with water first display active attempts to escape and vigorously swim around the cylinder, however within minutes they become less active and finally resort to passive floating, with minimal movement just enough to keep the head above the water. This response has been dubbed "behavioral despair", as it appears to reflect animals "giving up" on their chance to escape the water. Behavioral despair can also be triggered in a *tail suspension test* (TST), in which mice hung by the tail switch from initial struggling and climbing attempts to passive immobility (Steru et al. 1985). The validity claims of the three models rest mainly on their sensitivity to the antidepressant treatment. Indeed, antidepressants from most classes administered acutely and chronically have been shown to delay the onset of learned helplessness and behavioral despair in FST and TST (Cryan et al. 2005a; Cryan et al. 2005b). The foremost strength of these models is their ability to rapidly screen novel agents and for that reason they have become a gold standard for the early screening of novel molecules with putative antidepressant-like effects.

However, the construct and face validity of these models have been heavily criticized; some authors recommend to view behavioral despair tests solely as screening tests of antidepressant activity rather than stand-alone models (Nestler and Hyman 2010). Currently FST and TST are widely utilized as tests of depression-like behavior, to establish if more etiologically relevant chronic stress exposure facilitates display of behavioral despair.

While repeated stress models have been shown to induce depressive-like behavior, their construct validity is limited by the use of a single type of stress, while human experience is not limited to a single stressor. Moreover, repeated exposure to stressors removes the unpredictability factor and is likely to induce habituation, which can diminish behavioral outcome (Girotti et al. 2006).

There are a lot of other tests commonly used to "replicate" depression-like behavior in rodents' but this is beyond the scope of the present thesis.

Table 2.1 summarize the routinely behavioral assessment with vantages/disadvantages of the corresponding tests linked with symptoms they intend to model.

Table 2.1. Assessment of various behavior and their advantages and disadvantages

Behavioral Assessment	Emulated MDD Behavior	Suitable Strains	Test Advantages	Test Disadvantages
Shock Avoidance/Escape Latency (ET)	Hopelessness	Holtzman	Creates depression susceptible (LH) and resilient (NLH) groups for comparison	Requires additional rats due to the differentiation of LH and NLH rats; requires specific equipment and program administration and analysis
		cLH	Similar to human patients with treatment-resistant MDD	Does not allow for comparison of LH and NLH rats
Forced Swim Test (FST)	Despair	Most Rodents	Quick and inexpensive; simple drug screening test	Weak validity and high variability depending on factors like temperature of water, size of cylinder, depth of water; results can be compounded by motor deficits
Tail Suspension Test (TST)	Despair	Mouse models	Inexpensive; simple drug screening	Only tested in mice; results can be compounded by motor deficits
Open Field Test (OFT)	Anxiety/Motor-locomotion	Most Rodents	Quick and inexpensive; simple drug screening test	Does not specifically test depression - only anxiety
Sucrose Preference Test (SPT)	Anhedonia	Most Rodents	Inexpensive; simple drug screening test	Weighing or measuring methods may vary from one lab to another; spillage can occur
Elevated Plus Maze (EPM)	Anxiety	Most Rodents	Simple process for anxiety assessment	Does not specifically test depression - only anxiety
Intracranial Self-Stimulation (ICSS)	Reward Sensitivity/Anhedonia	Most Rodents	Precise administration and drug localization is known to the researcher	Requires surgery, canula and proper administration equipment
Operant Fear Conditioning	Fear/Anxiety	Most Rodents	Inexpensive; simple drug screening	Does not specifically test depression - generally for anxiety

Modified from Wang et al. 2017.

To assess the ability of kiwifruit to reduce immobility time in both TST and FST, and thus to exert antidepressant-like activity, mice were chronically treated for 10 days with fruit juices, through intragastric gavage (IG) administration. After

completing the behavioral tests mice were sacrificed to collect serum and brain samples for metabolomic analysis, and behavioral data were then compared to analytical data. Moreover, pharmacokinetic (PK) experiments were performed to estimate adsorption and clearance of kiwifruit selected compounds.

2.2 – Materials & methods

2.2.1 – Animals

Naïve male C57BL/6JOlaHsd mice (Envigo RMS Srl, San Pietro al Natisone, Udine, Italy) aged 5 weeks were used (n=12/16 for behavioral experiment, n=4 for PK experiment and n=6 for perfusion experiment). On arrival mice, weighing 20-25 gr were housed six per cage in Optimice® cages (36.3x29.2x15.5) with sawdust as a bedding material at constant room temperature (21 ± 1 °C) and relative humidity (60%) with a 12h light cycle (light on 7:00 AM-7:00 PM) with food (Mucedola NFM18) and water accessible *ad libitum*. Animals were allowed to adapt to laboratory conditions for at least two weeks before the beginning of the experimental procedures. Procedures involving animals were conducted at University of Verona which adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorization n.19/2008-A issued March 6, 2008 by Ministry of Health); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE).

2.2.2 – Reagents

Fluoxetine HCl (FLX) was obtained from Alomone labs (Jerusalem, Israel); Acetonitrile (ACN), methanol (MeOH) and waters were purchased from Honeywell (Seezle, Germany); formic acid (HCOOH) was from Biosolve (Dieuze, France) and leucine-enkephalin solution from Waters (Milan, Italy). All solvents were of liquid chromatography-mass spectrometry (LC-MS) grade.

2.2.3 – Fruit sampling and fresh juice preparation

Kiwifruits (*Actinidia deliciosa* cv. Hayward) were sourced from local producers, prepared by cutting peeled slices of each sample, freezing them immediately in liquid nitrogen and storing them at – 80 °C. The frozen material was powdered using an A11 basic analytical mill (IKA-Werke, Staufen, Germany) and the powder was stored at – 80 °C. Fresh juice was prepared by weighing and thawing 27 g of frozen homogenized powder and centrifuging at 3650× g for 15 min at 4 °C. The supernatant was then transferred to a fresh tube and centrifuged at 21,000× g for 15 min at 4 °C. The supernatant was passed through a 0.22-µm Millex PES filter (MilliporeSigma) to remove high fiber content of the phytocomplex. Kiwifruit was also split into other two different dilutions, starting from the most concentrated and undiluted called **Kiwi 1** and with a 1:2 and 1:3 dilutions in water MilliQ® (MilliporeSigma) for **Kiwi 2** and **Kiwi 3** respectively and given to animals at a volume of 10 ml/kg (Howard et al. 2011).

2.2.4 – NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was conducted in collaboration with the organic chemistry group of the University of Verona leaded by Professor Michael Assfalg. Kiwifruit juice samples were prepared by thawing 2–3 g of homogenized kiwifruit flesh powder as previously described (Section 2.2.3). The thawed fruit was first carefully mixed and then sonicated for 15 min in a cold-water bath. The suspension was then centrifuged at 15,000× *g* for 10 min at 4 °C, and the supernatant was transferred to a fresh tube and centrifuged at 18,000× *g* for 20 min at 4 °C to remove the remaining insoluble debris. We then diluted 0.56 ml of the soluble aqueous extracts to a final volume of 0.7 ml in 0.15 M potassium phosphate buffer (pH 6.0), 0.02% (w/v) sodium azide, 5% (v/v) D_2O (Cambridge Isotope Laboratories, Cambridge, UK), and 1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid-d_6 (DSS-d_6) (MilliporeSigma). NMR spectra were recorded at 298 K using a Bruker Avance III instrument (Bruker, Karlsruhe, Germany) equipped with

a triple resonance TCI cryogenic probe and operating at a ^{1}H Larmor frequency of 600.13 MHz. We acquired ^{1}H-NOESY spectra with a mixing time of 100 ms, a recycle delay of 10 s, 64 free induction decays (FIDs), 64,000 data points, and a spectral width of 20 ppm. Spectra were processed with Topspin v3.2 (Bruker) by multiplying FIDs with an exponential weighting function with line broadening of 0.3 Hz before Fourier transformation, phasing and baseline correction. Spectra were then referenced to the DSS-d_6 singlet signal and analyzed using Chenomx NMR Suite v8.0 (Chenomx, Alberta, Canada) and by comparison with the Biological Magnetic Resonance Bank (http://www.bmrb.wisc.edu/). The identified metabolites were quantified by integrating the NMR signals using DSS-d_6 as an internal standard. The Chenomx software was also used to quantify metabolites with overlapping signals, such as fructose. We then corrected the resulting values with the dilution factor and converted to milligrams per 100 grams of fresh fruit based on the starting weights and volumes.

2.2.5 – Vehicle and neutralized juice preparation

On the basis of our NMR analysis of kiwifruit and to exclude the possibility that bulk primary metabolites (sugars, organic acids and ascorbic acid) could have some effect on behavioral tests, we created a vehicle solution replicating the concentrations of these major components. The composition of the complete vehicle solution was 10.29 g/L sucrose, 33.5 g/L fructose, 35.94 g/L glucose, 0.7 g/L ascorbic acid, 9 g/L citric acid and 8.53 g/L malic acid, based on undiluted kiwifruit. In order to limit the number of candidate molecules of kiwifruit juice, we passed the undiluted kiwifruit juice (kiwi 1) through a Discovery® DSC-MCAX SPE C8 mixed-mode cation exchange cartridge (MilliporeSigma) according to the manufacturer's instructions to remove a fraction of the secondary metabolites. Briefly, columns were activated with 15 ml of ethanol for 15 minutes, then equilibrated with 25 ml of an acid aqueous solution with 1mM of malic acid (pH 3.3) and finally samples were loaded and eluted. The "composition" of kiwifruit and neutralized juice determined by LC-MS based untargeted metabolomics (see below) is shown in Appendix Table 1. M/Z, retention time and relative

quantification were reported for both identified and unidentified metabolites. Vehicle and neutralized juice were administered to animals at a volume of 10 ml/kg (Howard et al. 2011).

2.2.6 – Forced swim test

Mice were placed in a transparent plexiglass cylinder (46 cm height x 20 cm diameters) filled with 30 cm of water at a temperature of 25±1 °C for 6 minutes. The dimensions of the tanks were selected to ensure that the mice will not be able to touch the bottom of the tank, either with their paws or their tails, during the test. One rectangular wood divider is used between tanks to prevent mice from seeing each other during the test and potentially altering their behaviors. A white background was used in order to increase contrast in the video recorded between mice and wall. Test consist of 6 minutes, however only the last four minutes of the test are analyzed due to the fact that most mice are very active at the beginning of the FST (Petit-Demouliere et al. 2005). Immobility time was manually scored by two trained observers.

2.2.7 – Tail suspension test

Similar to the forced swim test, the tail suspension test involves recording immobility of mice exposed to an inescapable stressful situation. In this case mice were suspended above the ground by their tails through a tape of 17 cm that connect the tail at the fixed grid suspended 60 cm from the bench (Steru et al. 1985; Can et al. 2012). One rectangular wood divider is used between tanks to prevent mice from seeing each other during the test and potentially altering their behaviors. A white background was used in order to increase contrast in the video recorded between mice and wall. This test has been extensively validated pharmacologically (Cryan et al. 2005a) and can be used alongside, or as an alternative to, the FST. A further issue with this test is the curious ability of mice of the C57Bl/6J strain to climb up their own tails, therefore confounding assessment of the time spent immobile (Mayorga and Lucki 2001). Those mice that

excessively display this acrobatic attempt to escape usually are excluded from further analysis, which can lead to sampling bias. For that reason, we decided to use a climbstopper (4 cm length, 1.6 cm outside diameter, 1.3 cm inside diameter, 1.5 grams) to prevent this behavior according to Can and colleagues (2012) (**Figure 2.1**). Immobility time was manually scored by two trained observers.

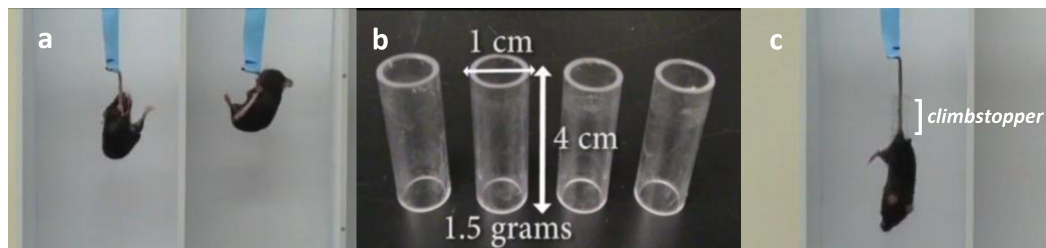


Figure 2.1. **a)** C57BL/6J in TST without climbstopper, **b)** climbstopper itself, **c**) C57BL/6J in TST with climbstopper that prevent the climbing behavior.

The behavioral tests were repeated 2 times and the sum of the two experiments were reported.

2.2.8 – Open field test

Since both FST and TST are basically test that involves locomotion, independent assessments of locomotor activity should be made to ensure the selectivity of any changes in immobility given by treatments. Mice were assessed for activity in a 40x40 cm open square arena for 5 minutes. During the test the arena was evenly lit with low light (~40 Lux). Each mouse was taken from its cage and placed in pre-room for acclimatation to the novel condition for at least 1 hour before the test. Total distance travelled and mean velocity by each mouse were recorded and then analyzed by a MATLAB® Toolbox (Tort et al. 2006).

2.2.9 – Sample preparation and extraction

After completing behavioral test mice were sacrificed to collect serum and brain samples. Under deep isoflurane anesthesia blood samples were collected from the retro-orbital sinus through a glass non-heparinized capillary. Blood was left clotting at room temperature for 20 minutes and then centrifuged at 6,800× g

for 5 minutes at 4°C. Supernatants were collected, frozen in liquid nitrogen and then stored at – 80°C. Brain samples were extracted form skull, washed in 0.9% saline, immediately frozen in liquid nitrogen and then stored at – 80°C until further analysis.

2.2.10 – Metabolite extraction for targeted and untargeted UPLC-MS analysis

Serum samples were thawed at room temperature and once defrosted 1 ml of serum were added with 10 volumes of cold (– 20°C) LC-MS grade methanol. The mixture was gently mixed using "vortex" mixer for 30 s and then centrifuged at 3650× g for 15 min at 4 °C. The supernatant was then transferred to a fresh tube and centrifuged at 21,000× g for 20 min at 4 °C. The supernatant was passed through an Oasis® PRiME HLB 1 cc Vac 30 mg Sorbent per Cartridge (Waters, Milan, Italy) attached to a Waters 20-Position Extraction Manifold (Waters, Milan, Italy) to remove common matrix interferences such as proteins and phospholipids (**Figure 2.2**) according to manufacturer's instructions. Briefly these cartridges don't need to be activated or equilibrated, simply samples are loaded and collected immediately with a pass-through method.

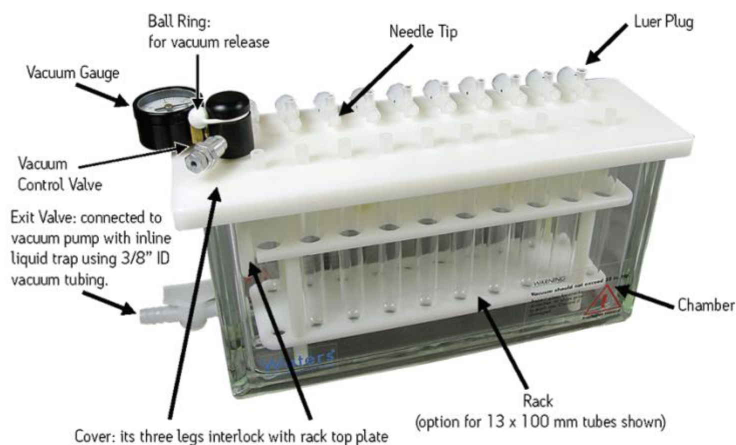


Figure 2.2. **Waters 20-Position Extraction Manifold**. This method, alongside with the Oasis® PRiME HLB cartridge, allows to remove protein and lipid components of samples.

For the metabolomics analysis, the methanol extracts were diluted 1:2 (v/v) with LC-MS grade water (Honeywell) for C18 analysis and 1:2 with methanol LC-MS

grade for HILIC analysis. Extracts were then passed through Minisart RC4 filters with 0.2 µm pores (Sartorius, Göttingen, Germany) and 3 µL were injected into the UPLC device.

Brain samples were weighted and then 10 volumes of cold (– 20°C) LC-MS grade methanol was added. The mixture was homogenized with a Precellys cryolys® evolution (Bertin, Montigny-le-Bretonneux, France) at 4 °C, then sonicated at 40 kHz in a Sonica Ultrasonic Cleaner ultrasonic bath (SOLTEC, Milano, Italy) for 20 min before two rounds of centrifugation, first at 3650× *g* for 15 min and second at 21,000× g for 15 min at 4° each. The supernatant was passed through an Oasis® PRiME HLB 1 cc Vac 30 mg Sorbent per Cartridge (Waters, Milan, Italy) as detailed before, diluted and filtered like before for the injection in the UPLC-ESI-MS.

For kiwifruit and neutralized juice, samples were prepared as already described in section 2.2.3, then 100 µl of sample were added with 900 µl of cold (– 20°C) LC-MS grade methanol. The mixture was gently mixed and centrifuged at 21,000× g for 10 min at 4 °C. The resulting supernatant was collected, diluted and filtered like before for the injection in the UPLC-ESI-MS.

2.2.11 – UPLC-MS untargeted metabolomics

An ACQUITY I CLASS UPLC system (Waters, Milford, MA, USA) equipped with refrigerated autosampler was connected to a Xevo G2-XS qTOF mass spectrometer (Waters, Manchester, UK) featuring an electrospray ionization (ESI) source operating in either positive or negative ionization mode and was controlled by MassLynx v4.1. All extracts were injected into a both Waters ACQUITY UPLC BEH C18 and HILIC column (2.1 mm × 100 mm, 1.7 µm) kept at 30°C. C18 mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B), the initial conditions were 99% A and 1% B and the following elution profile was applied: 0-1 min, 1% B; 1-10 min, 1-40% B; 10-13.50 min, 40-70% B; 13.50-15.00 min, 70-90% B; 15.00-16.50 min, 90-100% B, 16.50-20 min, 100% B, 20-20.1 min, 100-1% B (initial conditions). Subsequently, the system was equilibrated in 99% A and the

elution was complete after 25 min. HILIC mobile phases consisted of 20 mM of ammonium formate in water (A) and 5% of water containing 10 mM of ammonium formate and 95% of acetonitrile (B). The initial conditions were 0% A and 100% B, and the following elution profile was applied: 0–3 min, 100% B; 3–7 min, 100–85% B; 7–10 min, 85% B; 10–15 min, 85–50% B; 15–20 min, 50% B; 20–20.10 min, 50–100% B (initial conditions). Subsequently, the system was equilibrated in 100% B and the elution was complete after 30 min. The flow rate was set to 0.350 mL/min for both columns. Samples were kept at 8 ◦C and analysis were randomized. A quality control (QC) sample was prepared by mixing equal parts of all samples in order to check the UPLC-qTOF performance along the whole experiment. QC was injected after nine samples had been analyzed. The ion source parameters were capillary voltage 0.8 kV, sampling cone voltage 40 V, source offset voltage 80 V, source temperature 120 ◦C, desolvation temperature 500 ◦C, cone gas flow rate 50 L/h and desolvation gas flow rate 1000 L/h. Nitrogen gas was used for the nebulizer and in desolvation whereas argon was used to induce collision-induced dissociation. An MS method was created to acquire data in continuum mode using a fixed collision energy in two scan functions. In function 1, the low energy was disabled, whereas in function 2 the high energy was set to 35 V. For some samples, the high energy was increased to 45 V in order to achieve the better fragmentation of certain metabolites. In both functions, the Xevo G2-XS was set to perform the analysis in sensitivity mode, within the range 50–2000 m/z and with a scan time of 0.3 s. The lock mass solution used as "calibrator" to verify the accuracy of the mass spectrometer consisted of a 100 pg/µL leucine-enkephalin solution (Waters) injected with a flow rate of 10 µL/min, generating a signal of 556.2771 in positive mode and 554.2615 in negative mode.

2.2.12 – Data processing and metabolite identification

Statistical analysis of behavioral experiment was carried out using Prism v9.0 (GraphPad Software, San Diego, CA, USA). Significant differences between

samples were determined by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test.

The raw data generated during the untargeted metabolomics analysis were processed using Progenesis QI software (Waters). An absolute ion intensity for peak picking was set at 300 and a minimum chromatographic peak width at 0.03 min. An automatic online search of public databases (MassBank, PlantCyc, Plant Metabolic Network and Human Metabolome Database) was used for tentative metabolite identification, by comparing *m/z* ratios, isotope similarities and fragmentation patterns, using the individual compound fragmentation extrapolated by Progenesis QI software. Further identifications were achieved using Metlin (https://metlin.scripps.edu) with a tolerance of 0.003 Da, and an in-house library of authentic standards. Finally, data reported in literature was used to support the putative annotations. Since internal standards were not applied, the relative quantitation (i.e. comparison between samples) was based on the area of each of the signals extracted from the chromatograms and expressed in arbitrary intensity units.

2.3 – Results

2.3.1 – Kiwifruit chronic treatment decrease mouse immobility time in TST and FST

The possible antidepressant activity of different kiwifruit extract dilutions (kiwi 1, kiwi 2 and kiwi 3) were investigated with both TST and FST through the evaluation of the ability to reduce immobility time. Animals, once arrived, were allowed to adapt to the laboratory conditions for at least 2 weeks. After this period animals were weighted and handled every day to get them habituated and thus to reduce stress by human manipulation, indeed IG administration is very stressful since elicits immediate changes in physiological parameters indicative of stress (elevation of blood corticosterone, glucose, growth hormone and increase hearth rate and blood pression)(Howard et al. 2011). For that reason, 1 week before

starting animal procedure, mice were allowed to adapt to IG administration (IG of water twice a day) since is reported in literature that after 4 days there is a decrease in all parameters mentioned above (Howard et al. 2011). For kiwifruit extracts, vehicle and neutralized juice, mice were treated chronically for 10 days through IG administration and tested after 90 min whereas Fluoxetine (Prozac®) was used as positive control drugs and administered intraperitoneally (IP) 30 min prior the test at a concentration of 20 mg/kg (Lucki et al. 2001; Tang et al. 2014). All groups were compared against our negative control, the vehicle-treated group. Kiwi 1 strongly reduce immobility time in both tests compared to vehicle-treated group [$F_{(6,88)}$=15.39, p<0.0001; $F_{(6,88)}$=11.79, p<0.0001 for TST and FST respectively] whereas kiwi 2 and kiwi 3 were less active, with kiwi 3 activity that completely disappear in FST (**Figure 2.3**). Fluoxetine, our positive control didn't work in FST paradigm, but this problem is reviewed below in the discussion paragraph. Locomotor activity to evaluate possible locomotor impairment were assessed on day 8 in an OFT, as previously described. No motor impairment was found between different groups of animals (**Figure 2.4**).

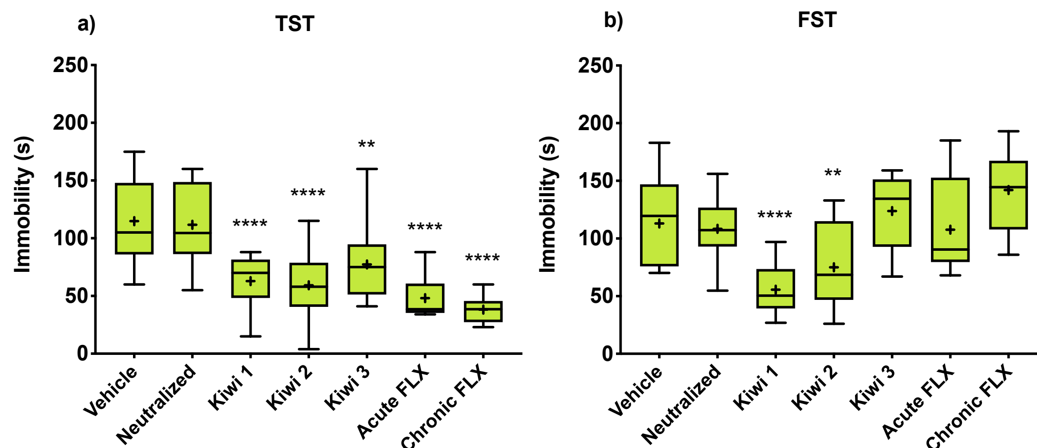


Figure 2.3. **Boxplot representing the effect of extracted Kiwi at different dilutions on immobility time in TST and FST.** The immobility time of TST (**a**) and FST (**b**) were assessed 90 min after administration of Vehicle, Neutralized juice, Kiwi 1, Kiwi 2, Kiwi 3 at a volume of 10 ml/kg and 30 min after administration of 20 mg/kg of fluoxetine (FLX) at a volume of 10 ml/kg. Values were evaluated by one-way ANOVA followed by Dunnett's post hoc test and expressed as mean ± SD (n=12/16 per group). **p≤0.01, ****p ≤0.0001 *vs.* vehicle-treated group. FLX=Fluoxetine

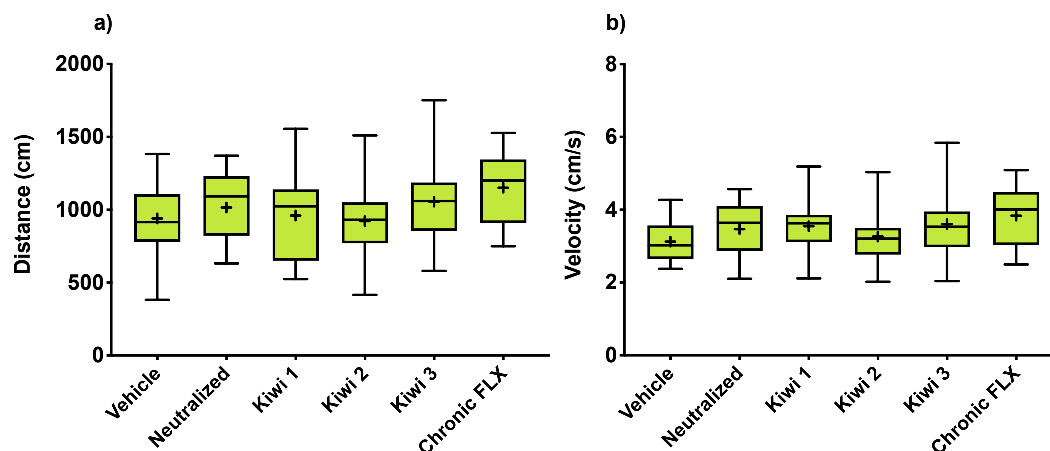
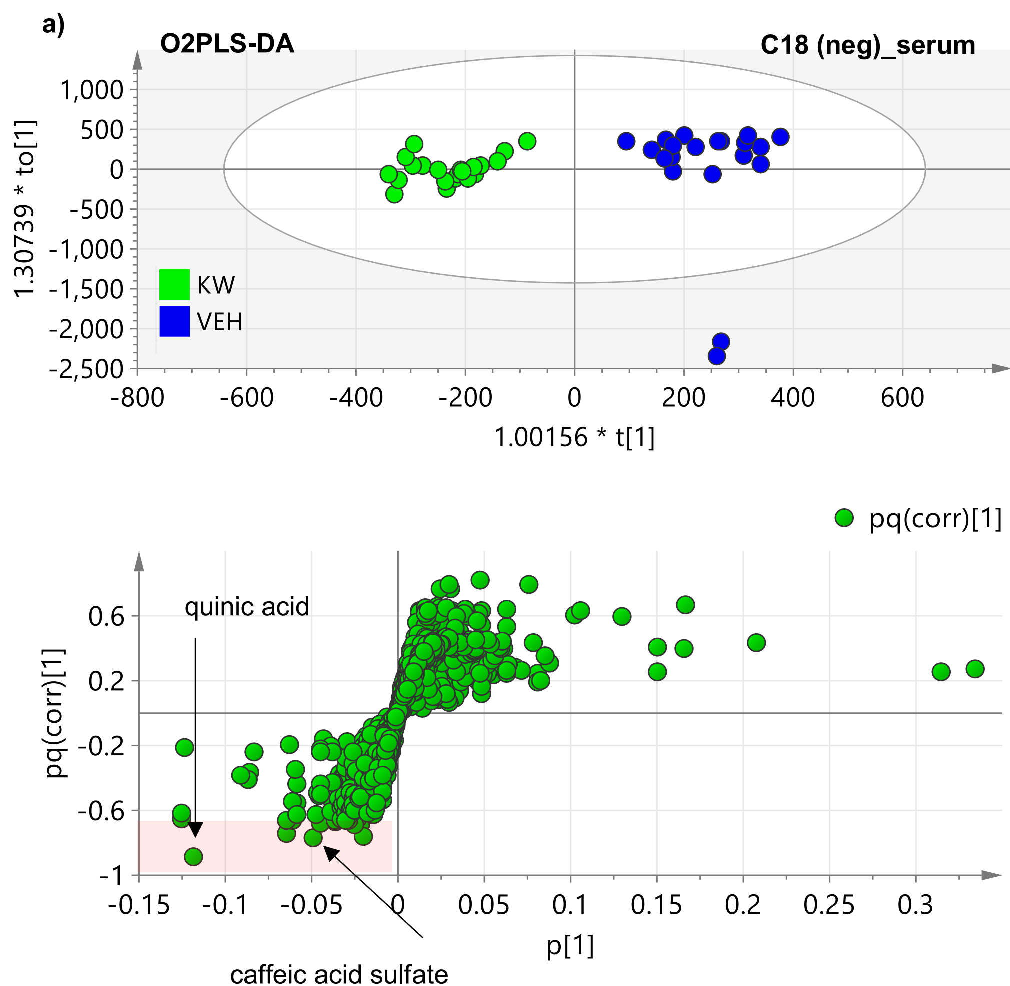


Figure 2.4. Boxplot representing the effect of extracted Kiwi at different dilutions on distance travelled and mean velocity of mice in OFT. Distance (**a**) and mean velocity (**b**) were evaluated in 5-min open field test 90 min after administration of Vehicle, Neutral, Kiwi 1, Kiwi 2, Kiwi 3 at a volume of 10 ml/kg and 30 min after administration of 20 mg/kg of fluoxetine (FLX) at a volume of 10 ml/kg. No differences were found between different treated groups. Values were evaluated by one-way ANOVA and expressed as mean ± SD (n=12/16 per group). FLX=Fluoxetine

2.3.2 – Only few specific kiwifruit metabolites can be recovered in mouse serum and brain after kiwifruit juice administration

In an attempt to identify possible kiwifruit candidate molecules for the observed neuroactivity, the bioavailability of kiwifruit metabolites in serum and brain was investigated. After behavioral experiments, serum and brain samples were collected for further UPLC-ESI-MS analysis to evaluate kiwifruit metabolites bioavailability in these tissues/organs in the different experimental groups. An untargeted metabolomics approach was followed, and in order to identify putative kiwifruit metabolites (both known and unknown) from the endogenous molecules, kiwi 1 treated group was compared with vehicle-treated one. A multivariate data analysis of the feature quantification matrix was used to discriminate differences between these two groups. The feature quantification matrix obtained by using Progenesis QI, including *m*/*z* features and their relative abundances in each of the samples, was submitted to orthogonal two-block partial least squares-discriminant analysis (O2PLS-DA) through SIMCA 13.0 (Umetrics) after Pareto scaling and centering. In serum we found, in both kind of

chromatographic analysis (based on C18 and HILIC chemistry, to include as many metabolites as possible, from the high polar to the medium and low polar ones), only few putative kiwifruit metabolites i.e., metabolites detectable only in kiwi 1 treated group compared to the vehicle-fed mice (**Figure 2.5**). Most of them were putatively identify and two resulted to be quinic acid, an acidic secondary metabolite abundantly present in kiwifruits, and caffeic acid sulfate, which is probably the mouse catabolite of the caffeic acid-based molecules present in kiwifruit (caffeic acid glucosides and esters, see Appendix Table 1). In the following chapter of this thesis the parameters used for the identification of these two metabolites are discussed in detail.



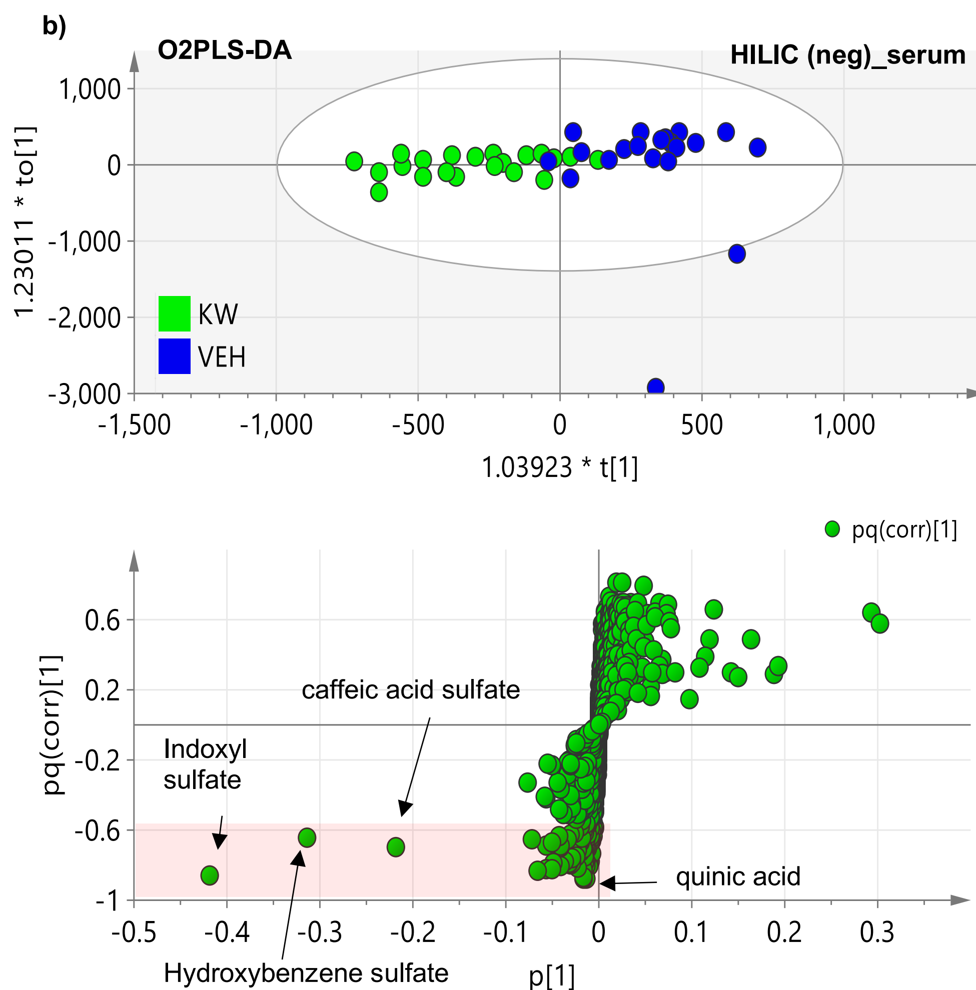


Figure 2.5. **O2PLS-DA overview score plot and S-loading plot of serum samples analyzed in negative ionization mode with C18 (a) and HILIC (b) columns.** In the score plot each dot represents one sample, in the S-loading plot each dot represents one metabolite.

Other two metabolites found only in kiwi treated mice, putatively identified as indoxyl sulfate and hydroxybenzene sulfate, are not kiwifruit metabolites; they could be either catabolites of some fruit metabolites or endogenous mice metabolites that increase after treatment.

The same two metabolites, quinic acid and caffeic acid sulfate, were also found in brain samples. The relative quantification of quinic acid and caffeic acid sulfate in serum and brain tissue of mice treated with all the kiwifruit juice dilutions and with the “neutralized” kiwifruit juice obtained by the above UPLC-ESI-MS analysis, is reported in **Figure 2.6**. We detected quinic acid only with C18

chromatographic condition while caffeic acid sulfate is better detected with HILIC condition. Since the levels of the detected caffeic acid sulfate in the brain using the HILIC technique was lower than the Limit of Detection (LOD, the lowest quantity of a substance that can be determined with a level at least 5 time higher that the background), we decided to not evaluate them.
As expected, both molecules levels linearly decrease with increasing dilutions of the kiwifruit extracts [$F_{(3,20)}$=46.35, $p<0.0001$; $F_{(3,20)}$=9.644, $p<0.0004$ of quinic acid for serum and brain respectively and of caffeic acid sulfate only for serum $F_{(3,20)}$=16.34, $p<0.0001$]. Furthermore, comparing the kiwifruit and "neutralized" kiwifruit metabolome, previously characterized, it was possible to see that caffeic acid derivatives was nearly absent in the kiwifruit "neutralized" juice, and quinic acid abundance was lowered (Appendix Table 1). These observations are in line with the very low recovery of these metabolites in "neutralized" juice treated mice. Moreover, the low level of these metabolites in "neutralized" juice, which was unactive in behavioral TST and FST tests, strengthens the hypothesis that one or both of these molecules may be at least partially responsible for the behavioral activities of the kiwifruit juice on mice.

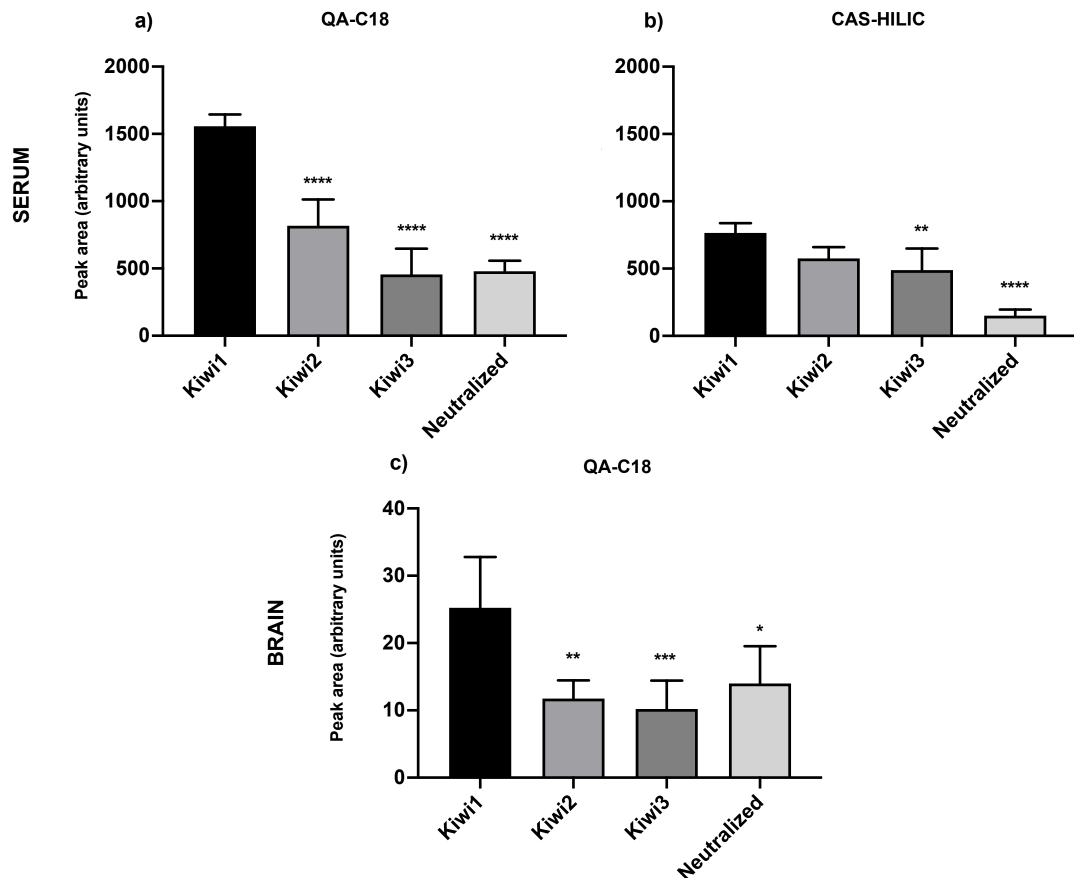


Figure 2.6. **Relative quantification of quinic and caffeic acid sulfate.** Relative quantification of quinic acid (**a**,**c**) under C18 chromatographic condition in serum and brain respectively, and caffeic acid sulfate (**b**) in serum under HILIC condition. Values were evaluated by one-way ANOVA followed by Dunnett's post hoc test and expressed as mean ± SD (n=12/16 per group). *p≤0.05 **p≤0.01, ***p≤0.001 ****p ≤0.0001 *vs.* Kiwi1-treated group. QA=quinic acid, CAS=caffeic acid sulfate.

The appearance and elimination kinetics of quinic acid and caffeic acid sulfate in serum and brain of mice after kiwifruit administration were followed using a pharmacokinetics approach (PK) on different group of mice treated only with the undiluted kiwifruit (kiwi 1) and sacrificed at different time points. Time points used in this experiment are the following: 15min, 30min, 45min, 1h, 1h15 min, 1h30min, 2h, 4h, 8h and 24h. **Figure 2.7** shows the relative quantification in arbitrary units of quinic acid and caffeic acid sulfate in both serum and brain samples. Not surprisingly, relative levels of the two molecules decrease along the time.

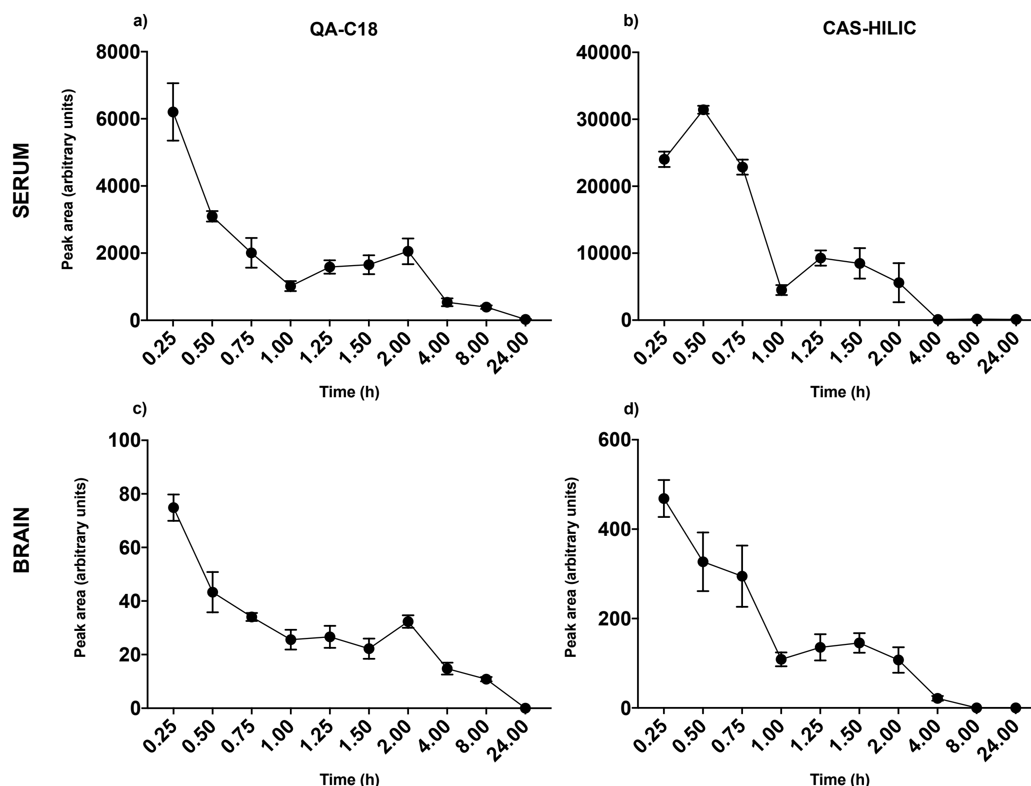


Figure 2.7. **Relative quantification of quinic acid and caffeic acid sulphate in serum and brain of mice after kiwifruit juice administration.** Relative quantification of quinic acid (**a**,**c**) under C18 chromatographic condition in serum and brain and caffeic acid sulfate (**b**,**d**) under HILIC chromatographic condition in serum and brain. Each time point represents the mean ± SD of n=4 per group. QA=quinic acid, CAS=caffeic acid sulfate.

The recovery of quinic acid and caffeic acid sulfate in the whole homogenate of brain tissue can be due to the metabolites contained in the blood vessels of the brain, and thus is not necessarily indicative of the ability of the molecule to pass the blood brain barrier (BBB) and thus on their real presence in brain parenchyma. In order to verify the real ability of quinic acid and caffeic acid sulfate to cross the BBB, we use the technique of perfusion. This technique allowed us to remove the blood from the brain, and thus to evaluate the real presence of quinic acid and caffeic acid sulfate in the tissue. Basically, animal after treatment (kiwifruit juice administration) were anesthetized and the entire blood volume was washed up with cold 0.9% saline and after 5 minutes of perfusion the brain samples were collected and frozen in liquid nitrogen (Gage et al. 2012). Samples were stored at

– 80°C until LC-MS analysis. **Figure 2.8** shows the relative quantification in arbitrary units of brain quinic acid and caffeic acid sulfate after perfusion: the post-perfusion levels of both the metabolites are lower compared to non-perfused samples (about half), but still detectable, proving that the two molecules pass the blood brain barrier and are able to enter in the brain parenchyma [$F_{(5,5)}$=6.112, p=0.0020 and $F_{(5,5)}$=18.61, p=0.0014 for brain quinic acid and brain caffeic acid sulfate respectively].

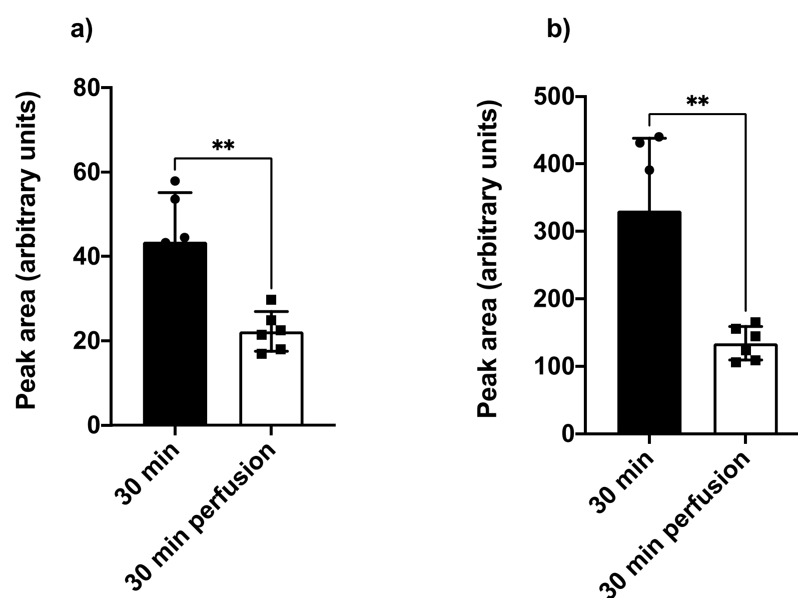


Figure 2.8. Relative quantification of quinic and caffeic acid sulfate in PK experiments after perfusion technique. PK relative quantification in brain of quinic acid under C18 chromatographic condition (**a**) and caffeic acid sulfate under HILIC conditions (**b**). Values were evaluated by unpaired t-test and expressed as mean ± SD (n=6 per group). **p≤0.01.

2.3.3 – Kiwifruit indolalkaloids bioavailability

Serotonin and tryptamine bioavailability were evaluated after chronic treatment in both serum and brain samples of mice through UPLC-ESI-MS analysis. In general, serotonin and tryptamine in the diet are usually degraded by MAO isozymes present in the gut and for that reason they are not bioavailable, but a role for these molecules when eaten within a composite phytocomplex cannot be excluded due to synergic activity between different components. Thus, in

principle, possible MAO-A and B inhibitory components of kiwifruits could change the fate of fruit serotonin and tryptamine making them more bioavailable.
In our data tryptamine were not found in both samples analyzed while for serotonin no differences were found between kiwi 1-fed mice and control group. To exclude definitively the bioavailability of kiwifruit serotonin, PK analysis of mice treated with kiwi 1 dilution added with a detectable deuterated serotonin (Serotonin-d4) were performed.
No differences in administered serotonin-d4 were found in PK analysis (data not shown).

2.4 – Discussion

Our results indicate that oral administration of kiwifruit could have antidepressant-like effect. Moreover, possible kiwifruit candidate metabolites for this activity have been identified. In our behavioral experiments data from different treatment-groups were compared against negative control, that in our case was a vehicle solution, a mix of bulk primary compounds of kiwifruit without any activity.

We decided to adopt TST and FST as behavioral paradigm to assess depressive-like behavior even if there are many other tests commonly used to evaluate this topic since these tests are highly sensitive and are regularly used to evaluate the potency of antidepressant drugs. Moreover, we chose IG administration because it allows to control exactly the administered dose, and because it mimics the real adsorption of the phytocomplex compared to other types of administration.
Kiwifruit highly reduce immobility time in both TST and FST in mice also compared to positive control, which were drugs currently used in clinical treatment of depression, without causing locomotor impairment, as reported by OFT data.

We decided to use FLX at 20 mg/kg as positive control, since this dose is comparable to plasma levels of FLX-treated patients according to Dulawa and colleagues (2004) in both acute and chronic regimes.

However, in FST our positive control did not work as reported in literature with this strain of mice (Mayorga and Lucki 2001). This is probably due to the so-called serotonin (5-HT) syndrome, a serious disease that in humans commonly appears after antidepressant over-dose or after combining several psychotropic medications. This acute 5-HT toxicity has been characterized in mice by expression of certain behavioral and physiological responses as hind limb abduction, forepaw treading, backward movement, straub tail, head weaving, tremor and low flat posture (Diaz and Maroteaux 2011). Strangely we did not observe any locomotor impairment in the OFT test carried out two day before the end of the chronic treatment (Day 8) even if it is reported in literature that motor impairment, if happens, could appear after 3-4 days of chronic treatment (Da-Rocha et al. 1997; Dulawa et al. 2004). For that reason, in the following experiments we decide to change our positive control to another SSRI drug (chapter 3).

Furthermore, analytical data from UPLC-ESI-MS reveal two potential kiwifruit molecules candidate for the observed behavioral activity, present in the serum of kiwi-fed mice. The possible role of these molecules has been tested, and the results are reported and discussed in deep details in the chapter 3 and 4. As expected, these two molecules show serum and brain levels that decrease with linearity with the increase of the kiwifruit dilutions administered (**kiwi1**>**kiwi2**>**kiwi3**).

In addition, “pharmacokinetics” analysis of these kiwifruit metabolites was performed in mice treated with the highest kiwifruit dilution (kiwi1), and relative quantification in brain and serum samples were assessed by UPLC-ESI-MS technique over 24 h period. The accumulation was very rapid, since levels of both metabolites decrease around the baseline between 8 and 24 h.

The approach of studying single molecules bioavailability after whole fruit administration/consumption as a tool to identify possible candidates of

neuroactivities was already positively used by Wang and collaborators (2018) whereby they were able to identify two active molecules, the dihydrocaffeic acid (DHCA) and malvidin-3'-O-glucoside (Mal-gluc), after the administration of Concord grape juice solution.
We therefore analyze a group of animals treated with the same kiwifruit dilution (kiwi1) that underwent a perfusion approach to assess possible brain penetration of the two metabolites of interest; not surprisingly brain levels after perfusion were about the half of the level of non-perfused animals. These data confirm the passage of both molecules into the brain parenchyma.
The technique of perfusion with cold saline is widely used to remove blood from brain or other target tissues in various brain investigation, mainly because in many cases blood could interfere with the following brain analysis (Tsuda et al. 1999; Schinella et al. 2002; Velazquez et al. 2003; Vingtdeux et al. 2010; Wang et al. 2014; Wang et al. 2018) or, in the case of homogenized brain, molecules present in the brain vessels could biases the analysis.
As in this thesis, Wang and collaborators (2013), in another article used this technique to assess the ability various polyphenol metabolites to really pass the BBB.

2.5 – References

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Chapter 3 – Quinic acid is able to mimic kiwifruit juice effects on murine models of depression

3.1 – Introduction

Secondary or specialized metabolites are defined as those metabolites in plants that are not associated with basic life functions such as respiration, growth, and cell division (McGhie 2013). They are less abundant than primary metabolites and have roles that determine plant fitness and define the way that plants interact with an ecosystem. Secondary metabolites have biological activities as diverse as UV absorption (phytoprotection), toxicity to animals and insects (antifeeding properties), color (reproduction) and may be antibiotic, antifungal, and antiviral to provide protection from pathogens (Bourgaud et al. 2001).

In our previous *in vivo* experiments on animal models after chronic administration of kiwifruit juice we found that only very few new metabolites can be found both in mouse serum and brain after juice administration. Between them we could recognize quinic acid (QA), a specialized secondary metabolite of kiwifruit, and caffeic acid sulfate (CA-sulfate), a mouse metabolite produced from the kiwifruit caffeic acid. The latter is abundantly present in kiwifruit juice metabolome in form of various glucosides and esters. Thus, we decided to go further and analyze in deep details the possible effects of these two molecules.

3.1.1 – D-(−)-quinic acid

Quinic acid [(3*R*,5*R*)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid] and its derivatives are compounds containing a quinic acid moiety, which is composed of a cyclohexane ring bearing a carboxylic acid at position 1 and four hydroxyl groups at positions 1,3, 4, and 5 as illustrated in **Figure 3.1a**.

The initial discovery of quinic acid can be traced back to a French chemist named Nicholas-Louis Vauquelin that described it for the first time in the medicinal plant

Chincona officinalis L. (Vauquelin 1806). After that, quinic acid has been widely studied and in the year 1932 Fischer and Dangschat found the structure and stereochemistry of it. Fischer and Dangschat studied also the relationship between the biogenetic origin of quinic, gallic and shikimic acids (**Figure 3.1**).

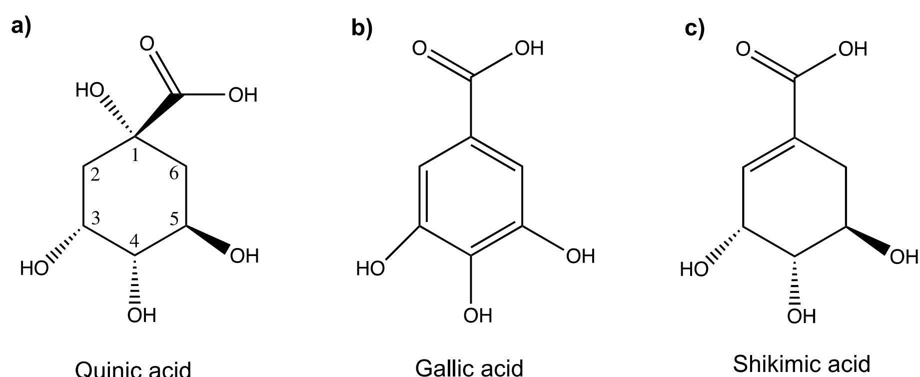

Figure 3.1. Natural products with common biogenetic origin. Chemical structure of Quinic acid (**a**), Gallic acid (**b**) and Shikimic acid (**c**).

They found out that these three acids have a common biogenetic origin and that quinic acid presence can be related to shikimate pathway as a precursor, which is an important multi-step process of chemical reactions within a cell. Shikimate pathway is used in biosynthesis by bacteria, plants, archaea, and others and provides precursors for aromatic amino acids (**Figure 3.2**) (Herrmann 1995; Barco et al. 1997; Ghosh et al. 2012). Interestingly, quinic acid can constitute an alternate or the only source of carbon in several microorganisms (e.g., *Aerobacter aerogenes* and *Klebsiella pneumonia*). In nature, quinic acid is found widely from plants such as coffee beans, *Cinchona* (L.) bark, *Eucalyptus globulus* (Labill.) bark, tobacco leaves and *Urtica dioica* L. (Barco et al. 1997; Mulzer et al. 2008). However, quinic acid free form is rare in vegetables and fruit indeed is mainly and abundantly found in plants as an ester with other secondary metabolites, especially hydroxycinnamic acids such as caffeic acid, producing Chlorogenic acid (CGA) (**Figure 3.2**). In this form it is widespread within the plant kingdom with a protecting activity against microbial attack (Clifford et al. 2017).

In nature, quinic acid can be produced from D-glucose with many reactions in common with the shikimate pathway, through a four steps enzymatic reaction. The pathway starts from by converting D-glucose to D-erythrose-4-phosphate by transketolase. Phosphoenol pyruvate is added to the compound and they react and form 3-desoxyheptulosonic acid (DAHP). With DHQ synthase, the DAHP is converted to 3-dehydroquinic acid (DHQ) which is then converted to quinic acid by DHQ dehydrogenase enzyme. Quinic acid can also be produced from shikimic acid by quinate hydrolyase enzyme (**Figure 3.2**) (Herrmann 1995; Barco et al. 1997; Clifford et al. 2017).

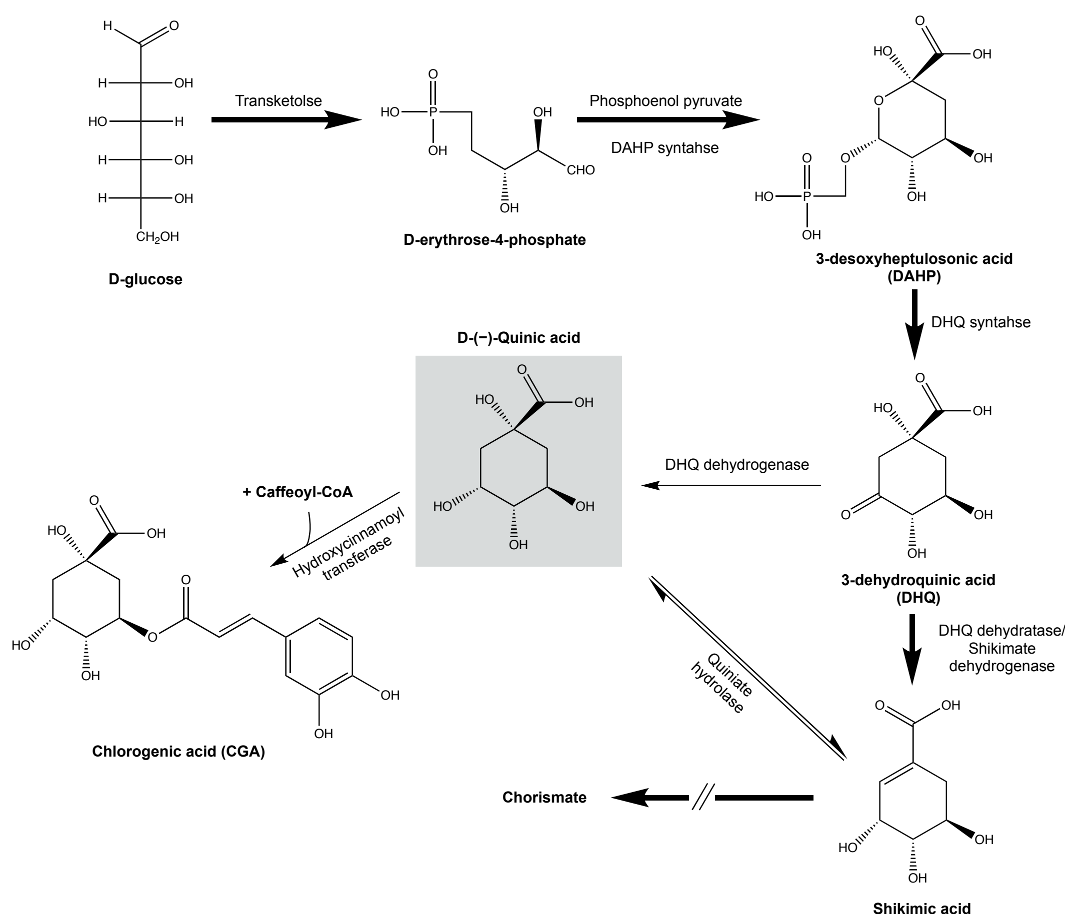

Figure 3.2. Biotransformation of quinic acid and plant shikimate pathway. Bold arrows indicate reactions of the main trunk of the shikimate pathway resulting in the production of chorismate (not shown in figure, see figure 3.3). Quinic acid is the precursor of the CGA via the adding of caffeoyl-CoA or via the p-coumaroyl quinic acid (not shown in figure).

Quinic acid and its derivatives, especially phenolic acid derivatives, have shown a broad-spectrum of antitumor, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective actions in treatment for various types cancer as well as central nervous system-related diseases (Pereira et al. 2003; Chung et al. 2009). For instance, (-)-4-O-(4-O-β-D-glucopyranosylcaffeoyl)quinic acid is demonstrated to display anticancer capability against human colon cancer (Liu et al. 2015).

3.1.2 – Caffeic acid

Caffeic acid (3-(3,4-Dihydroxyphenyl)-2-propenoic acid) (**Figure 3.3**) is a natural phenolic compound belonging to the class of hydroxycinnamic acids (coumaric acid, caffeic acid, ferulic and sinapic acid and their derivatives), a class of aromatic acids which has a phenylpropanoid (C_6-C_3) structure with a 3,4-dihydroxylated aromatic ring attached to a carboxylic acid through a transethylene wire (Espindola et al. 2019). These compounds are hydroxy derivatives of cinnamic acid (Clifford et al. 2017).

It is produced through the secondary metabolism of vegetables and widely distributed in plants, such as coffee, thyme and argan oil (Verma and Hansch 2004).

This phenolic compound is found in the simple form (monomers) as organic acid esters, sugar esters, amides and glycosides (e.g. caffeic acid 3-β-D-glucoside), or in more complex forms such as dimers, trimers and flavonoid derivatives, or they may also be bound to proteins and other polymers in the cell wall of the vegetables (Silva et al. 2014; Tpaovj 2017). CA participates in the defense mechanism of plants against predators, pests and infections, as it has an inhibitory effect on the growth of insects, fungi and bacteria and also promote the protection of plant leaves against ultraviolet radiation B (UV-B) (Espindola et al. 2019).

The biosynthesis of this compound in plants occurs through the endogenous shikimate pathway that is responsible for the production of aromatic amino acids from glucose (Lin and Yan 2012). The reaction starts with shikimic acid and undergoes three enzymatic reactions: the first is a phosphorylation mediated by

the enzyme shikimate-kinase, followed by the conjugation of a molecule of phosphoenolpyruvate, mediated by 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase and finally by the enzyme chorismate synthetase, reaching one of the most important intermediary metabolites of this pathway, chorismic acid. This is transformed into prephenic acid through the enzyme chorismate mutase (a precursor of L-phenylalanine). L-phenylalanine formation is mediated by pyridoxal phosphate (PLP) as a coenzyme in the deamination process and by nicotinamide adenine dinucleotide (NAD) as an electron exchanger. The deamination of L-phenylalanine by the enzyme phenylalanine ammonia lyase (PAL), forms cinnamic acid. It is then converted to p-coumaric acid by the cinnamate-4-hydroxylase (C4H) and finally to caffeic acid through the enzyme 4-coumarate 3-hydroxylase (C3H) (Espindola et al. 2019) (**Figure 3.3**).

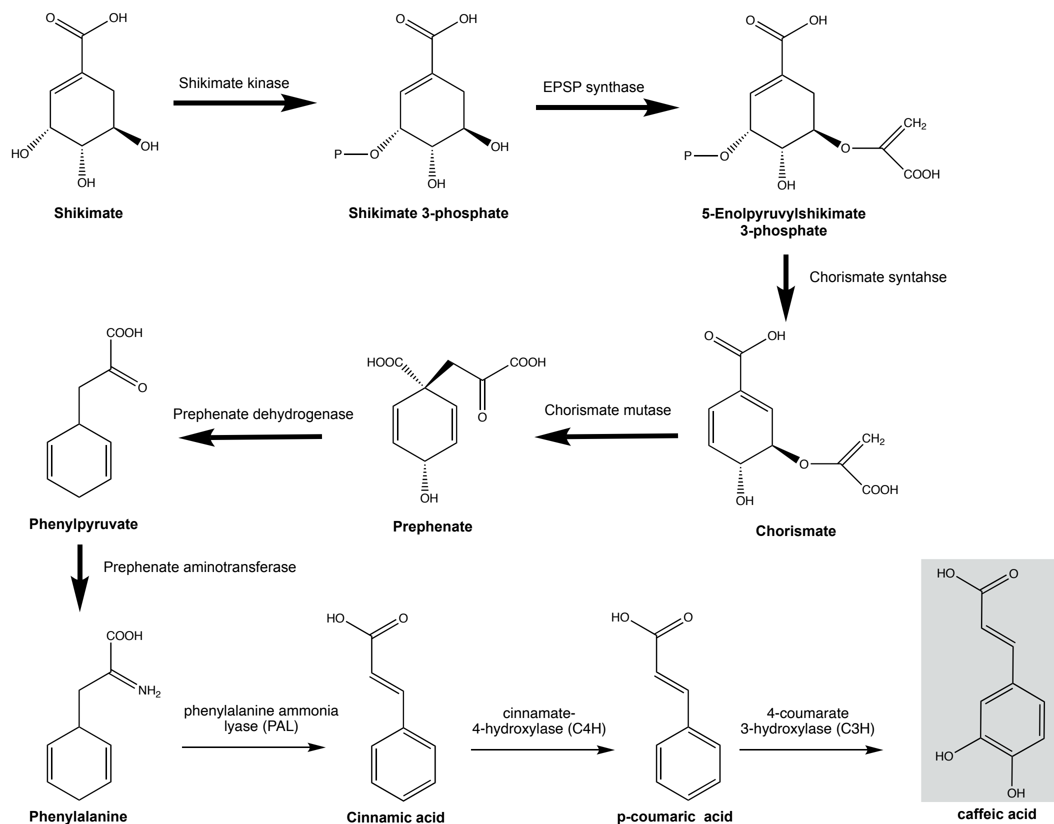

Figure 3.3. Biotransformation of caffeic acid. Bold arrows indicate reactions of the main trunk of the shikimate pathway resulting in the production of phenylalanine.

Caffeic acid has a wide variety of health benefits including anti-inflammatory, anti-cancer, anti-thrombosis, anti-hypertensive, anti-fibrosis, anti-viral, anti-oxidative, and anti-tumor properties (Roos et al. 2011; Scapagnini et al. 2011; U. Rehman and Sultana 2011). It displays antioxidant activity probably by the free radical scavenging properties (Khanduja et al. 2006). Moreover, it inhibits lipid peroxidation and protects DNA from oxidative damage (Khanduja et al. 2006). Caffeic acid has been also shown to have promising beneficial effects in animal models of Alzheimer disease (Kim et al. 2015).
Caffeic acid was also demonstrated to prevent neurotoxicity induced by acrolein *in vitro*. This insult has the ability to mimic many mechanisms that underlie neurodegenerative pathways, such as those activated in AD. Indeed, pretreating cells with caffeic acid decreased neurotoxicity, ROS, and it also exerts indirect antioxidant activity by protecting the essential endogenous antioxidant glutathione (Huang et al. 2013).

3.2 – Materials & Methods

Animals care and treatment, ethical permission, behavioral models involving animals, fresh juice preparation (including vehicle and neutralized juice), sample collection (serum and brain) and extraction for analytical analysis and UPLC-ESI-MS methods are presented in detail in M&M of chapter 2.

3.2.1 – Reagents

Escitalopram was obtained from MedChemExpress (Monmouth Junction, NJ, USA); D-(−)-quinic acid and caffeic acid 3-β-D-glucoside were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All chromatographic solvents (ACN, MeOH, Water and HCOOH) were “liquid chromatography-mass spectrometry” (LC-MS)-grade and purchased from Honeywell (Seezle, Germany); leucine-enkephalin solution from Waters (Milan, Italy).

3.2.2 – Quinic and Caffeic acids quantification in kiwifruit

The absolute quantification of metabolites by LC-MS requires a careful evaluation of the possible matrix effects that can results in ion suppression or enhancement. Using the dilution method shown by Cavaliere et al. (2008) and Toffali et al. (2011), we determined the dilution in which matrix effects for the metabolite of interest disappeared.

Kiwifruit juice (kiwi 1) was sequentially diluted, and we found that at a dilution of 1:1600 for quinic acid and at a dilution of 1:100 for caffeic acid matrix effect was not acting anymore but the two metabolites were still well detectable.

Afterwards, we further check the absence of matrix effect interference of both metabolites in kiwifruit juice by spiking method, according to Toffali and collaborators (2013), by comparison of the peak areas of three groups of samples: (1) diluted kiwifruit juice alone; (2) the same kiwifruit juice of the group 1 spiked with 1.5 ng/μl of D-(−)-quinic acid and 125 pg/μl for caffeic acid 3-β-D-glucoside; (3) solution of pure standard compounds, using the same compounds of the group 2 at the same concentrations. 1 μl of each diluted solution was analyzed three times by UPLC-ESI-MS and no matrix effect were found for both metabolites. The peak area of the metabolites of interest was normalized for the dilution factor and compared with a calibration curve obtained using authentic standards.

3.2.3 – Quinic acid quantification in serum

Quinic acid was also quantified in serum of animals treated with kiwifruit and sacrificed after 30 minutes in both perfused and non-perfused group of animals. A dilution of 1:80 was used. Matrix effect was determined by spiking method according to Toffali and collaborators (2013) using the following three groups of samples: (1) serum sample alone; (2) the same serum sample of the group 1 spiked with 18 pg/μl of D-(−)-quinic acid; (3) solution of pure quinic acid, using the same concentrations of the group 2. 1 μl of each dilution was analyzed three times by UPLC-ESI-MS and a little matrix effect were found, determined and used to obtain a correct absolute quantification.

3.2.4 – Data analysis

Statistical analysis was carried out using Prism v9.0 (GraphPad Software, San Diego, CA, USA). Significant differences between samples were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. UPLC-ESI-MS data processing was already described in chapter 2 (See section 2.2.12).

3.3 – Results

3.3.1 – Identification and quantification of the putative active metabolites of kiwifruit

In the previous experiments (chapter 2) just few metabolites of kiwifruits were found in sera and brains of the kiwifruit-treated mice and putatively identified, namely quinic acid and caffeic acid sulfate, a putative mouse metabolite of kiwifruit caffeic acids.

Quinic acid, abundantly present in kiwifruit in free form, has been identified using authentic commercially available quinic acid, on the basis of retention time (0.81 min) and mass to charge (m/z) ratio (expected m/z: 191.0555, detected m/z: 191.0550, mass error: 2.6 ppm) (**Figure 3.4**).

The caffeic acid sulfate, a putative mouse metabolite of kiwifruit caffeic acids, has been identified on sera and brains on the basis of mass to charge (m/z) ratio (expected m/z: 258.991, detected m/z: 258.990, mass error: 3.8 ppm) and fragmentation pattern (ms/ms), because commercial standard were not available: after fragmentation it generated a fragment of m/z 179.0330, corresponding to caffeic acid (m/z expected: 179.0344) and a fragment of m/z 135.0423 (m/z expected 135.0446), which is a typical and diagnostic fragment of caffeic acid, generated by its decarboxylation (caffeic acid-COOH-H^+).

As shown in the Appendix Table 1, the kiwifruit juice used in the behavioral tests contained four caffeic acid hexoses, one caffeic acid di-hexose, one caffeic acid glucuronide and two unidentified caffeic acid derivatives. The caffeic acid hexoses were identified based on their mass to charge (m/z) ratio (expected m/z:

341.0872, detected m/z: 341.0873, mass error 0.29 ppm) and fragmentation patterns (ms/ms, all generated the fragments with m/z 179.0344 +/-0.005, corresponding to caffeic acid, and 135.0446 +/-0.005, corresponding to caffeic acid –COOH-H^+). Three caffeic acid hexoses showed interesting amount in kiwifruit juice, and one of these, the more abundant caffeic acid hexose, was precisely identified as caffeic acid 3-β-D-glucoside using a commercially available authentic standard, based on retention time (4.6 min), mass to charge (m/z) ratio and fragmentation pattern (**figure 3.4**). The other two peaks showed retention time of 3.98 min and 5.29 min. On the base of their mass to charge (m/z) ratio and fragmentation patterns they can be putatively identified as structural isomers of caffeic acid 3-β-D-glucoside. By the way, the detection of one of the other two peaks in caffeic acid 3-β-D-glucoside commercial authentic standard suggests that the peak at 4.6 minutes and the other peak at 5.29 minutes are isoforms easily converting between each other's (**Figure 3.4**).

In order to check if the antidepressant-like activity of kiwifruit, investigated in chapter 2, could be addressed to quinic acid and caffeic acid glucosides, i.e., those kiwifruit molecules that were able to reach both the sera and the brains of the treated animals, behavioral tests could be performed with the pure molecules at the same concentration of the fruit.

Thus, quinic acid and the two putative isoforms of caffeic acid 3-β-D-glucoside in the fruit needed to be exactly quantified.

For quinic acid quantification in kiwifruit juice, a calibration curve was built with concentrations of the commercial standard ranging from 0.5 to 5 µg/µl with an R^2 of 0.9993. Three different measures of 1:1600 dilutions of kiwifruit juice were plotted against the calibration curve to obtain exact quantification that was 491.26 ± 1.80 mg/100 g fruit fresh weight.

The calibration curve of caffeic acid 3-β-D-glucoside ranged from 31.25 to 312.5 pg/µl with an R^2 of 0.9995. Three different measures of 1:100 dilutions of kiwifruit juice were plotted on the calibration curve to obtain exact quantification that was 6.475 ± 0.026 mg/100 g fruit fresh weight.

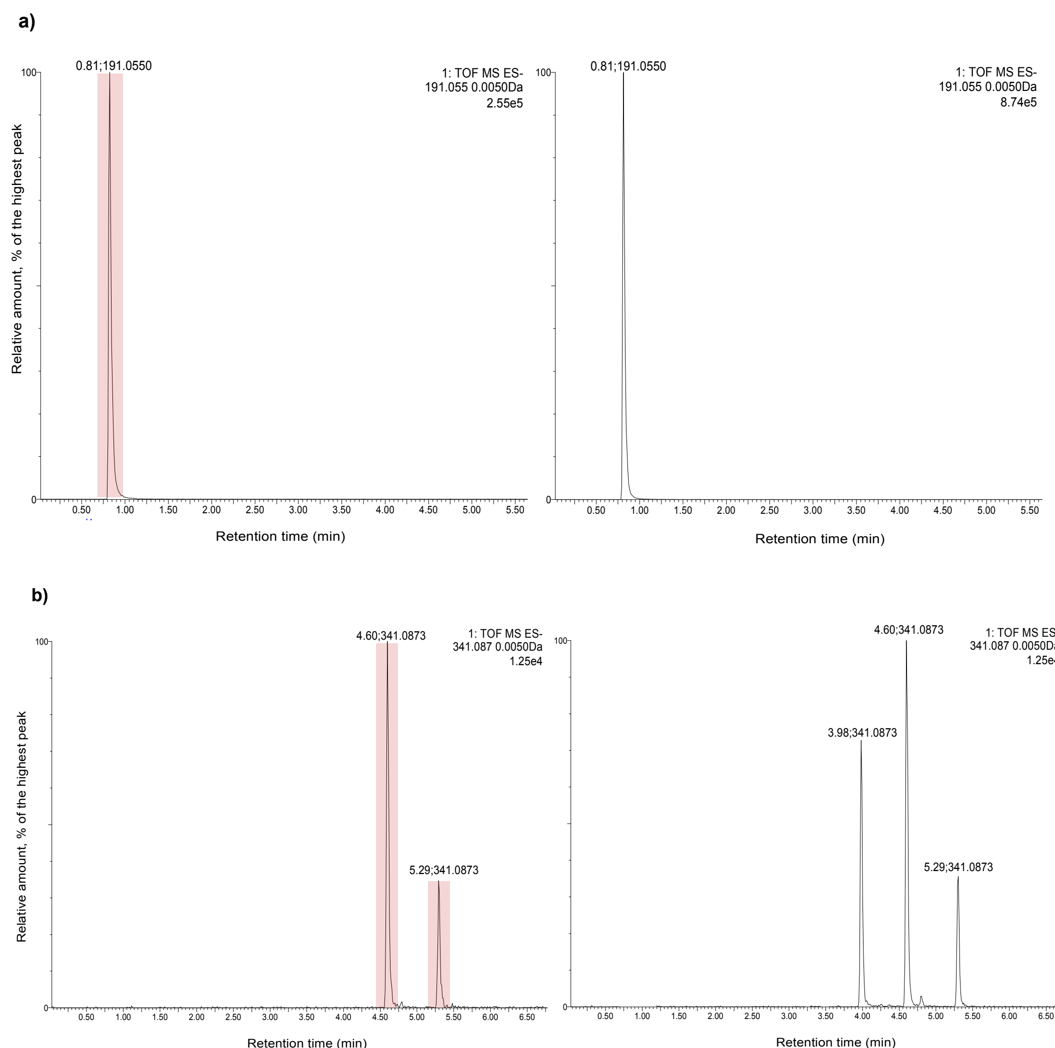

Figure 3.4. **UPLC-ESI-MS chromatograms of specialized metabolites of kiwifruit and their corresponding commercial standards.** Red shadow peaks represent authentic standards whereas non-shadow peaks represent the specialized metabolites found in kiwifruit. Both molecules are visible only in the negative ionization mode. In panel (**a**) is shown quinic acid while in panel (**b**) is shown caffeic acid 3-β-D-glucoside. Above each peak the retention time and the m/z (-) is reported.

3.3.2 – Quinic acid is able to partially mimic kiwifruit antidepressant activity in chronic treatment

The ability of the above-mentioned specialized kiwifruit metabolites to reduce immobility time were evaluated in both TST and FST paradigm using the exact concentration of the metabolites found in the fruit. Kiwifruit extract at the highest dilution (kiwi 1), vehicle (negative control), quinic acid and caffeic acid glucoside at the same concentration ok kiwi 1 and a combination of the two

metabolites were used to chronically treat mice for 10 days through IG administration. TST and FST were performed at day 10 after 90 min from the juice/metabolite administration, while Escitalopram (Cipralex®) was used as positive control drug and administered intraperitoneally (IP) 30 min prior the test at a concentration of 10 mg/kg (Mitchell et al. 2013; Zemdegs et al. 2016). As in the previous experiment (see section 2.3.1 and figure 2.3 in chapter 2), Kiwi 1 confirmed its ability to strongly reduce immobility time in both tests compared to vehicle-treated group; also quinic acid given alone and in combination with caffeic acid had strong ability to decrease immobility time whereas caffeic acid alone was not active even if in this group a high inter-subjects variability was observed [$F_{(5,66)}$=26.33; p<0.0001; F $_{(5,66)}$=11.54, p<0.0001 for TST and FST respectively] (**Figure 3.5**). Escitalopram (ESC), in this case, was a good positive control for both tests as opposed to Fluoxetine that works only on TST (see **Figure 2.3b** in chapter 2). Locomotor activity to evaluate possible locomotor impairment were assessed on day 8 in OFT, as previously described (see section 2.2.8 in chapter 2). No motor impairment was found between different groups of animals (**Figure 3.6**). Thus, quinic acid administration at the same concentration found in kiwifruit can partially mimic kiwifruit antidepressant-like activity on mice, indicating that quinic acid is one of the active anti-depressant molecules of the fruit.

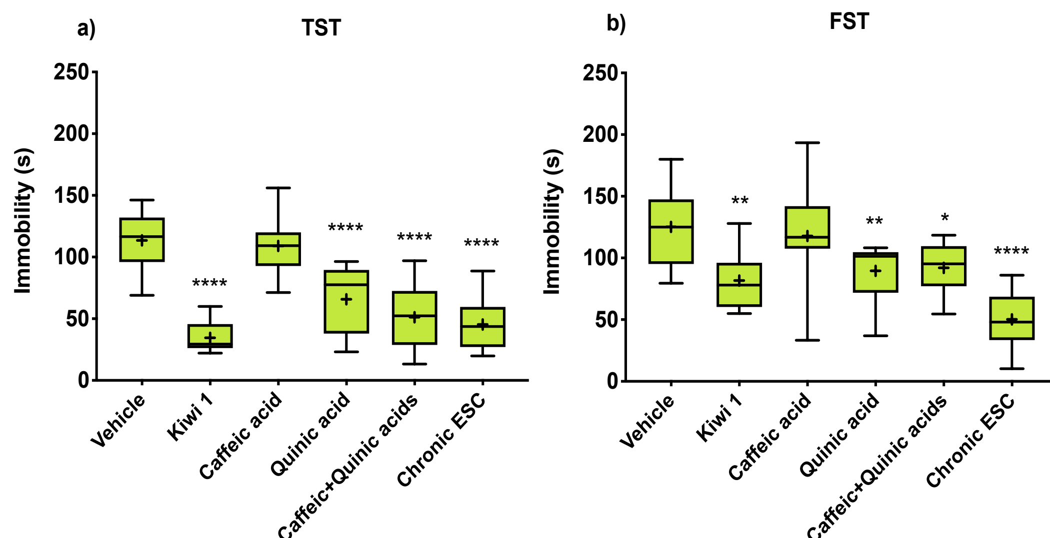

Figure 3.5. **Boxplot representing the effect of kiwifruit and its metabolites on immobility time in TST and FST.** The immobility time of TST (**a**) and FST (**b**) were assessed 90 min after administration of Vehicle (negative control), Kiwi 1, Quinic acid, Caffeic acid and Caffeic+Quinic acids solution at a volume of 10 ml/kg and 30 min after administration of 10 mg/kg of escitalopram (ESC, positive control) at a volume of 10 ml/kg. Values were evaluated by one-way ANOVA followed by Dunnett's post hoc test and expressed as mean ± SD (n=12 per group). * p≤0.05, **p≤0.01, ****p ≤0.0001 *vs.* vehicle-treated group.

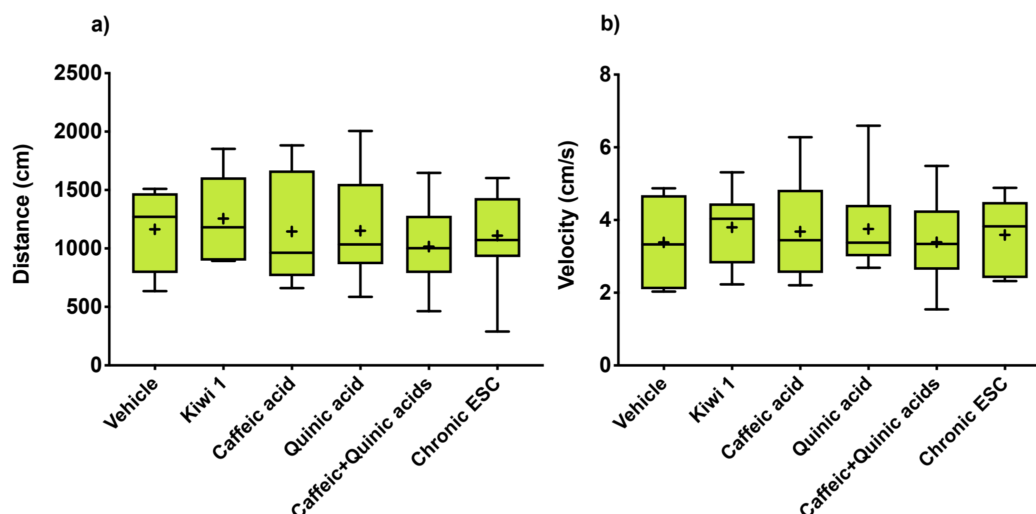

Figure 3.6. **Boxplot representing the effect of kiwifruit and its metabolites on distance travelled and mean velocity of mice in OFT.** Distance (**a**) and mean velocity (**b**) were evaluated in 5-min open field test 90 min after administration of Vehicle (negative control), Kiwi 1, Caffeic acid, Quinic acid and Caffeic+Quinic acids solution at a volume of 10 ml/kg and 30 min after administration of 10 mg/kg of escitalopram (ESC, positive control) at a volume of 10 ml/kg. No differences were found between different treated groups. Values were evaluated by one-way ANOVA and expressed as mean ± SD (n=12 per group).

After behavioral experiments serum and brain samples were collected and analyzed by UPLC-ESI-MS. **Figure 3.7** shows the relative quantification in arbitrary units of caffeic acid sulfate and and quinic acid in both serum and brain samples of mice treated with kiwi 1, quinic acid and a combination of quinic acid and caffeic acid glucoside, which were the effective treatments in TST and FST; both the results obtained with the two chromatographic conditions (C18 and HILIC) are shown [$F_{(2,33)}$=63.46, p<0.0001; $F_{(2,33)}$=35.96, p<0.0001 of quinic acid for serum and brain respectively and of caffeic acid sulfate only for serum $F_{(2,33)}$=18.86, p<0.0001].

As previously reported in chapter 2, quinic acid was detectable only in C18 chromatographic condition while caffeic acid sulfate is better detected with HILIC chromatography. However, levels of brain caffeic acid sulfate were not evaluated since they were still detectable but under the LOD (the lowest quantity of a substance that can be determined with a level at least 5 time higher that the background).

Interestingly, levels of both quinic acid and caffeic acid sulfate in sera and brains were lower in all the treatments with the pure metabolites (QA, CA or QA+CA) compared to the levels of the molecules in the undiluted kiwifruit (kiwi 1). Since quinic acid and caffeic acid glucosides were administered exactly at the same concentration of the phytocomplex, this suggests that in the whole fruit there are other molecules or components that could increase the metabolites adsorption and/or stability in the living organism. Thus, the lower activity of pure quinic acid and quinic acid-caffeic acid glucoside combination compared with kiwi1 treatment can be due to the activity of other low-level and undetectable kiwifruit molecules or to a limited absorption and/or stability of quinic and caffeic acid when they are taken as pure molecules.

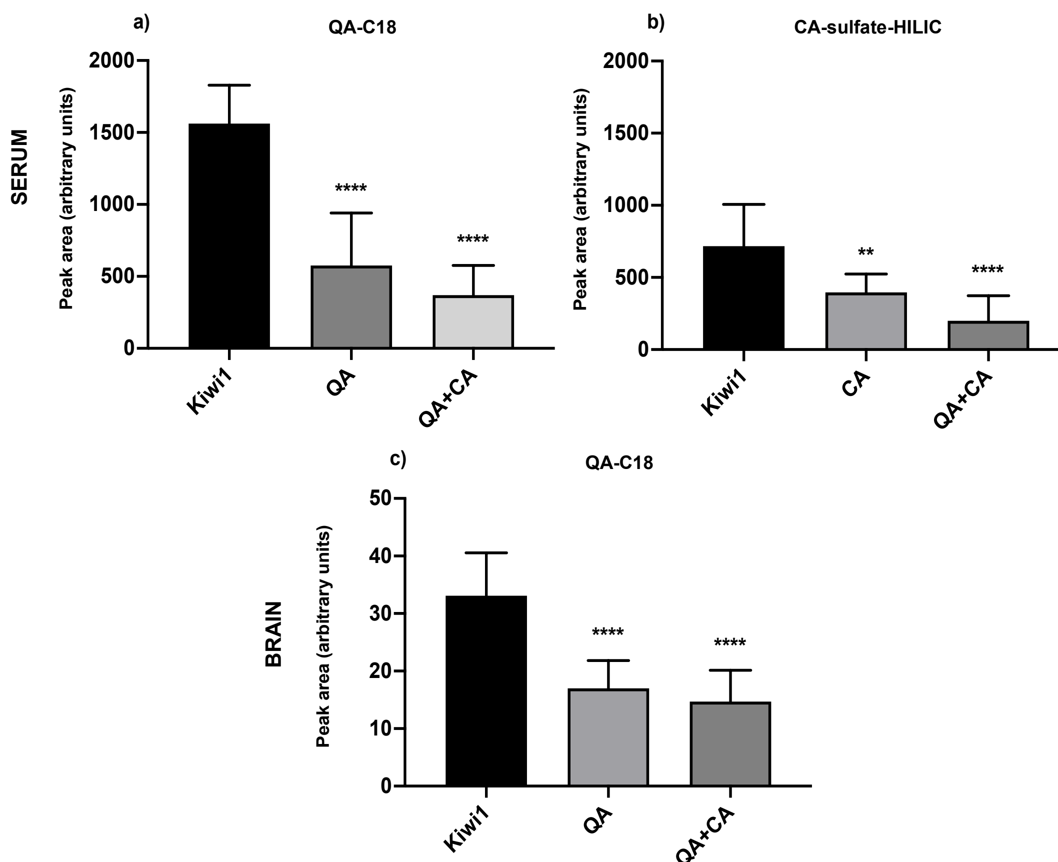

Figure 3.7. **Relative quantification of quinic and caffeic acid sulfate.** Relative quantification of quinic acid (**a**,**c**) under C18 chromatographic condition in serum and brain respectively, and caffeic acid sulphate (**b**) in serum under HILIC chromatography. Values were evaluated by one-way ANOVA followed by Dunnett's post hoc test and expressed as mean ± SD (n=12 per group). **p≤0.01, ****p ≤0.0001 *vs.* Kiwi1-treated group. QA=quinic acid, CA-sulfate=caffeic acid sulfate.

3.3.3 – Kinetics analysis and quantification of serum in mice treated with quinic acid

PK experiments were performed on different groups of mice treated with quinic acid and sacrificed at different time points. Time points used in this experiment are the following: 15min, 30min, 45min, 1h, 1h15 min, 1h30min, 2h, 4h, 8h and 24h. **Figure 3.8** shows the relative quantification in arbitrary units of quinic acid in both serum and brain samples. Obviously and as seen with kiwifruit treatment, relative levels of the quinic acid decreased along the time.

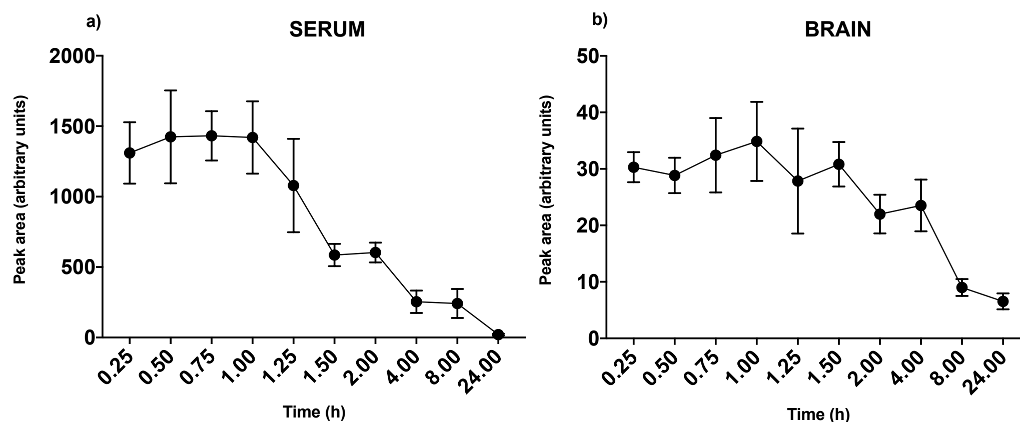

Figure 3.8. **Relative quantification of quinic acid in serum and brain of mice after quinic acid-single treatment.** Pharmacokinetics relative quantification of quinic acid in serum (**a**) and in brain (**b**) under C18 chromatographic condition. Each time point represents the mean ± SD of n=4 per time-point.

Also, in this case as with the whole kiwifruit phytocomplex we evaluated the real brain-penetration using the technique of perfusion in cold 0.9% saline buffer for 5 minutes (more details are presented in chapter 2). **Figure 3.9** shows the relative quantification in arbitrary units of brain quinic acid in C18 chromatographic conditions. Brain levels after perfusion were, as expected, lower compared to non-perfused samples (see also figure 2.8 in chapter 2). In addition, we compared the brain levels of mice treated with QA and with kiwifruit after perfusion technique. Levels of the QA-only treated mice were much lower compared to the other group [$F_{(5,5)}=6.189$, $p<0.0001$ for brain quinic acid (**a**) and $F_{(5,5)}=5.819$, $p=0.0017$ for the comparison of perfused kiwifruit and quinic acid groups (**b**)].

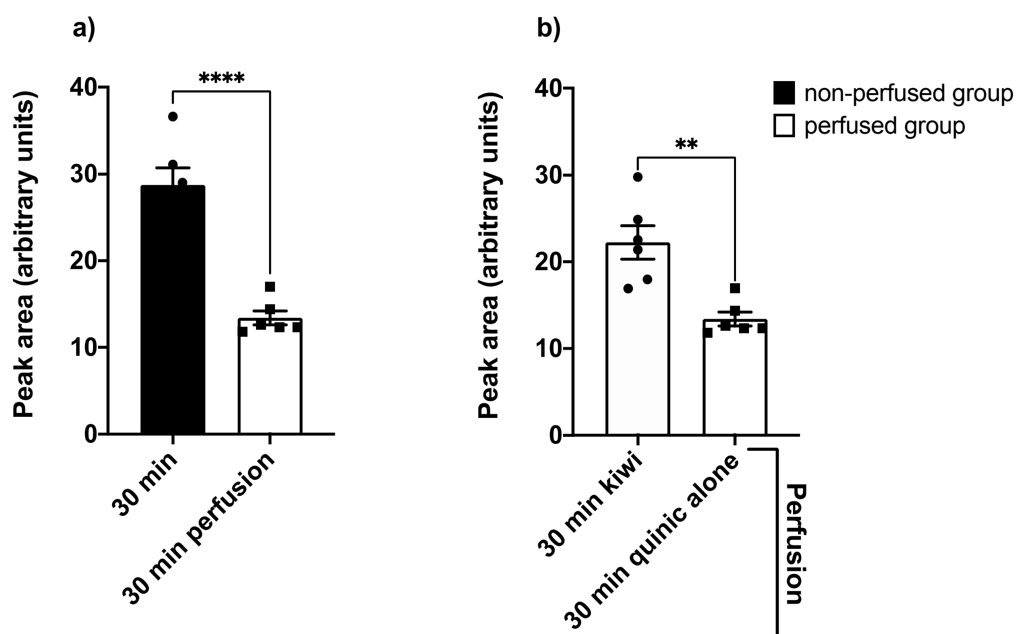

Figure 3.9. **Relative quantification of quinic acid in PK experiments after perfusion technique.** PK relative quantification in brain of quinic acid under C18 chromatographic condition (**a**) and comparison of perfused animals treated with kiwifruit and with quinic acid 30 min prior the sacrifice (**b**). Values were evaluated by unpaired t-test and expressed as mean ± SD (n=6 per group). $^{**}p \leq 0.01$ $^{****}p \leq 0.0001$

Moreover, we tried also to exactly quantify quinic acid in serum and brain of animals treated with an acute administration of quinic acid at the same concentration of kiwi 1 solution (undiluted kiwifruit). A calibration curve was created with concentrations of quinic acid authentic standard ranging from 6 to 60 pg/µl with an R^2 of 0.9969. Eight different serum samples of 1:80 dilutions in three replicates were plotted against the calibration curve. This dilution was chosen because it was the most diluted form that allow us to detect clearly the quinic acid in serum samples even if a little matrix effect was still present. For that reason, we determined matrix effect through spiking experiments, and we corrected data to obtain the exact quantification that was 1.451 ± 0.318 ng of quinic acid/ul of serum.

Quantification in brains were not possible because during the dilution of brain extracts the signal due to quinic acid disappeared at dilutions at which matrix effect was still too high to allow an accurate quantification. This is not surprising,

given that phospholipids are among the main responsible for the matrix effect and given that the brain contains very relevant amounts of phospholipids. For caffeic acid the accurate absolute quantification was not possible both in sera and brains.

3.4 – Discussion

Having found few specialized kiwifruit metabolites (quinic acid and caffeic acid hexoses) able to reach both blood and brain after chronic administration of kiwifruit juice in mice, some experiments were set up to verify whether these could be the active anti-depressant molecules of fruits. To do this, first the absolute amount of these metabolites was accurately quantified in kiwifruit juice and then these metabolites were administered to mice, alone or in combination, at the same concentration of the undiluted kiwifruit extract (kiwi 1).

The level of quinic acid in our kiwifruit juice was ~492 mg/100 g of fresh fruit weight, in line with that reported in literature [390 mg/100 g fresh weight (Lintas et al. 1991)], whereas for the three caffeic acid hexoses was ~6.5 mg/100 g of fresh fruit weight. To the best of our knowledge, this is the first report for the quantification of the caffeic acid 3-β-D-glucoside since in literature it was already detected but not quantified (Pinelli et al. 2013; Guo et al. 2017).

When QA and CA-glucoside were administered to mice, alone and in combination, at the same concentration of the undiluted kiwi extract, QA was able to partially mimic kiwi 1 effect since it displayed high effect, although not as high as that of kiwi 1, in reducing immobility time in both behavioral tests. Instead, CA-glucoside administered alone did not show any effect, while the combination of the two molecules seemed to have a synergistic effect only in TST but this effect completely disappeared in FST. Since in previous behavioral experiments we had some trouble with positive control FLX, in this experiment we decided to change it to escitalopram 10 mg/kg, a more selective SSRI drug and it worked on both tests. All treated group were also scored for motor impairment in OFT at day 8 and

no motor impairment were detected, suggesting that the reduction of immobility was due to antidepressant effect.

Free quinic acid is found at low levels (< 100 mg/100 g fresh weight) in vegetables and fruits such as lemon, strawberry, pear and leek (Hulme 1958; Flores et al. 2012; Priecina and Karklina 2015). It is found at higher levels (100–500 mg/100 g fresh weight, or more) in garlic bulbs and herbs such as celery, parsley and dill (Priecina and Karklina 2015). Ripe fleshy fruits generally accumulate citric and malic acids as major organic acids instead of quinic acid, and edible fruits with high levels of quinic acid are rare. Exceptions include black chokeberry (*Aronia melanocarpa*, Michx., Elliot) (Denev et al. 2018), Ponkan mandarin (*Citrus reticulata*, Blanco) and kiwifruit (Lintas et al. 1991; Batista-Silva et al. 2018). Free quinic acid is also found in some processed foods due to ester hydrolysis, for example during the roasting of coffee beans (Dawidowicz and Typek 2017).

Literature in recent year have extensively focused the attention on CGAs derivatives and their metabolism with both *in vitro* and *in vivo* studies while the metabolic fate of QA has been so far overlooked.

The bioavailability of pure QA is not reported in the literature, but its recovery in human serum after coffee consumption suggests it can reach the blood (Guertin et al. 2014; Guertin et al. 2015). Nowadays, in literature is present only one article that quantified the recovery of quinic acid after apple smoothie consumption in human (Hagl et al. 2011). Other articles are focused on the actions of this specialized metabolite with *in vitro* and *in vivo* approaches. For example, QA is found to be active in releasing insulin from pancreatic ß cells making it a possible therapeutic tool against diabetes (Heikkila et al. 2019), reduce the activation of the TNFα-stimulated adhesion molecule expression decreasing vascular inflammation (Jang et al. 2017), has an anti-hepatitis B action reducing its replication (Wang et al. 2009) and also have radioprotective activity against X-ray (Cinkilic et al. 2013). Besides, QA is already used in creating QA-conjugated nanoparticles as drug carrier to solid tumors since it interacts with endothelial selectins mimicking its ligands (Amoozgar et al. 2013; Xu et al. 2018).

Interestingly, when QA and CA-sulfate were quantified (relative quantitation) in serum and brain of mice, the levels of the single or the combination treatment (two molecules) were lower compared to that of kiwi 1 dilution, even if the concentrations of the metabolites administered to the animals were the same. This suggests that other molecules present in the whole fruit are necessary for the stability and/or the "correct" intestinal adsorption of the two metabolites. However, perfusion experiments proved that quinic acid given alone was able to reach the brain parenchyma but showed lower brain penetration compared to the whole kiwifruit treatment.

Furthermore, "pharmacokinetics" analyses over 24 h period on serum and brain of mice treated with QA reveal that the accumulation was very rapid, since levels of the metabolite decrease around the baseline between 8 and 24 h. These data are in line with the previous data of kiwi-fed mice even if the relative quantifications are lower.

The effects of secondary metabolites in fresh fruits and vegetables are difficult to unravel not only due to the large number of different molecules but also because their activity could depend on bioavailability of each individual metabolites and on additive, synergistic and antagonist effects between them (Liu 2003; Phan et al. 2018). There are a number of factors influencing the bioavailability of metabolites in an individual; regarding polyphenols, for instance, it can be influenced by many factors including colonic microbial composition, the dose consumed and the presence of other polyphenols and macronutrients within the food matrix (Bondonno et al. 2017). In addition, there is limited attention given to studying the impacts of bioaccessibility and bioavailability interferences on bioactivity interactions between phytochemicals.

Synergy and antagonism are notoriously difficult to study in a rigorous fashion since natural products chemistry research methodology is typically devoted to reducing complexity and identifying single active constituents for drug development (Caesar and Cech 2019).

Synergy in biological activities can result only from phytochemical combinations that can promote solubility, safety, absorption, stability or bioavailability of the principal active compounds (Phan et al. 2018).
A less-discussed phenomenon is antagonism, in which effects of active constituents are masked by other compounds in a complex mixture.
This explains, in general, why no single compound can replace the combination of natural phytochemicals in fruits and vegetables to achieve the health benefits (Liu 2004).
In our case, data display that QA administered alone had high effect in line with the undiluted kiwifruit extract but the minor activity well correlate with the lower levels in both serum and brain samples. This confirm that the whole phytocomplex could be important in stability and adsorption but not necessarily in activity.

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Chapter 4 – Kiwifruit extract and some of its metabolites, quinic acid included, are able to inhibit human Monoamine Oxidase enzyme

4.1 – Introduction

MAO is a flavin-containing enzyme (EC 1.4.3.4) that catalyzes the oxidative deamination of primary and some secondary amines, including the catecholamines, serotonin (5-hydroxytryptamine or 5-HT), and trace amines (Tipton 2018). Humans possess two isozymes of MAO, named MAO-A and MAO-B, both located on the outer membrane of mitochondria (Schnaitman et al. 1967; Shih 2018). The two isozymes have 70% homology in their amino acid sequence and the shared pentapeptide sequence (Ser-Gly-Gly-Cys-Tyr) binds the cofactor FAD (**Figure 4.1**).

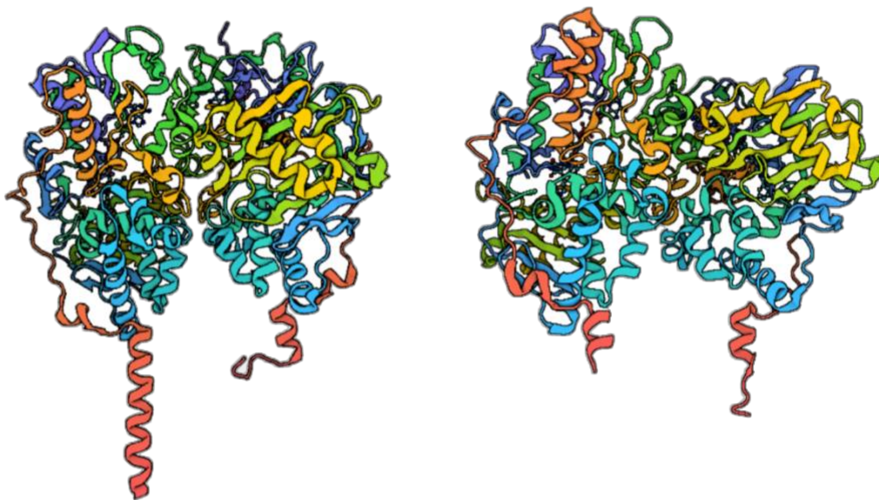

Figure 4.1. Cartoon structures of MAO-A and MAO-B respectively. Different colors represent different sequences.

The isozymes were originally defined according to differences in substrate and inhibitor sensitivity: MAO-A has a higher affinity for serotonin and norepinephrine

and is sensitive to low concentrations of clorgyline, whereas MAO-B prefers β-phenethylamine as a substrate and is inhibited by low concentrations of deprenyl (selegiline) (Youdim et al. 2006). MAO-A is predominantly expressed in the placenta, intestine, liver and lungs, whereas MAO-B is the prevalent form in platelets (Saura et al. 1997; Shih 2018), but both appear to play a protective role by catalyzing the oxidative deamination of biogenic and xenobiotic amines (Ramsay and Albreht 2018). In the brain, MAO-A is predominantly found in catecholaminergic neurons whereas MAO-B is more abundant in serotonergic and histaminergic neurons and especially in glial cells (Shih et al. 1999). In addition, there are some differences in distribution of MAOs through rodents and humans, indeed there is more type A than type B enzyme throughout rat brain, whereas in the human brain is the opposite. Serotonergic neurons therefore contain the form of MAO that does not preferentially metabolize 5-HT. This has led to the hypothesis that type B MAO in serotonergic neurons prevents the cell from accumulating various natural substrates (e.g., dopamine) that could interfere with the storage, release, and uptake of 5-HT (Tipton 2018). Furthermore, treatment of rats with clorgyline, a selective inhibitor of type A MAO, raises the brain content of 5-HT and reduces the conversion of 5-HT to 5-HIAA in brain. Thus, 5-HT may well be oxidized preferentially by type A MAO *in vivo*, just as it is *in vitro*, even though serotonergic neurons do not contain much of this form of the enzyme (Siegel et al. 2012). Recently, techniques have been developed that permit the selective elimination or "*knockout*" of genes encoding specific proteins in mice. Using such methodology, mice that have either type A-or type B MAO knocked out have been developed (Cases et al. 1995). In the brains of mice deficient in type A MAO, the content of 5-HT is markedly elevated for about 12 days after birth and then slowly declines, reaching values comparable to those in normal mice after about seven months. In the type A MAO–deficient mice, the selective inhibitor of type B MAO, deprenyl, had a greater effect on serotonin metabolism than it did in normal mice. Such observations indicate that in the absence of type A MAO, the type B isoform can metabolize 5-HT *in vivo*. However, mice lacking the MAO B

isoenzyme do not have elevated levels of 5-HT in brain. Of interest are the aggressive behaviors exhibited by the mice deficient in type A MAO, consistent with a postulated role of serotonergic neurons in human aggressive behaviors. Given the role of MAOs in the metabolism of neuroactive amines, MAOI inhibitors (MAOIs) have extensively been used in clinical states, such as depression, with the aim to increase dysregulated monoamine levels. Nowadays, MAO-A inhibitors have limited therapeutic use due to potentially dangerous side effects while MAO-B inhibitors are still in use as adjunct therapy in Parkinson's disease to elevate dopamine levels (Tipton 2018).

Older MAOIs such as phenelzine and iproniazid are irreversible, and the effect lasts until new enzyme is synthesized, which may take days to months (Tipton 2018). Newer MAOIs such as moclobemide are reversible. Some MAOIs are active against both isoenzymes whereas others are selective; for example, moclobemide selectively inhibits MAO-A, whereas safinamide selectively inhibits MAO-B. Some MAOIs are associated with side effects linked to hypertensive crisis (e.g., the cheese reaction caused by high levels of tyramine) or drug interactions, and there is great interest in the development of alternatives, including natural ones.

In spite of side effects, these medications are still a good option in the treatment of depression (both isozymes inhibitors), especially for non-responsive patients; in fact, in certain cases, they relieve depression when other treatments have failed. With the appropriate dietary restrictions and attention to potential drug interactions with 5-HT and noradrenaline agents this class of drugs can be used effectively and safely.

Actually, the use of MAOIs, especially reversible ones, appears to be safer and the number of patients with atypical, treatment-resistant or bipolar depression who may potentially benefit from MAOIs is substantial. In conclusion the MAOIs still represent an important element in our therapeutic equipment (Shulman et al. 2013; Duarte et al. 2020; Sub Laban and Saadabadi 2020).

4.1.1 – MAOs Inhibitory effect by plant extracts

Numerous plant extracts have been tested for their ability to inhibit MAO, including a large-scale screen that identified four herbs (*Phellodendron amurense*, Rupr.; *Cyamopsis psoralioides*, L., Taub.; *Glycyrrhiza glabra/uralensis*, L./C.DC. and *Psoralea corylifolia,* L., Medicus seed) used in traditional medicines as plant extracts with the higher ability to inhibit MAO-B (Mazzio et al. 2013). The analysis of numerous foods and herbal remedies has revealed a large number of plant metabolites with the ability to inhibit one or both isoforms of MAO (Clarke and Ramsay 2011; Vina et al. 2012; Carradori et al. 2014). One example is the flavonol quercetin, which inhibits both forms of MAO but shows greater activity against MAO-A, as supported by molecular docking studies (Chimenti et al. 2006). Other flavonoids preferentially inhibit MAO-B, such as (+)-catechin, (–)-epicatechin and naringenin (Hou et al. 2005; Olsen et al. 2008), whereas kaempferol and apigenin are more potent inhibitors of MAO-A (Sloley et al. 2000). Many investigators have focused on β-carbolines, which are bioactive alkaloids found in hallucinogenic plants, tobacco and coffee (Herraiz 2004; Callaway 2005). For example, harman is a selective inhibitor of MAO-A, whereas norharman strongly inhibits both forms of the enzyme (Herraiz and Chaparro 2006). The alkaloid piperine and related compounds, found in long pepper plants (*Piper longum*, L.), are more potent inhibitors of MAO-B (Lee et al. 2005). Anthocyanins, the abundant red and blue pigments found in plants such as cherry and red chicory, can also inhibit MAO, although their rarer aglycosylated counterparts (anthocyanidins) are more potent (Dreiseitel et al. 2009).

In the previous chapters *in vivo* investigation on mouse models and metabolomics were used to investigate kiwifruit antidepressant-like activity and the fruit molecules involved. However, the *in vivo* approach did not allow to unravel the possible molecular targets involved in the overall observed activity.

It is important to cover the topic with both *in vivo* tests in animal models and humans, which can demonstrate efficacy at the organism level but generally

cannot identify molecular targets, and *in vitro* tests, which can identify molecular targets but cannot provide information about efficacy at the organism level.

To evaluate a possible mechanism of action of kiwifruit, we investigated its ability to inhibit MAO. We refined the MAO-A and MAO-B inhibition assays to avoid effects caused by the acidic pH typical of plant organs, we then tested extracts of kiwifruit together with its individual metabolites.

4.2 – Materials & Methods

4.2.1 – Reagents

Clorgyline, R-(−)-deprenyl, human enzymes MAO-A and MAO-B expressed in baculovirus-infected insect cells (BTI-TN-5B1-4), caffeic acid, (+)-catechin hydrate, L-ascorbic acid, L-Malic acid, Citric acid, D(+)-Sucrose, D(-)-Fructose, D(+)-Glucose, Bradford reagent, and bovine serum albumin (BSA) were obtained from MilliporeSigma (Milan, Italy), D-(−)-quinic acid was purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and esculin was obtained from Extrasynthese (Genay, France).

4.2.2 – Protein concentration

The protein concentration in fresh and neutralized kiwifruit juice was determined using Bradford reagent (MilliporeSigma) in a 96-well plate format (Bradford 1976). A standard curve was prepared using BSA standards in the concentration range 0.1–1.4 mg/ml. Absorbance was measured on an Infinite 200 Pro microplate reader (Tecan Italia, Cernusco sul Naviglio, Italy).

4.2.3 – MAO-Glo assay

The ability of each sample to inhibit MAO *in vitro* was evaluated using the two-step bioluminescent MAO-Glo Assay (Promega, Milan, Italy) in 96-well flat bottom white opaque plates (Thermo Fisher Scientific, Rodano, Italy) as previously described (Valley et al. 2006). The reaction buffer comprised 100 mM HEPES (pH

7.5) and 5% glycerol for MAO-A, and additionally 10% (v/v) dimethyl sulfoxide (DMSO) for MAO-B. In each well, 25 µl of the MAO enzyme (20 µg/ml) were mixed with 12.5 µl the model substrate (40 and 4 µM for MAO-A and MAO-B, respectively) and 12.5 µl of each candidate inhibitor. The final reaction volume is 50 µl. The plate was then incubated at room temperature for 1 h. The luminescent signal was then generated by adding 50 µl of the luciferin detection reagent, incubating the plate for 20 min at room temperature, and measuring the signal using an Infinite 200 Pro microplate reader. For the blank, the reaction buffer was used instead of the MAO enzyme. Each experiment also included a positive control with a known selective inhibitor (clorgyline for MAO-A and deprenyl for MAO-B) and a negative control with buffer in place of the candidate inhibitor.

4.2.4 – Data analysis

Statistical analysis was carried out using Prism v9.0 (GraphPad Software, San Diego, CA, USA). Significant differences between samples were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. IC_{50} values were calculated by nonlinear regression analysis.

4.3 – Results

4.3.1 **– MAO inhibition assay optimization and kiwifruit activity analysis**

Microplate assays to test the ability of compounds to inhibit MAO-A and MAO-B were optimized by testing freshly extracted kiwifruit juice. Freshly extracted fruit and vegetable juice is acidic (pH ~3) due to the abundance of organic acids (mainly citric and malic acids, but also ascorbic acid). In contrast, secondary metabolites are heterogeneous low-molecular-weight compounds found at much lower concentrations, but they are responsible for many of the beneficial properties of fruits and vegetables, including antioxidant activity. To optimize the assay, we adjusted the pH of the fresh juice to 4, 5 and 7.5 and tested its ability to inhibit MAO-A and MAO-B. Low pH strongly inhibited MAO activity

[$F_{(4,10)}$=2424, p<0.0001; $F_{(4,15)}$=3373, p<0.0001 for MAO-A and B respectively] so all the subsequent experiments were adjusted to pH 7.5 to ensure that inhibitory effects were directly caused by the metabolites rather than the low pH. At H 7.5, which is also the pH of the enzyme buffer, kiwifruit juice caused a consistent inhibition of both the enzymes compared with the negative control (**Figure 4.2**).

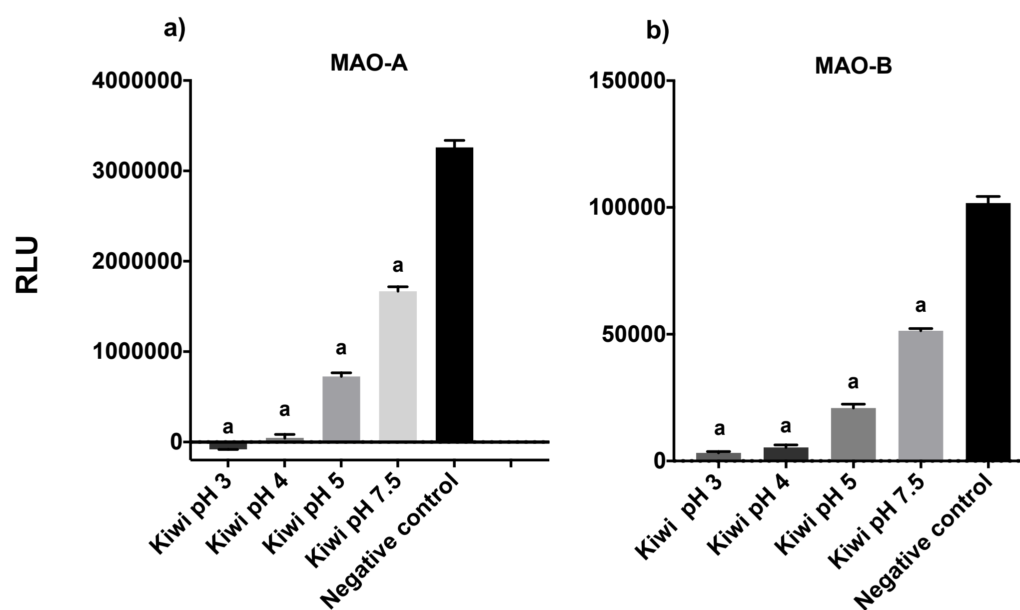

Figure 4.2. **Effects of pH on MAOs activity**. MAO-Glo assay of kiwifruit juice (at different pH values) to test for the inhibition of human MAO-A (**a**) and MAO-B (**b**). We used 12.5 µl of kiwifruit juice corresponding to 16.8 mg of fresh fruit. Activity was measured in relative light units (RLUs) compared to the uninhibited enzyme (negative control). Values were evaluated by one-way ANOVA followed by Dunnett's test and are expressed as means ± SD (n=4 per group; [a]p≤0.0001 vs. negative control).

In order to limit the number of candidate inhibitors, we passed the fresh kiwifruit juice over a cation exchange cartridge to remove small, polar metabolites (See Section 2.2.5 in M&M of chapter 2 for further details). The relative composition of the fresh kiwifruit juice and its "neutralized" derivative was then compared by untargeted metabolomics (Appendix Table 1). To exclude the possibility that bulk primary metabolites such as sugars, organic acids and ascorbic acid were responsible for the inhibitory activity, we test vehicle solution replicating the concentrations of these major components as determined by NMR spectroscopy (See Section 2.2.4 in M&M of chapter 2 for further details details). We created additional vehicle solutions containing only the sugars or one or both organic acids

or ascorbic acid. We then tested the inhibitory activity of the complete and partial vehicle solutions against the fresh and neutralized juice, all adjusted to pH 7.5. We found that only the fresh juice shows significant inhibition activity, with greater activity against MAO-A than MAO-B (**Figure 4.3**).

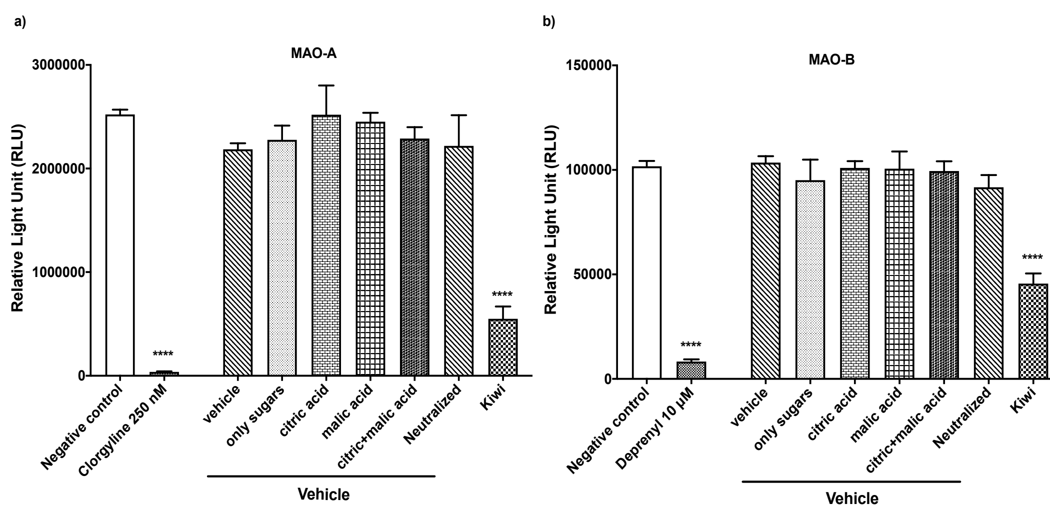

Figure 4.3. Effect of fresh and neutralized kiwifruit juice and matching vehicle solutions on MAOs activity.
We used 12.5 µl of kiwifruit juice corresponding to 16.8 mg of fresh fruit, and equivalent amounts of the other samples. The samples were tested for their ability to inhibit (**a**) MAO-A and (**b**) MAO-B activity using the bioluminescent MAO-Glo assay, with values expressed as relative light units (RLUs) compared to 250 nM clorgyline and 2.5 µM deprenyl as positive controls for MAO-A and MAO-B, respectively, and a negative control with buffer in place of the inhibitor. Data were evaluated by one-way ANOVA followed by Dunnett's test and are expressed as means ± SD (n=4 per group; ****p≤0.0001 vs negative control group).

We next evaluated the possible role of fruit proteins in the observed inhibitory effect, because kiwifruits contain proteolytic enzymes such as actinidin that could digest the MAO-A and B enzymes resulting in a fake enzyme "inhibition" (Chalabi et al. 2014). The protein concentration in kiwifruit (active) and neutralized juice (inactive) was found to be the same, suggesting that proteins are not responsible for the inhibition of MAOs. The protein concentration in the fresh juice was 0.548 ± 0.099 mg/ml and that in the neutralized juice was 0.573 ± 0.215 mg/ml (**Table 4.1 and Figure 4.4**).

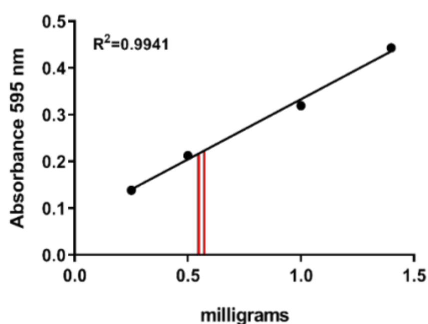

Table 4.1. Bradford Assay

Compound	Protein concentration (mg/ml)	Optical Density (Entered)
	X	Y
Kiwifruit juice	0.548 ± 0.099	0.217
Neutralized	0.573 ± 0.215	0.223

Figure 4.4 and Table 4.1. Bradford Assay
Absorbance was measured in kiwifruit and neutralized solution by Bradford Assay. Values were evaluated by unpaired t-test and expressed as mean ± SD (n=4 per group).

To confirm that kiwifruit proteins do not inhibit MAO, we prepared a protein-free derivative of the fresh juice by passing it through filers with molecular weight cut-off (MWCO) values of 3 and 10 kDa before repeating the inhibition assays. As expected, there were no significant differences between the fresh juice and the two protein-depleted filtrates in terms of their ability to inhibit MAO-A and MAO-B, suggesting that active inhibitory substances are probably low molecular weight molecules, i.e. the metabolites (**Figure 4.5**). Since a role of the bulk primary metabolites was already excluded (see above), the best candidates for the kiwifruit inhibitory activity are the secondary specialized metabolites.

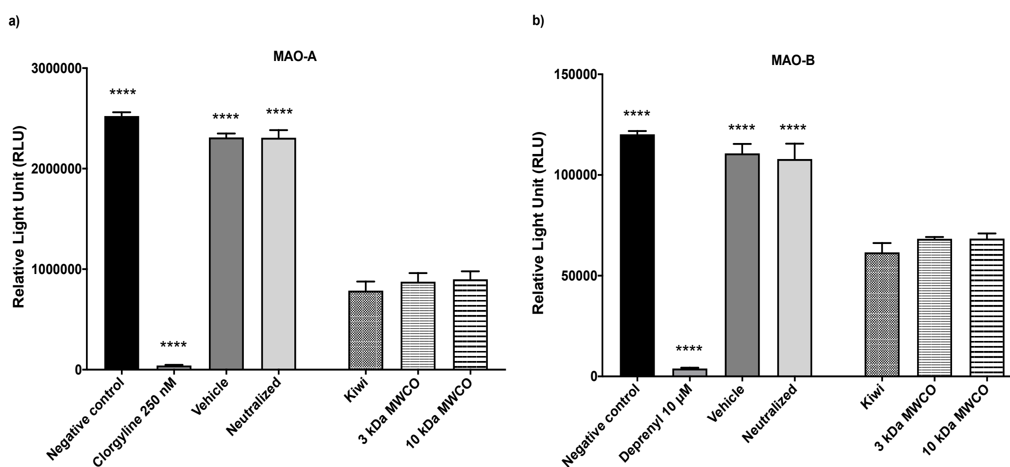

Figure 4.5. Effect of fresh and protein-depleted kiwifruit juice on MAOs activity.
We used 12.5 µl of fresh or protein-depleted kiwifruit juice corresponding to 16.8 mg of fresh fruit. The samples were tested for their ability to inhibit (**a**) MAO-A and (**b**) MAO-B activity using the bioluminescent MAO-Glo assay, with values expressed as relative light units (RLUs) compared to 250 nM clorgyline and 2.5 µM deprenyl as positive controls for MAO-A and MAO-B, respectively, and a negative control with buffer in place of the inhibitor. Data were evaluated by one-way ANOVA followed by Dunnett's test and are expressed as means ± SD (n=4 per group; ****$p \leq 0.0001$ vs fresh kiwifruit juice group).

Having excluded proteins, sugars, bulk organic acids and ascorbic acid as the source of the inhibitory activity, we prepared a list of metabolites present in the active fresh juice but not in the inactive "neutralized" juice, based on the heat map from the untargeted metabolomic analysis (Appendix Table 1). The list of putatively identified specialized metabolites that were more abundant in the fresh juice included caffeic acid hexoses and di-hexoses, other caffeic acid derivatives, esculin, catechin, quinic acid and quinic acid derivatives.
The effect of different concentrations of kiwifruit juice was compared to clorgyline and deprenyl standards in the MAO inhibition assay, revealing that the juice has an IC_{50} of 86 ± 2.6 mg/ml against MAO-A and 122.9 ± 5.5 mg/ml against MAO-B (**Table 4.2, Figure 4.6 and 4.7**). We prepared a list of metabolites that were promising candidate inhibitors according to their abundance in fresh kiwifruit juice or a high ratio between fresh and neutralized kiwifruit juice. Based on these criteria, we selected caffeic acid, quinic acid, catechin and esculin for further experiments. Caffeic acid and quinic acid were considered of high interest because their bioavailability, in form of caffeic acid hexoses and free quinic acid, was already proved (see chapter 2 and 3), and quinic acid was considered of very high interest because it was able alone to mimic at least partially the antidepressant kiwifruit effect (see chapter 3).
The effects of the above-mentioned molecules were compared to clorgyline and deprenyl standards in the MAO inhibition assay. We found that catechin and esculin inhibited both enzymes similarly, caffeic acid inhibited both enzymes but was more active against MAO-A, whereas quinic acid showed lower but very selective activity against MAO-B (**Table 4.2, Figure 4.6 and 4.7**).

Table 4.2. IC_{50} values of kiwifruit and its secondary metabolites

Compound	IC_{50} value			
	MAO-A[a]		MAO-B[a]	
	(mg/ml)[b]	M[c]	(mg/ml)[b]	M[c]
Clorgyline	$5.49\times10^{-6} \pm 2.4\times10^{-7}$	0.0178 ± 0.0007 µM	-	-
R-(-)-Deprenyl	-	-	$1.96\times10^{-5} \pm 8.2\times10^{-7}$	0.1048 ± 0.004 µM
Kiwifruit[b]	86 ± 2.6	-	122.9 ± 5.5	-
D-(-)-Quinic acid	-	-	6.60 ± 0.25	34.37 ± 1.307 mM
Caffeic acid	0.49 ± 0.02	2.747 ± 0.126 mM	0.08 ± 0.005	0.439 ± 0.03 mM
Catechin	1.88 ± 0.04	6.464 ± 0.121 mM	1.17 ± 0.04	4.029 ± 0.122 mM
Esculin	3.93 ± 0.15	11.55 ± 0.4305 mM	4.10 ± 0.13	12.06 ± 0.3721 mM

The ability of kiwifruit juice, quinic acid, caffeic acid, catechin and esculin to inhibit MAO-A and MAO-B was determined using the MAO-Glo assay compared to clorgyline and deprenyl as positive controls for MAO-A and MAO-B, respectively.

[a] Each inhibitory activity is expressed as the mean of 50% inhibitory concentration (IC_{50}) of quadruplicate determinations obtained by interpolation of the concentration-inhibition curves.
[b] mg fruit fresh weight/ml.
[c] for metabolite standards, data are shown also in molarity (M) for comparative purposes.

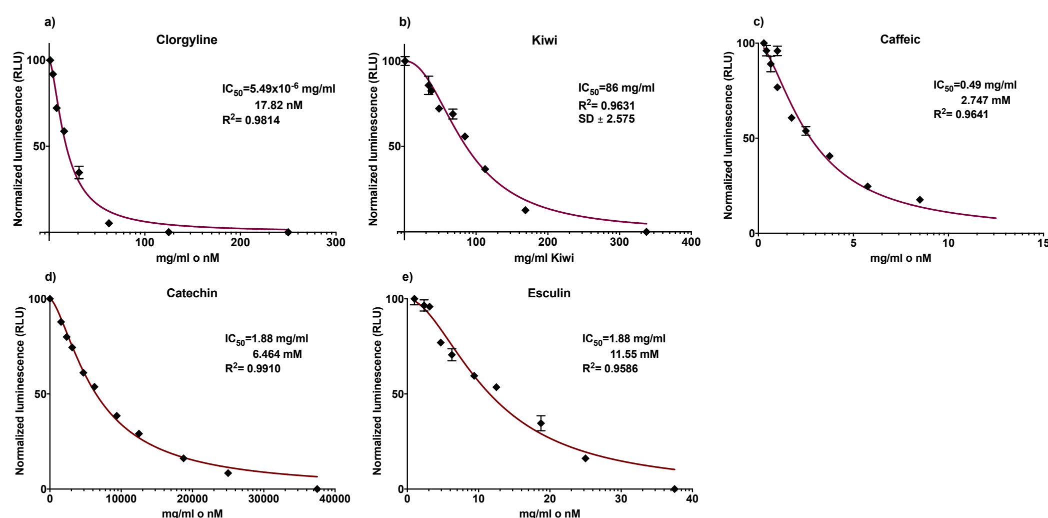

Figure 4.6. IC_{50} curves for the inhibition of MAO-A.
(a) Clorgyline, (b) kiwi, (c) caffeic acid, (d) catechin and (e) esculin were evaluated using the bioluminescent MAO-Glo assay, with values expressed as relative light units (RLUs). Values were evaluated by nonlinear regression analysis and expressed as mean ± SD (n=4 each concentration/dot per curve). Data are expressed both in mg fruit fresh weight/ml and in molarity (M) for comparative purposes.

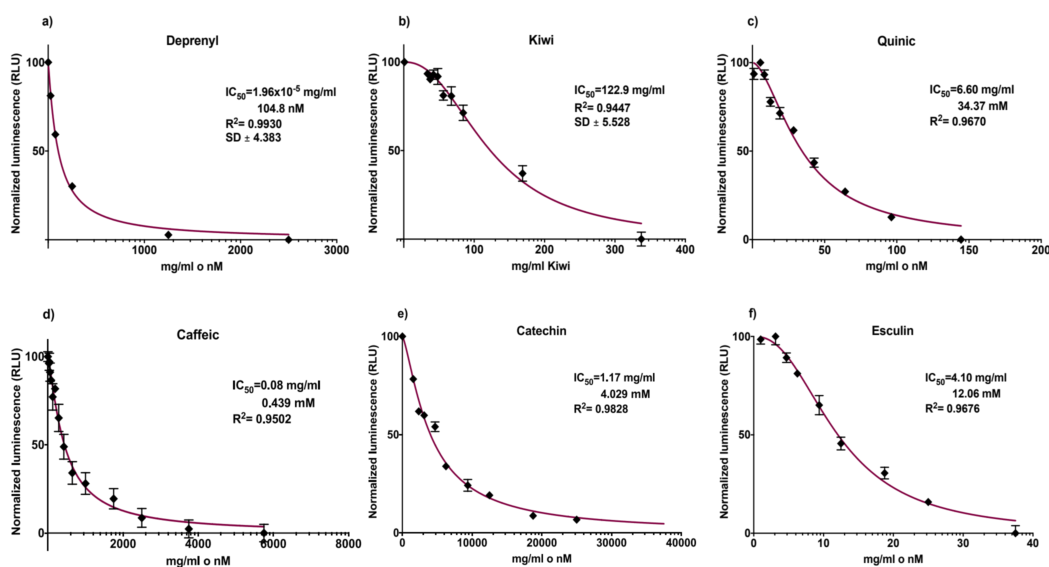

Figure 4.7. IC_{50} curves for the inhibition of MAO-B.

(a) Deprenyl, (b) kiwi, (c) quinic acid, (d) caffeic acid (e) catechin and (f) esculin were evaluated using the bioluminescent MAO-Glo assay, with values expressed as relative light units (RLUs). Values were evaluated by nonlinear regression analysis and expressed as mean ± SD (n=4 each concentration/dot per curve). Data are expressed both in mg fruit fresh weight/ml and in molarity (M) for comparative purposes.

4.4 – Discussion

To identify possible molecular targets to explain the kiwifruit antidepressant-like activity seen in behavioral experiments (chapter 2 and 3), we tested various dilutions of kiwifruit extract together with its primary and specialized secondary metabolites for the ability to inhibit the human MAO-A and MAO-B at pH 7.5. Data confirmed the presence of inhibitory activity of kiwifruit and all of its secondary specialized metabolites tested.

Fruit and vegetable tissues are complex mixtures of insoluble macromolecules (polysaccharides, structural proteins, lignin, cutin and suberin), lipophilic molecules, soluble proteins and carbohydrates, and a complex array of small-molecule metabolites, many with bioactive properties (Butnariu and Butu 2015). The insoluble components comprise most of the dietary fiber, which is largely

removed during the juice preparation process (see section 2.2.3 of chapter 2), whereas the soluble portion still retains a complex mixture of molecules, including proteins, abundant primary metabolites (e.g., sugars, amino acids, and organic acids) and specialized secondary metabolites of various classes, some of which at low level (Pott et al. 2019). In principle, all these components could inhibit MAO-A and/or MAO-B.

Fruits and vegetables are acidic due to the high content of organic acids in the vacuolar sap (Tan et al. 2019), with citric and malic acids predominant in many fleshy fruits such as kiwifruit (Etienne et al. 2013). It was therefore necessary to evaluate the effect of kiwifruit pH on the MAO-A and MAO-B activity assays, revealing that both enzymes are inhibited per se by the low pH of fruit juice, with a linear decrease in the degree of inhibition as the pH approaches neutrality. We therefore adjusted the pH of our extracts to 7.5, which is also the pH of the enzyme buffer, before further inhibition assays because the effect of fruit metabolites should be tested in vitro under the physiological conditions as found in vivo (e.g., physiological pH). The pH of extracts was also adjusted in the previous large-scale screen of herbs, spices, leaves and seeds (Mazzio et al. 2013), but other authors have neglected this aspect and their data may therefore include false positive results due to enzyme inhibition caused by pH rather than the bioactive molecules in their extracts. Since kiwifruits, as other fruits as fig, papaya and pineapple contain proteases that could damage MAO-A and B enzymes, it was also necessary to exclude the possibility that proteases in the fruit extracts were responsible for MAO inhibition, because again this would generate false positive results. We found that the protein-free juice adjusted to pH 7.5 retained the ability to inhibit both enzymes, confirming that proteases were not responsible for the inhibitory activity observed *in vitro*.

To investigate the inhibitory activity in more detail, the composition of the active kiwifruit juice was evaluated by NMR-based targeted metabolomics and UPLC-QTOF untargeted metabolomics. We also tested neutralized juice devoid of many but not all small, polar metabolites by passing the fresh juice over an ion exchange

cartridge and so-called 'vehicle solutions' which replicated the profile of one or more major primary metabolites (sugars, organic acids and ascorbic acid) but none of the specialized metabolites. We found that the neutralized juice and the vehicle solutions were inactive, indicating that the remaining molecules of the juice, mainly specialized metabolites, were responsible for the inhibitory activity.
The comparison of fresh and neutralized juices allowed us to select a list of candidate specialized metabolites most likely to inhibit MAO-A and MAO-B, specifically those present in large quantities and those exclusively present in the fresh juice. This resolved the list of candidates to caffeic acid glucosyl derivatives, catechin, esculin and quinic acid.

When tested individually, caffeic acid, catechin and esculin showed non-selective inhibitory activity against both MAO-A and MAO-B, whereas quinic acid showed a lower but very selective inhibitory activity against MAO-B.

These metabolites were selected as candidates due to their abundance and representation in active fresh juice (compared to inactive juice eluted from an ion-exchange cartridge). Caffeic acid, caffeic acid derivatives and catechin (all representing the phenylpropanoid family) have already been reported for their ability to inhibit MAO, but this is the first report of the inhibitory activity of esculin. For quinic acid, one previous study reported the ability of this molecule to protect rats from aluminum chloride-induced dementia and suggested non-selective inhibition of MAO as a potential mechanism (Liu et al. 2020). In addition, high levels of quinic acid were detected in ileostomy patients after the consumption of an apple smoothie (~70%) (Hagl et al. 2011), although bioavailability has been assessed only *in vitro* (Lopez-Froilan et al. 2016) and our *in vivo* and thus more resolutive study (see chapter 2 and 3).

Caffeoylquinic acids, the more abundant and widespread caffeic acid derivative in plant kingdom, as their derivatives, show higher bioavailability (Clifford et al. 2017) but their selectivity for MAO-A and MAO-B is unclear. Two previous articles (Mei et al. 2019; He et al. 2020) provide evidence for selective activity against MAO-B, whereas another (Andrade et al. 2016) also showed an

effect against MAO-A. Probably the bioavailability of caffeoylquinic acids is controversial because the degree of utilization and excretion is unclear (Nabavi et al. 2017).

Esculin, like other metabolites, has low oral bioavailability compared to its acylated derivatives (Zhang et al. 2017) as shown in animal studies (Rehman et al. 2015).

Catechin has been shown to achieve the nonselective reversible inhibition of MAO-A and MAO-B in vitro although with greater potency toward MAO-A, but this effect was not replicated in an *in vivo* model, maybe due to low bioavailability (Reinheimer et al. 2020).

All polyphenols, including catechin, have poor bioavailability and a limited ability to cross the blood–brain barrier, which hinders their clinical development (Dhir 2020; Martinez Pomier et al. 2020). For that reason, nanotechnology could help to overcome this challenge by improving penetration and absorption (Giacomeli et al. 2019).

Nevertheless, our data, including also behavioral results, indicate that both quinic and caffeic acids can pass the blood brain barrier, though at lower level, and are active in reducing depressive like behaviors.

4.5 – References

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Chapter 5 – General discussion & concluding remarks

The beneficial effects of a diet rich in fruits and vegetables are well known and generally recognized (Oyebode et al. 2014; Wang et al. 2014).

Over the past decade, there has been a steady increase in epidemiological studies investigating the relationships between dietary patterns and mental state since diet plays an important role in the onset and severity of a various mental health disorders, including depression (Berk and Jacka 2019; Marx et al. 2020). Earlier observational and small intervention studies pointed mainly on possible effects of diet rich in the above dietary items or of the entire dietetic patterns containing them (Vitiello et al. 2016). Afterward, the possible role of various specific dietary fruits or vegetables has been suggested through observational and interventional investigations. However, observational and interventional studies cannot elucidate single molecules activity that must be proven through *in vitro* assays and confirmed with controlled preclinical trials *in vivo*.

The primary purpose of this dissertation was to evaluate the antidepressant-like activity of kiwifruit juice and to further advance our understanding of the effect of nutraceutical products on depression. To accomplish this, I used two gold-standard animal models of depressive behavior in mice together with analytical analysis and an *in-vitro* inhibition assay of MAOs.

At the very beginning, kiwifruit (i.e. the fruit of *Actinidia deliciosa*, the “green” kiwifruit) was chosen based on previous reports on antidepressant activity of the fruit of its close relative *Actinidia chinensis* (the “gold” kiwifruit) (Carr et al. 2013). Moreover, other investigations suggested the ability of green kiwifruit to display activity at brain level, as the improve of sleep quality in youngs and adults with sleep problems (Lin et al. 2011; Nødtvedt et al. 2017). Moreover, green kiwifruit contains relevant amount of serotonin (Feldman and Lee 1985) and its plant precursor, tryptamine (data not shown in this thesis). In principle, both serotonin and its derivative, melatonin, could be involved in sleep improve (Binks et al.

2020), even though melatonin have never been detected in our analysis. Also the plant-precursor of serotonin, the indolamine tryptamine, could act in the same pathway, since it can act as serotonin agonist (Kay and Martin 1978; Radulovacki et al. 1983).

Dietary serotonin and tryptamine are generally not bioavailable because they are substrates of gut MAO-A and B enzymes, but still a role for these molecules when eaten within a composite phytocomplex such as a fruit or vegetables, or a combination of them, cannot be excluded due to synergic activity between different components. As an example, the shamanic beer ayahuasca retain the hallucinogens properties of the dimethyltryptamine (DMT) contained in the herb *Psychotria viridis*, Ruiz & Pav. thanks to the MAO-inhibiting activity of the β-carbolines (harmine, harmaline and tetrahydroharmine) of *Banisteriopsis caapi*, (Griseb.) Morton (McKenna et al. 1984; Martin et al. 2012). Thus, in principle, possible MAO-A and B inhibitory components of kiwifruits could change the fate of fruit serotonin and tryptamine making them more bioavailable.

A completely different story emerged as the project unfolded: kiwifruit resulted to display quite strong antidepressant-like activity; MAO-A and B inhibitory components resulted to be really present in kiwifruit but, nevertheless, fruit serotonin and tryptamine were not able to reach the blood or the brain; one metabolite, previously not considered, quinic acid, resulted to be both able to reach blood and brain, passing the blood brain barrier, and to be at least partially responsible for the antidepressant activity of kiwifruit.

Here's the whole story.

In **Chapter 2**, I examined the effect of 10-days chronic treatment with different kiwifruit dilutions on TST and FST in mice. Kiwifruit at highest dilution, the so-called Kiwi 1, strongly reduced immobility time in above-mentioned tests without causing any locomotor impairment. Moreover, few kiwifruit metabolites were found in kiwifruit-fed mice serum and brain compared with controls, and two of these were identified: one, quinic acid, is a kiwifruit metabolite, while the other,

caffeic acid sulfate, is a mouse metabolite of kiwifruit molecules. Nor serotonin or tryptamine were found to be different in serum or brain of animals treated with kiwifruit compared to control group. Quinic acid and caffeic acid sulfate levels were evaluated by mass spectrometry. Additionally, PK experiments were performed in animals fed with kiwifruit to evaluate adsorption and clearance of the two compounds. Furthermore, brain penetration was evaluated in animals treated with the two metabolites after transcardiac perfusion technique. The post-perfusion brain level of both QA and CA-sulfate were lower compared to non-perfused samples (about half), but still detectable, proving that the two molecules pass the blood brain barrier and are able to enter in the brain parenchyma.

In **Chapter 3**, I evaluated the two single specialized metabolites of kiwifruit in the same behavioral paradigms after 10-days chronic treatment regime. Only QA but not CA-glucoside was active in reducing immobility time in both tests, although it was less efficient of the whole kiwifruit phytocomplex. Additionally, when given in combination, the two molecules seemed to have a synergistic effect that is displayed only in TST. PK and perfusion experiments showed that QA administered alone had lower adsorption and brain penetration compared to the whole fruit. Thus, QA administered alone at the same concentration found in the whole fruit can partially mimic kiwifruit antidepressant-like activity in mice, indicating that QA is one of the active anti-depressant molecules although its activity is lower in comparison of kiwifruit. The full activity, bioavailability and brain penetration of the two kiwi metabolites could require other molecules present in the whole fruit whose presence might be fundamental for the correct intestinal adsorption and/or stability in mice. In addition, a combination of additional molecules, that we have to presume to be at very low level since they are not detected by the very sensitive UPLC-ESI-MS platform, could be responsible for part of the antidepressant activity of the whole fruit, maybe acting in synergy with each other's clarifying the lower behavioral and analytical effects seen in single-molecule treatments. Secondary metabolites synergy activity is well-known

in literature and could explain our data obtained administering QA and CA-glucoside together.

Based on the effective dose of kiwifruit juice and the exact quantification of QA and CA-sulfate obtained by mass spectrometry, we could hypothesize a translational antidepressant-like effect in humans of approximately 700 grams of kiwifruits/day.

To identify possible molecular targets that could explain the *in-vivo* kiwifruit antidepressant-like activity, in **chapter 4** we optimized a MAOs inhibition assay showing that kiwifruit and four of its metabolites, quinic acid, caffeic acid, catechin and esculin, displayed good inhibitory activity. Interestingly only quinic acid showed a selective-MAO inhibitory effect since it is active only against MAO-B, whereas other molecules exhibit non-selective inhibition.

All these data confirm that kiwifruit has potent antidepressant-like effect and is a potent inhibitor of both MAOs. These effects could be addressed to its specialized metabolites, especially quinic acid. For that reason, kiwifruit or the single metabolites could be used as coadjuvant in the treatment of depression in patient's refractory to other treatments or to reduce the long lag-time present in current antidepressant pharmacotherapies.

One main limitation using plant secondary metabolites in therapy is the possible low stability and bioavailability of these molecules in animals and humans, in fact quinic acid, caffeic acid as well as CGA are poorly absorbed through alimentary tract in human and animals (Farah and de Paula Lima 2019; Lu et al. 2020). Specifically, regarding quinic acid, gut flora played a crucial role in conversion of quinic acid to benzoic and hippuric acids (Naranjo Pinta et al. 2018; Sova and Saso 2020), probably greatly decreasing its bioavailability. Thereby, development of novel fruit metabolite delivery systems, maybe using nanoparticles, as dietary supplements, could overcome this problem.

In addition, since the above nutraceuticals products display a long history of safe consumption, they could be promptly used as coadjuvant in the treatment of

depression, since they do not require control by regulatory agencies such as FDA and EMA.

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Appendix

Table 1. Untargeted metabolomics data matrix. The color code ranges from blue (lowest relative abundance) to red (highest relative abundance)**.** ui=unidentified compounds

id	RT	m/z (-) detected	m/z (-) expected mass	Elemental formula	Putative identification	Main fragments	kiwifruit juice/neutral solution ratio	mean	
								kiwifruit juice	neutral
36	0,030	474,093			ui		6,384322526	3815,098264	597,5729216
75	0,030	519,110			ui		1,413838574	658,4026694	465,6844717
72	0,041	357,103			ui		1,071010382	4029,285282	3762,134662
94	0,041	146,045			ui		1,361012177	1356,652357	996,7966341
106	0,051	377,085			ui		1,876433063	10345,3577	5513,31028
112	0,051	379,085			ui		1,706124413	3961,381434	2321,859652
69	0,051	367,064			ui		1,245553152	2639,532943	2119,165238
113	0,051	579,176			ui		1,126881487	1358,487671	1205,528432
70	0,062	165,040			ui		1,068957853	3209,892569	3002,824256
114	0,062	192,022			ui		10,30925555	19737,26831	1914,519261
67	0,083	586,048			ui		1,837551213	9051,899684	4926,066615
102	0,083	129,018			ui		2,354967138	1213,379356	515,2425849
91	0,093	609,187			quinic acid derivative/adduct	191,056	1,012894147	2371,45026	2341,261687

88	0,093	191,234	191,055	C7H12O6	quinic acid	191,056	1,156941056	2041,852275	1764,87148
90	0,125	391,066			quinic acid derivative/adduct	191,056	1,93734486	25620,76701	13224,68062
93	0,125	347,023			ui		1,487967862	7288,108901	4898,028438
95	0,177	389,033			ui		9,420480907	2348,328762	249,2790745
107	0,187	391,061			ui		5,570418615	18064,72512	3242,974428
142	0,187	549,167			ui		1,687488181	4805,087826	2847,479396
139	0,187	605,194			piruvic acid derivative	87,007	-	4984,342766	0
62	0,219	420,995			citric acid derivative/adducts	191,019	1,027442742	14792,43276	14397,33054
63	0,240	649,964			citric acid derivative/adducts	191,019	1,273505257	15372,09061	12070,69271
148	0,240	457,939			citric acid derivative/adducts	191.109, 111.007	2,266140543	8252,094135	3641,475001
150	0,250	111,008			ui		1,845078262	26348,52791	14280,43918
152	0,250	191,235	191,019	C6H8O7	citric acid	111,007	1,137584715	1973,961491	1735,221531
151	0,250	129,018			ui		1,066023394	1005,682061	943,395864
154	0,261	448,990			citric acid derivative/adducts	191.019, 111. 007	1,161523844	12174,65805	10481,6256
155	0,261	641,016			citric acid derivative/adducts	191.019, 111. 007	1,842152553	9598,277827	5210,359919
2	0,615	516,895			ui		3,2413124	4099,719112	1264,83307
1	0,615	418,893			ui		3,473134614	3823,678618	1100,930152
65	0,625	288,935			ui		2,42474644	5839,69625	2408,373987
4	0,625	532,872			ui		2,523762711	2829,505971	1121,145803

66	0,625	402,916			ui		4,525283372	4797,722086	1060,20368
64	0,625	272,958			ui		5,365386023	4944,901817	921,6302044
19	0,625	616,835			ui		2,802792554	2202,383685	785,7819096
158	0,625	434,870			ui		1,741232408	655,5080061	376,4620984
159	0,625	500,918			ui		7,747142058	638,1036252	82,36632561
68	0,667	296,881			ui		1,136581596	1272,261951	1119,375816
11	0,667	466,803			ui		1,061682767	966,8796227	910,7048292
71	0,678	632,727			ui		1,043802096	1979,84303	1896,760926
121	0,678	548,766			ui		1,147955675	1808,499164	1575,408531
76	0,678	380,843			ui		1,20618015	1671,753644	1385,990015
73	0,678	464,805			ui		1,179996821	1364,372012	1156,250582
8	0,678	1390,380			ui		1,062300144	1168,593579	1100,059702
130	0,678	1306,418			ui		1,148249157	970,3434191	845,0634716
74	0,678	1474,343			ui		1,166520598	887,6354439	760,9256495
80	0,678	1558,306			ui		1,129383172	696,1555019	616,4032893
77	0,709	225,061	225,061	C6H12O6	glucose		1,026299304	1544,81346	1505,227037
132	0,730	245,035			ui		1,100272176	6342,461698	5764,447958
17	0,730	277,033			ui		1,07925489	5371,106989	4976,680709
84	0,730	367,063			ui		1,318776722	964,9382112	731,6918741
82	0,730	96,966			ui		1,712675084	1172,484512	684,5924968

81	0,730	355,087	355,066	$C_{15}H_{16}O_{10}$	caffeic acid glucuronide	193,033	1,167593764	782,9690636	670,5834575
78	0,740	421,075			ui		1,124578598	1805,594933	1605,574689
137	0,740	517,140			ui		1,139272198	732,6387427	643,0761183
79	0,740	146,045			ui		1,151308097	603,5556178	524,2346676
83	0,751	195,050			ui		1,166086903	3438,812797	2949,019312
13	0,751	367,105			ui		1,28489975	3353,574662	2609,989348
61	0,751	217,029			ui		1,116685807	2783,951795	2493,048428
136	0,751	601,138			ui		1,174744257	2462,877905	2096,522619
57	0,751	237,061			ui		1,216759392	2109,048065	1733,332061
147	0,751	209,030	209,029	$C_6H_{10}O_8$	glucaric acid		1,138344542	1943,754258	1707,52719
24	0,751	357,103			ui		1,200576907	1753,161234	1460,265663
29	0,751	424,031			ui		1,069125901	867,8995372	811,7842216
135	0,761	165,039			ui		1,182497024	1336,971398	1130,634049
33	0,761	379,084			ui		1,10010951	944,6989783	858,7317627
26	0,761	223,045			ui		1,240784448	945,1163354	761,7087216
134	0,761	412,030			ui		1,372790662	700,5067417	510,2793609
31	0,772	565,047			ui		1,243225119	3786,835824	3045,977567
32	0,772	221,029			ui		1,104953182	1082,590703	979,7616052
133	0,772	207,050			ui		1,153524334	768,801645	666,4806476
28	0,772	579,182			ui		1,457789187	930,5369681	638,3206685
59	0,782	439,084			ui		1,231262065	13085,24944	10627,50962
27	0,782	267,071			ui		1,100739106	1143,084201	1038,469693

131	0,793	191,059	191,055	$C_7H_{12}O_6$	quinic acid	173.044, 127.039, 111.043	1,141425187	40834,57838	35775,08086
12	0,793	383,119			quinic acid derivative/adduct	191,057	1,069536676	14945,65235	13973,95029
25	0,793	405,101			ui		1,197531832	6797,722367	5676,443985
30	0,793	377,085			ui		1,134875735	3315,324868	2921,310912
128	0,793	586,068			ui		1,389617091	1766,311858	1271,078105
127	0,793	191,302			ui		1,040043803	1035,169209	995,3130881
60	0,793	613,128			ui		1,233584196	978,1187658	792,9079906
126	0,793	191,374			ui		1,059159652	772,9325197	729,7601623
23	0,803	533,404			quinic acid derivative/adduct	191,057	1,038531121	4121,523661	3968,608718
34	0,803	609,187			quinic acid derivative/adduct	191,057	1,167386605	1639,097838	1404,074563
125	0,803	533,470			quinic acid derivative/adduct	191,057	1,037743359	957,8813454	923,0426168
124	0,814	533,176			quinic acid derivative/adduct	191,057	1,127003065	83605,74293	74184,13093
122	0,814	695,223			sucrose derivative/adduct	341.1088, 179.0550	1,02254831	2608,302733	2550,786803
123	0,814	533,586			quinic acid derivative/adduct	191,057	1,036627213	2337,448425	2254,859215
21	0,824	605,192			sucrose derivative/adduct	341.1088, 179.0550	1,076481895	6333,249893	5883,285097
35	0,824	549,166			sucrose derivative/adduct	341.1088, 179.0550	1,048924451	3286,349346	3133,065821
138	0,824	683,224			sucrose derivative/adduct	341.1088, 179.0550	1,007727582	2687,269586	2666,66273
20	0,824	387,314	387,113	$C_{12}H_{22}O_{11}$	sucrose derivative/adduct	341.1088, 179.0550	1,059252976	2018,272241	1905,373209

141	0,824	342,112			sucrose derivative/adduct	341.1088, 179.0550	1,166493069	1628,832056	1396,349537
157	0,824	903,268			sucrose derivative/adduct	341.1088, 179.0550	1,296686432	1699,060979	1310,309831
18	0,824	663,197			sucrose derivative/adduct	341.1088, 179.0550	1,090770263	1413,057697	1295,467749
37	0,824	965,255			sucrose derivative/adduct	341.1088, 179.0550	1,092620516	1214,413393	1111,468598
140	0,824	387,466			sucrose derivative/adduct	341.1088, 179.0550	1,014546383	939,692259	926,2191207
16	0,824	781,198			sucrose derivative/adduct	341.1088, 179.0550	1,068042372	939,3309356	879,4884548
156	0,824	1074,329			sucrose derivative/adduct	341.1088, 179.0550	1,086303071	879,5207578	809,6458359
22	0,835	173,008			ui		1,088193712	12427,56012	11420,35649
56	0,835	736,135			sucrose derivative/adduct	341.1088, 179.0550	1,201325762	4768,220919	3969,132328
47	0,835	517,141			sucrose derivative/adduct	341.1088, 179.0550	1,238820329	1686,961267	1361,74813
55	0,835	839,225			ui		1,120772654	1148,734957	1024,94913
54	0,835	637,182			ui		1,016524079	626,547433	616,3626083
153	0,835	455,101			ui		1,731042957	777,90401	449,3845788
53	0,845	133,014	133,013	$C_4H_6O_5$	malic acid		1,180092602	9127,44322	7734,514397
52	0,845	475,130			ui		1,101431105	7077,11359	6425,380181
149	0,845	1145,288			ui		1,697215869	1460,909183	860,7680439
51	0,845	555,117			ui		1,55720337	924,9736872	593,9967157
119	0,937	275,021			ui		1,024954401	1511,677131	1474,87257
146	0,969	391,070			ui		1,097376813	9598,904492	8747,136238
50	1,084	391,069			ui		1,027572651	10304,23041	10027,73906
145	1,084	965,259			ui		1,040585828	697,9498376	670,7277948
144	1,168	306,076	306,075	$C_{10}H_{17}N_3O_6S$	glutathione		1,221966486	880,4514217	720,5201056

3	1,281	405,029			ui		1,627705728	45101,40396	27708,5736
143	1,281	435,958			citric acid derivative/adducts	191,018	1,165287055	15727,40195	13496,59029
9	1,281	649,966			ui		1,379068857	8021,432714	5816,557074
6	1,281	635,002			ui		1,220047202	6650,624601	5451,120736
120	1,281	619,036			citric acid derivative/adducts	191,018	2,15315734	6478,167176	3008,682671
49	1,281	613,020			citric acid derivative/adducts	191,018	2,033640039	5543,056044	2725,681998
118	1,281	420,996			citric acid derivative/adducts	191,018	1,412977209	2572,592166	1820,689074
98	1,281	665,932			citric acid derivative/adducts	191,018	1,056026824	708,3397417	670,7592324
96	1,281	885,953			citric acid derivative/adducts	191,018	1,722657212	1138,494092	660,8941608
48	1,291	448,985			citric acid derivative/adducts	191,018	1,133628603	2671,093519	2356,233348
99	1,291	191,267			ui		1,677920257	2447,901997	1458,890544
92	1,291	191,234			ui		1,276471047	976,2057663	764,7692193
14	1,302	191,020			ui		1,265772208	109864,9791	86796,80151
58	1,302	111,008			ui		1,649436171	6552,191883	3972,382805
5	1,302	641,017			ui		1,553386419	5340,50067	3437,973067
86	1,302	191,158			ui		1,738844364	5564,628062	3200,187537
85	1,302	191,338			ui		1,31205352	714,086172	544,2507956
87	1,302	857,957			ui		1,514196812	718,1355474	474,2682998
89	1,302	597,042			ui		2,484907334	643,5371212	258,9783178

46	1,323	849,008			ui		2,833276224	2300,418988	811,9289497
15	1,333	855,023			ui		2,504718862	3096,556094	1236,288887
45	3,053	351,130			ui		6,940013066	700,7742436	100,9759257
44	3,386	341,087	341,087	C15H18O9	caffeic acid hexose	179.034, 135.044	76,45862285	7278,693158	95,19780617
42	3,480	316,150			caffeic acid derivative	179,033	22,73484202	843,6442145	37,10798667
43	3,480	549,144	549,145	C21H28O14	caffeic acid di-hexose		602,9587376	1296,12739	2,149612086
111	3,585	323,134			ui		3,541738105	2248,760557	634,9313501
117	3,648	339,070	339,071	C15H16O9	esculin (esculetin-6-O-glucoside)		2036,248206	1025,814295	0,503776648
7	3,991	341,087	341,087	C15H18O9	caffeic acid hexose	201.015, 179.033, 135.044	274,4696194	15620,2417	56,91063999
41	3,991	477,060			caffeic acid hexose derivative	179.033, 135.044, 341.0866	-	1886,475476	0
105	4,023	363,164			ui		7861,375417	737,2152434	0,093776878
104	4,138	449,239			ui		-	1591,145203	0
40	4,209	323,134			ui		11,61545974	1024,636537	88,21317107
109	4,387	289,071	289,071	C15H14O6	catechin		-	1503,506546	0
10	4,615	341,087	341,087	C15H18O9	caffeic acid hexose	135,044	41,34951935	4149,9543	100,3628183
108	4,773	427,180			ui		697,0089829	1004,281645	1,440844623
115	5,014	441,196			ui		14621,99886	1172,773854	0,080206124
39	5,116	449,238			ui		776,880078	966,1107877	1,243577761
38	5,305	341,087	341,087	C15H18O9	caffeic acid hexose	135,043	29,7804075	659,3703191	22,14107779
100	6,033	461,165			ui		14,3444641	635,3570455	44,29283947
103	6,117	509,222			ui		13,03112822	1150,837408	88,31448738

110	7,827	469,227			ui		10829,35014	1122,193519	0,103625195
116	15,057	353,211			ui		7,245505649	3249,96017	448,5484282
101	15,288	387,153			ui	177,096	15,64426211	1392,998843	89,04215698
97	16,111	339,230			ui		2,465473198	13679,07104	5548,2538
129	16,423	484,319			ui		14,47662176	5659,362832	390,9311802
160	17,757	421,224			ui		3,919418998	2100,649883	535,9595093

Product of research

Posters presented at International congresses

- "Antidepressant-like effect of kiwifruit (*Actinidia deliciosa*) extract on mouse models of depression". 11th FENS Forum of Neuroscience. Berlin, 7-11 July 2018.
  Authors: ***Marzo C.M.****, Bianconi M., Commisso M., Chiamulera C., Guzzo F.*
- "Evaluation of antidepressant-like activity of kiwifruit (*Actinidia deliciosa*) extract in mice". Neuroscience 2018 - Society for Neuroscience. San Diego, 3-7 November 2018.
  Authors: ***Marzo C.M.****, Bianconi M., Commisso M., Chiamulera C., Guzzo F.*
- "Evaluation of antidepressant-like activity of kiwifruit (*Actinidia deliciosa*) extracts on mouse models of depression". HPLC 2019. Milan, 16-20 June 2019.
  Authors: ***Marzo C.M.****, Bianconi M., Chiamulera C., Guzzo F.*
- "Evaluation of antidepressant-like activity of kiwifruit (*Actinidia deliciosa*) extracts on mouse models of depression". MNS 2019. Marrakech, 23-27 June 2019.
  Authors: ***Marzo C.M.****, Bianconi M., Chiamulera C., Guzzo F.*
- "Evaluation of antidepressant-like activity of kiwifruit (*Actinidia deliciosa*) extracts on mouse models of depression". Neuroscience2020. Kobe, July 29, 2020 - August 1, 2020 (Virtual Meeting).
  Authors: ***Marzo C.M.****, Bianconi M., Chiamulera C., Guzzo F.*

Publications in the pipeline

- The content of Chapter 1 reporting the state of the art on depression, treatments and nutraceutical approaches will the object of future review publication.
- The content of chapter 2 and 3 with further experimental evidence related to the inhibitory effect of the various treatments on brain MAOs of mice, will be the object for future publication.
- The content of chapter 4 is already being used as a draft for a submitted article: “Inhibition of human monoamine oxidases A and B by specialized metabolites present in fresh common fruits and vegetables” to the journal *Foods*.

Other Publications

During these 3 years I had the opportunity to publish, as a coauthor, 2 paper of previous completed experiments:

- Moro F, Giannotti G, Caffino L, **Marzo CM**, Di Clemente A, Fumagalli F, Cervo L. "Lasting reduction of nicotine seeking-behavior by chronic N-acetylcysteine during experimental cue-exposure therapy". Addict Biol. 2020 Jul;25(4):e12771. https://doi.org/10.1111/adb.12771. Epub 2019 May 27.
- Lucchetti J, **Marzo CM**, Passoni A, Moro F, Di Clemente A, Bagnati R, Cervo L, Gobbi M. “Brain disposition, metabolism and behavioral effects of the synthetic opioid AH-7921 in rats.” Neuropharmacology. 2018 May 1;133:51-62. https://doi.org/10.1016/j.neuropharm.2018.01.023. Epub 2018 Jan 31.