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TEREZA RAQUEL PEREIRA TAVARES

DEVELOPMENT OF A PLANT-BASED BEVERAGE WITH ANXIOLYTIC-LIKE EFFECTS EVALUATED IN ZEBRAFISH (*DANIO RERIO*) MODEL

FORTALEZA
2023

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Dissertation presented to the Graduate Program in Food Science and Technology at the Federal University of Ceará, as a partial requirement toobtain the title of Master in Food Science and Technology. Concentration area: Science and Technology of Products of Vegetable Origin.

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ABSTRACT

With the scenario of COVID-19 pandemic, cases of depression, anxiety and other stress-related problems have increased, leading to an increase in the consumption of controlled-use drugs, such as antidepressants and anxiolytics. On the other hand, many studies indicate that some foods and medicinal plants can be used as an alternative to improve neurological and cognitive properties, alleviating the effects of stress and promoting a sense of well-being. Several studies attribute these effects to the composition of the raw materials used, due to the presence of bioactive compounds, amino acids and other constituents with this potential. The development of foods, which in addition to the nutritional function, seek to contribute with aspects directly related to health, is one of the main trends for the coming years, with an important emphasis on functional beverages. Based on the above, this research aimed to develop a composite functional beverage, with anxiolytic-like effect, produced with matcha (*Camellia sinensis*) and sugarcane juice, with the addition of medicinal plants (chamomile and lemon balm). For this, the work was carried out in two stages, the initial stage being dedicated to the scientific study of the raw materials involved and the intended market, which makes up the first chapter of this dissertation, and, in the second stage, which comprises the second chapter, are the analyzes to obtain the product. Initially, the beverage's base formulation was created from different combinations of matcha and sugarcane juice in order to test the total soluble solids content, the total antioxidant activity (by the ABTS method) and the anxiolytic-like effect through the *in vivo* test, using the anxiolytic model for the adult zebrafish (*Danio rerio*). After choosing the promising base formulation, the medicinal plants chamomile and lemon balm were added to it and the analyzes of bioactive compounds (total phenolic compounds, profile of phenolic compounds by HPLC, total antioxidant activity and bioaccessibility of phenolic compounds), as well as how the *in vivo* tests were carried out with adult zebrafish to prove the anxiolytic effect of the developed drink.

Keywords: functional beverage; bioactives compounds; mental health; zebrafish.

RESUMO

Com a pandemia de COVID-19, os casos de depressão, ansiedade e outros problemas relacionados ao estresse aumentaram, levando a um incremento no consumo de medicamentos de uso controlado, como antidepressivos e ansiolíticos. Por outro lado, muitas pesquisas indicam que alguns alimentos e plantas medicinais podem ser utilizados como alternativa para melhorar as propriedades neurológicas e cognitivas, aliviando os efeitos do estresse e promovendo a sensação de bem-estar. Vários estudos atribuem estes efeitos à composição das matérias-primas utilizadas, devido à presença de compostos bioativos, aminoácidos e outros constituintes com este potencial. O desenvolvimento de alimentos, que além da função nutricional, buscam contribuir com aspectos diretamente ligados à saúde, constitui uma das principais tendências para os próximos anos, havendo um destaque importante para as bebidas funcionais. Com base no exposto, esta pesquisa teve como objetivo desenvolver uma bebida funcional composta, com potencial ansiolítico, à base de matcha (*Camellia sinensis*) e caldo de cana, adicionada de plantas medicinais (camomila e erva cidreira). Para isto, o trabalho foi executado em duas etapas, sendo a etapa inicial dedicada ao estudo científico das matérias-primas envolvidas e do mercado pretendido, que compõe o primeiro capítulo desta dissertação e, na segunda etapa, que compreende o segundo capítulo, foram executadas análises para obtenção do produto. Inicialmente, foi criada a formulação base da bebida, a partir de diferentes combinações de matcha e caldo de cana a fim de testar o conteúdo de sólidos solúveis totais, a atividade antioxidante total (pelo método ABTS) e o efeito ansiolítico através do teste *in vivo*, utilizando o modelo ansiolítico para o zebrafish adulto (*Danio rerio*). Após a escolha da formulação base promissora, à ela foram acrescentadas as plantas medicinais camomila e erva cidreira e foram realizadas as análises de compostos bioativos (compostos fenólicos totais, perfil de compostos fenólicos por HPLC, atividade antioxidante total e bioacessibilidade de compostos fenólicos), bem como foram executados os testes *in vivo* com zebrafish adulto para comprovar o efeito ansiolítico da bebida desenvolvida.

Palavras-chave: bebida funcional; compostos bioativos; saúde mental; zebrafish.

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1 GENERAL INTRODUCTION

According to WHO - World Health Organization (2020), Brazil was considered the country with the most anxious population in the world. And in 2020, due to the great change in the daily lives of Brazilians, due to the COVID-19 pandemic, there was a 17% increase in the consumption of controlled-use medications, such as antidepressants and mood controllers (CNN BRASIL, 2021), as well as such as the 88% increase in the search for psychological care, in the first weeks of quarantine in the country (GOOGLE TRENDS, 2021).

On the other hand, research shows that some foods, including medicinal plants, can be essential in combating the damage caused by stress, such as insomnia, anxiety and depression (VASCONCELOS *et al*., 2007; ARAÚJO *et al.,* 2020; NASERI *et al.* 2021; ESMAEILPOUR-BANDBONI *et al*., 2021), as bioactive compounds (such as antioxidants) and fatty alcohols, such as octacosanol, are present in its composition, which help in neurological, cognitive and memory properties (SCHRÖDER *et al.,* 2019; KAMBLE *et al.*, 2021).

According to Whole Foods (2021), foods allied to well-being are a strong trend for 2022, among them ready-to-eat functional drinks. According to NAZIR *et al*. (2019), it is estimated that by 2025 this type of product will correspond to 40% of total consumer demand. Tireki (2021) shows that consumers will look for functional products that improve mood and stimulate more brain health, such as beverages with relaxing properties of the central nervous system and beverages that help relieve stress.

In this sense, the medicinal plants, such as matcha (*Camellia sinensis*), chamomile (*Matricaria chamomilla*) and lemon balm (*Melissa officinalis*), and sugarcane juice (*Saccharum officinarum*) were investigated as raw materials to obtain a functional beverage, considering that these separate ingredients contain bioactive compounds that can actve in combat disorders of the central nervous system, such as anxiety and insomnia. Based on the hypothesis that raw materials together in the composition of a beverage can provide greater antioxidant potential and, consequently, a desired anxiolytic effect.

Sugarcane juice is a drink commonly consumed in Brazil in its natural form to provide energy. Studies indicate that in addition to the sugar content present in the drink, it may have great potential to combat damage caused by stress, such as insomnia (KAMBLE *et* *al*., 2021). Matcha is a type of powdered green tea originating from Japan, which was commonly used by Japanese people in meditation ceremonies, because this tea promotes relief from anxiety and mindfulness (JAKUBCZYC, 2020). Nowadays, matcha is one of the trending raw materials for composing functional foods, as in addition to its umami flavor (which facilitates its introduction into different food matrices), it has a high content of antioxidants and amino acids (KOLÁČKOVA *et al*., 2020; JAKUBCZYC, 2020, DEVKOTA *et al*., 2021). However, in Brazil, matcha is not yet commonly known, which motivated the innovative opportunity to work with this raw material alongside sugarcane juice, which is typically Brazilian.

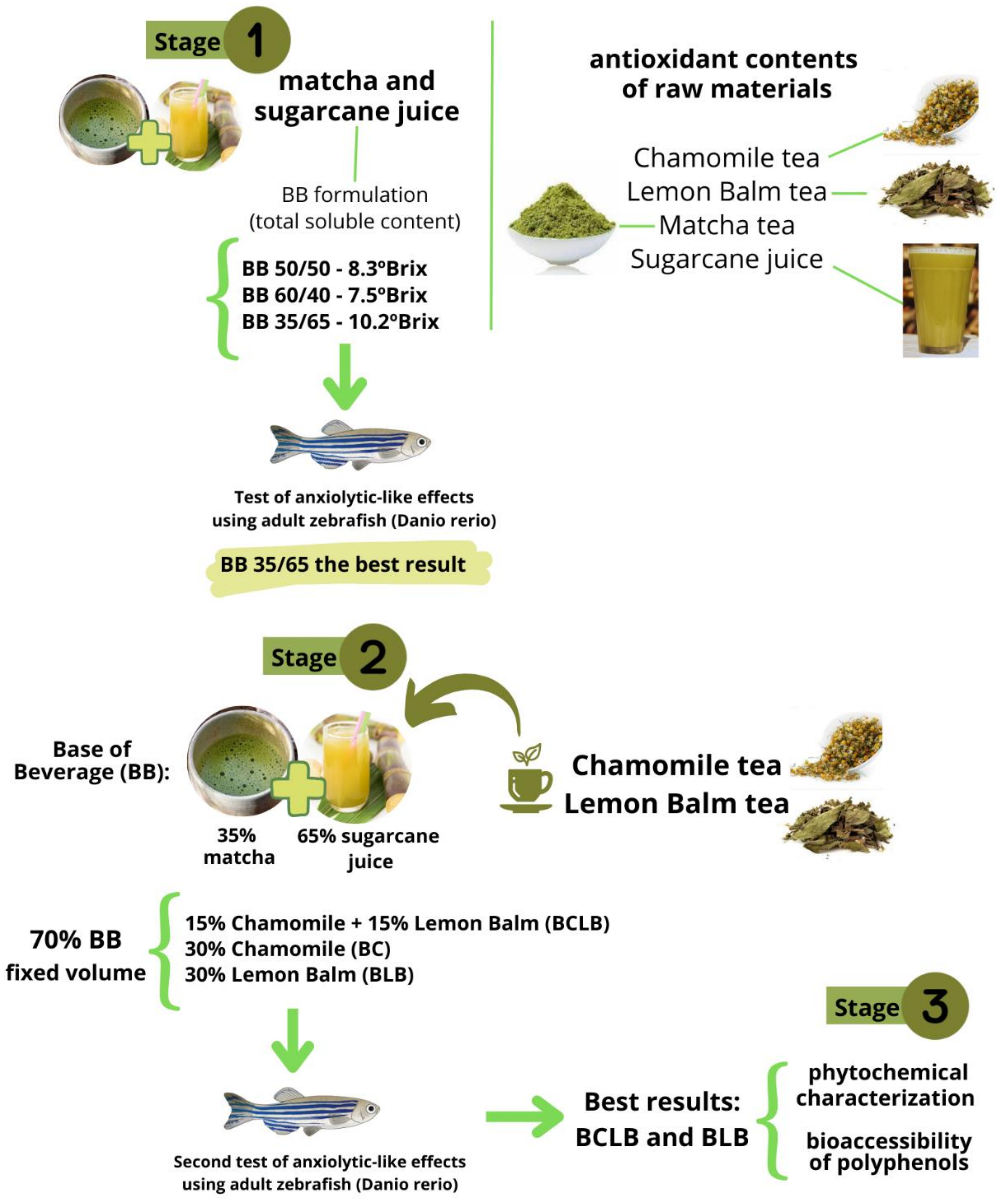
In addition, lemon balm and chamomile teas are commonly used to promote relaxation (NASERI *et al*., 2021; AMSTERDAN *et al*., 2009). Both have antioxidants that can help combat anxiety, among other damages caused by stress, in addition to being allies to cognitive functions (ARAÚJO *et al*., 2019; PETKOVA *et al.,* 2017). Inserting these raw materials into the developed beverage aimed to associate the new product with something already known by the target consumers, as well as differentiating the content of bioactive compounds in the final product.

The zebrafish model is increasingly gaining ground as a practical tool for evaluating neurological behaviors, because this animal provides a favorable compromise between neurobiological complexity and practical simplicity (SYED *et al.,* 2023). Initially, zebrafish models were mainly used in the medical and pharmaceutical fields, for example, to test the action of drugs. However, recently this animal is an important tool for the food industry to test, for example, the neurological effects of food and beverage products and ingredients, such as food preservatives (LEONARD *et al.,* 2023; XU *et al.,* 2022) and raw material used to compose food packaging (YUAN *et al.,* 2023).

Therefore, this present study is divided in two chapters. The first one consists of scientifically studying the context of stress-related diseases, the raw materials involved in the production of the beverage, the market for functional beverages and an explanation about the use of zebrafish in behavioral studies. The second chapter aimed the beverage's development. For this, the work was separeted in three steps, being the first one dedicated to beverage's base formulation was created from different combinations of matcha and sugarcane juice in order to test the total soluble solids content, as well were evalueted the total antioxidant activity (by the ABTS method) of all raw materials and the anxiolytic-like effect through the *in vivo* test, using the anxiolytic model for the adult zebrafish (*Danio rerio*). The second step comprehend the development of final products (BLB and BCLB), where the base formulation chosen was added from medicinal plants chamomile and lemon balm, isolated and combinated to evaluate the anxiolytic-like effect with the *in vivo* tests in adult zebrafish. And in the third step were tested analyzes of bioactive compounds (total phenolic compounds, profile of phenolic compounds by HPLC, total antioxidant activity and bioaccessibility of phenolic compounds), to characterize the final product.

These steps for the development of the functional beverage are shown in Figure 1 below.

Figure 1. Stages of the beverage's development.



Source: Author, 2023.

2 OBJECTIVES

The main objective of this research is to develop a beverage based on matcha tea and sugarcane juice, incorporated with medicinal plants (chamomile and lemon balm), aiming to obtain in this final product the nutritional and phytochemical characteristics that may help in the mental health and well-being of its consumers, based on the study of the anxiolytic-like effect in adult zebrafish.

2.1 Specific objectives

- Identify the most promising base formulation (matcha and sugarcane juice) for the drink, based on the total soluble solids content;
- Determine the anxiolytic activity of the bases formulated and final product using the adult zebrafish model (*Danio rerio*);
- Carry out the phytochemical characterization of the final product, after added chamomile and lemon balm, through analysis of antioxidant activity and total phenolic compounds;
- Evaluate, through *in vitro* digestion analysis, the bioaccessibility of phenolic compounds presented in the final product.

3 THEORETICAL REFERENTIAL

3.1 The mental health in Brazil

The According to WHO - World Health Organization (2020), Brazil was considered the country with the most anxious population in the world. And globally, there are nearly a billion people living with mental disorders such as anxiety and depression. Meanwhile, WHO and PAHO - Pan American Health Organization (2020) indicated that mental health is one of the most neglected areas.

In 2020, due to the COVID-19 pandemic, there was a big change in the daily lives of Brazilians with the practices adopted to combat the disease, such as social isolation. Given this scenario, Barros *et al.* (2020) interviewed 45,161 people to study the impact of the pandemic on anxiety, depression and insomnia in the Brazilian adult population and found that the frequent feeling of sadness or depression reached 40% of Brazilian adults, and the frequent feeling of anxiety and nervousness was reported by more than 50% of them. In addition, among those who did not have sleep-related problems, more than 40% started to have them and in almost 50% of those who already had insomnia, this problem got worse. Data from this survey were collected when confirmed cases of COVID-19 in Brazil rose from 45,757 to 330,890 and deaths from 2,906 to 21,048. In October 2021, confirmed cases and deaths from COVID-19 in Brazil exceeded 21 million and 600,000, respectively, according to data from the Ministry of Health (2021).

As was reported by CNN Brasil (2021), after a survey by the National Council of Pharmacy, in 2020 in the country, the sale of antidepressants and mood controllers increased by 17%, compared to the previous year. In the states of Ceará and Amazonas this increase corresponded to 29%, being higher than the Brazilian average.

On the report of Google Trends (2021), the number of people who began to seek psychological care in Brazil grew by 88% in the first weeks of quarantine in the country, still in 2020, causing an emerging demand for online therapies.

Before the pandemic in Brazil, an article by De Paula (2019) for Health Care Journal disclosed the analysis of the Assistance Map published by the Institute of Supplementary Health Studies (IESS) for the years 2013 to 2018, which presented an increase in the number of consultations with psychiatrists from 3.4 million to 4.9 million, this corresponded to an increase of 44.5%, the total number of sessions with psychologists almost doubled, going from 9.1 million to 17.6 million, representing a difference of 93.8%, while consultations with occupational therapists increased by 137.8%, going from 818.6 thousand to 1.9 million consultations.

These facts reflect the needs of a large public that suffers from stress and its consequences. These people yearn for an improvement in their quality of life, representing the search for everyday health and well-being, a concern that has grown in recent years.

3.2 Nutraceuticals, functional foods and medicinal plants in combating the damage caused by stress, depression and anxiety

3.2.1 Nutraceuticals

The term nutraceutical was first mentioned 30 years ago to describe a union between nutrition and pharmaceuticals, both important contributors to human well-being (HALLER, 2010; BERNAL *et al.,* 2011).

In their research, Bernal *et al.* (2011) explain that the boundary between nutraceuticals and functional foods is not always clear, the main difference being the format in which they are consumed: nutraceuticals are consumed in capsules or tablets, while functional foods are always consumed as normal foods. Thus, if a phytochemical is included in a food formulation it is considered a functional food. If the same phytochemical is included in a capsule, it will constitute a nutraceutical (ESPÍN *et al.,* 2007; BERNAL *et al.,* 2011).The interest in plant-derived healthy foods, nutraceuticals, functional foods and dietary supplements has increased in recent times as potential agents in maintaining health and preventing and treating diseases (DEVKOTA *et al*., 2021).

3.2.2 Functional foods and beverages

According to the Brazilian Ministry of Health (2009), functional foods are characterized by offering several health benefits, in addition to the nutritional value inherent to their chemical composition, and may play a potentially beneficial role in reducing the risk of diseases. In Brazil, foods under the authority of ANVISA that convey these claims must be classified and registered in the category of foods with claims of functional or health properties, in accordance with Resolution No. 19, de 30 de abril de 1999, of ANVISA, in the category of bioactive substances and isolated probiotics, according to ANVISA Resolution No. 02, of January 07, 2002.

Although there is no universal definition, a consensus on four main aspects to define functional foods has been reported in the literature, which are: the health benefits, the nature of the food, the level of function and the consumption pattern (MAURICE; JOANNE, 2008). The most active category of functional foods is beverages, with one of the factors being the greater possibility of incorporating nutrients and desirable bioactive components (NAZIR *et al.,* 2019). Thus, well-being foods, which focus on health, along with “superfoods” and supplements, which seek to strengthen the immune system, were a strong trend since 2022, according to Whole Foods research (2021). Among these foods are functional, ready-to-drink beverages that provide nutrients or other substances, such as flavanoids, specific amino acids, antioxidants or phytonutrients in their composition. According to Dini *et al.* (2019), examples of functional beverages are yogurt-based beverages (beverages fortified with pre- or probiotics), functional milk (extra calcium, omega-3 and vitamin-fortified beverages), juices (vitamins and beverages fortified with omega-3 -3), functional waters (fortified vitamin and mineral drinks), energy sports

drinks, herbal drinks, and health and wellness drinks.

3.2.2.1 Sugarcane juice (Saccharum officinarum)

Sugarcane (*Saccharum officinarum*) is a grass, which belongs to the Poaceae family, and represents an important crop cultivated in tropical and subtropical countries such as Brazil, India, Thailand, China and Australia (SHI *et al*., 2021). From it it is possible to extract the juice to manufacture sugar and by-products, such as molasses, molasses and rapadura; produce cachaça; ethanol, used as biofuel; and bioelectricity, from its bagasse. The sugar cane plantation is represented in Figure 2.

Figure 2. Sugarcane plantation (*Saccharum officinarum*).

Source: União Nacional da Bioenergia – UDOP (2021).

In Brazil, sugarcane was introduced in the colonial period and, since then, it has become one of the main crops of the Brazilian national economy (AMARAL *et al*. 2015). According to CONAB - COMPANHIA NACIONAL DE ABATECIMENTO (2021), Brazil is the world's largest producer of sugarcane and, in the 2020/21 harvest, it was responsible for the production of 654.5 million tons destined for the production of 41.2 million tons of sugar and 29.7 billion liters of ethanol. The sugar-energy complex, sugar and ethanol, occupies a prominent role in the export basket, and in 2020 the sector had a national share of 9.9% (US$9.9 billion), being the fourth most representative sector in the country (ANGELO *et al*., 2020). The largest sugarcane producers in the country are concentrated in the center-south and north-northeast, with emphasis on the states of São Paulo, Goiás and Alagoas (CONAB, 2021).

The distinctive characteristics of sugarcane culture among other crops include high sucrose content, high biomass content and high efficiency in accumulating solar energy (SHI *et al.,* 2021).

The plant is formed by culms, characterized by being erect, fibrous and rich in sugar (BEAUCLAIR, 2016). The reserve carbohydrates are stored in the stem (RIBEIRO *et al.*, 1999). Part of the sugarcane cultivated is destined for the extraction of sugarcane juice and used as a beverage (KAMBLE *et al.,* 2021). Thus, after harvesting, the sugarcane stalk is transported for the extraction of sugarcane juice (SHI *et al.,* 2021).

3.2.2.2. Sugarcane juice composition

In Brazil, sugarcane juice is commonly sold in direct consumption establishments such as snack bars and kiosks, which are distributed throughout most of the national territory. Along with its thirst-quenching properties, sugarcane juice is an instant source of energy (KAMBLE *et al.,* 2021; YUSOF *et al.,* 1998).

As described by Ribeiro *et al.* (1999), sugarcane juice consists of 80% water and 20% soluble solids, which are grouped into organic and inorganic sugars and non-sugars. The sugars are mainly represented by sucrose, glucose and fructose. Among the compounds classified as organic non-sugars are nitrogenous substances, such as proteins and amino acids, fats, waxes, acids (malic, succinic and aconitic) and coloring materials (chlorophyll, saccharine and anthocyanin) and inorganic non-sugar compounds are mainly represented by minerals such as potassium, phosphorus, calcium, sodium, magnesium, iron, chlorine, aluminum, sulfur and silica.

Another constituent of sugarcane juice of great importance is octacosanol. According to Taylor *et al.* (2003), octacosanol is a primary aliphatic alcohol of high molecular weight, the main component derived from the constituent wax of plants and seeds, such as sugarcane, and the main constituent of the policosanol present in it.

Policosanol from sugarcane has proven to be an ideal source of 1-octacosanol. It was reported that the filter sludge from sugarcane juice clarification contains 6.85 g / 100 g of waxes, and a high content (29.65 g / 100 g) of 1-octacosanol was observed in the waxes (KAMBLE *et al*., 2021; OU *et al.,* 2012).

This constituent has been highlighted in scientific research due to its potential beneficial effects (KAMBLE *et al*., 2021), such as cholesterol reduction, antiplatelet properties, cytoprotective use and ergogenic properties (TAYLOR *et al.,* 2003), as well as antinociceptive and anti-inflammatory (OLIVEIRA *et al.,* 2012). According to S. Kato *et al.* (1995), octacosanol may affect lipid metabolism, thus causing increased energy production necessary to improve motor endurance. Kaushik *et al.* (2017) evaluated the effect of octacosanol on sleep regulation in mildly stressed mice by oral administration and found that this compound reduced the level of corticosterone in blood plasma, which is a stress marker, as well as improved the animals' sleep. Therefore, Kaushik *et al.* (2017) found that octacosanol has the ability to reduce stress and increase sleep, potentially being useful for treating insomnia caused by stress.

3.2.3. Medicinal plants

Also according to ANVISA (2014), medicinal plants in the form of herbal drugs, hereinafter referred to as medicinal teas, are included in the category of traditional herbal products, which, according to this legislation, are considered those obtained with the exclusive use of raw materials. herbal actives, whose safety and effectiveness are based on safe and effective use data published in the technical-scientific literature and which are designed to be used without the supervision of a physician for diagnostic, prescription or monitoring purposes.

Herbal drinks, such as teas, have been consumed by man since ancient times, legends from China and India indicate that the habit of drinking tea began more than five thousand years ago (GUTMAN; RYU, 1996; DUFRESNE; FARNWORTH, 2001). Traditionally, tea was drunk to improve blood flow, eliminate toxins and increase resistance to disease (BALENTINE *et al.,* 1997; DUFRESNE; FARNWORTH, 2001). And, currently, many treatments have been carried out through phytotherapy, which consists of the use of freshly harvested medicinal plants or their natural extracts (PACÍFICO *et al.,* 2018).

After water, tea is the second most consumed beverage in the world (BINDES *et al.,* 2018), with several chemical compounds, it is of great importance for human health, in addition to having a pleasant flavor and aroma to the palate (SILVA; VILELLA, 2019). Green tea production in 2023 is expected to be twice as high as 10 years ago (SAKURAI *et al.,* 2020; CHANG, 2015). In this sense, many studies show that some teas, such as green tea (*Camellia sinensis*), chamomile (*Matricaria chamomilla*) and lemon balm (*Melissa officinalis*), have central nervous system relaxing properties, combat depression, anxiety, insomnia, and can even be used to improve cognitive functions (VASCONCELOS *et al.,* 2007; ARAÚJO *et al*., 2021; NASERI *et al*. 2021; ESMAEILPOUR-BANDBONI *et al*., 2021).

3.2.4. Medicinal plants with anxiolytic properties

3.2.4.1. Matcha tea (Camellia sinensis)

Matcha is a powdered type of green tea (*Camellia sinensis*) originating in Japan (JAKUBCZYC, 2020). Matcha tea powder is obtained from the leaves of the tea plant (*Camellia sinensis* (L.) Kuntze) grown under specific conditions using about 90% shade (DEVKOTA, 2021). This method of sun protection allows the plant to create large amounts of bioactive compounds, including chlorophyll and L-theanine (JAKUBCZYC, 2020), and a high concentration of amino acids, which provide a higher level of umami flavor and are also associated with its quality (KOLÁČKOVA *et al.,* 2020). Substrate concentrations are higher in matcha than in other green teas because the leaves are ground with a ceramic mill until a fine powder is obtained, which is subsequently dissolved in hot water and fully consumed. Figure 3 corresponds to an image of the plantation, the matcha obtained from green tea leaves and its main components.

Figure 3. Matcha plantation, visual characteristics of the powder and its main constituents.

Source: Devkota *et al.* (2021).

Fujioka *et al.* (2016) proved that powdered tea, compared to leaf tea, is characterized by a higher concentration of polyphenols when using the same amount of leaves and powder. It was also observed in this same study that the protective effect of matcha against oxygen radicals was significantly greater than that of tea leaves due to the increased amount of catechins.

Koláčkova *et al*. (2020) confirmed that the most abundant free amino acid in matcha was theanine, followed by Glu (glutamic acid), GABA (gamma aminobutyric acid), Met (methionine) and Thr (threonine), as well as high concentrations of the minerals Mg, P, K, Ca, Fe, Zn, Mn and Cu. Indicating that the daily consumption of 5 g of matcha does not pose a health risk, but offers valuable benefits.

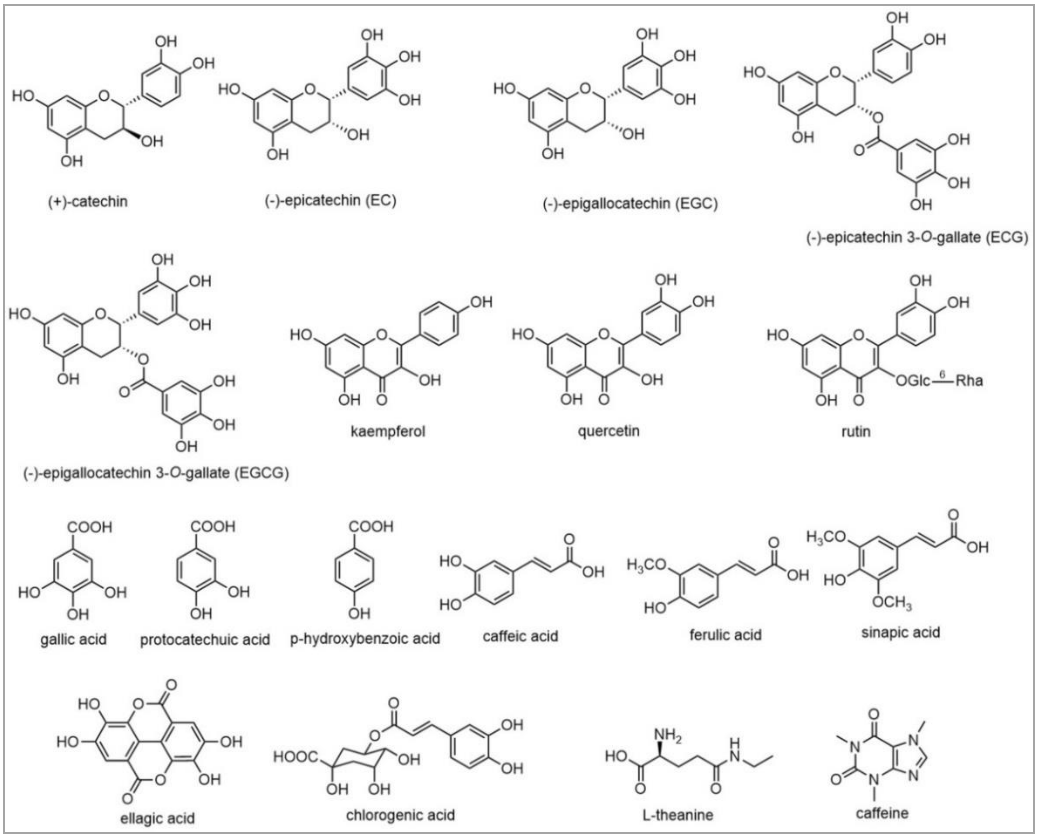
Clinical studies have shown that green tea consumption has a positive effect on relieving stress and reducing anxiety in individuals already suffering from psychopathological symptoms (SCHOLEY *et al*., 2012; GIESBRECHT *et al.,* 2010; SAKURAI *et al*., 2020), as well as having properties that help fight depression (ESMAEILPOUR-BANDBONI *et al*., 2021).

Sakurai *et al.* (2020), carried out a clinical study with elderly people aged between 60 and 84 years, where they subjected these participants to a cognitive psychological function test at the beginning of the trial and after 12 weeks of daily intake of matcha, in order to assess cognitive capacity. and memory, as well as the impulsivity of these participants. At the end of the experiment, an improvement in cognitive function was observed in women. Matcha green tea powder contains several bioactive compounds, including catechins, EGCG (epigallocatechin gallate) and others such as caffeine and theanine, which have been recognized as functional compounds related to cognitive function. In a previous study, Vignes *et al*. (2006) attributed anxiolytic properties to EGCG.

In the clinical study by Esmaeilpour-Bandboni *et al*. (2021), 72 elderly people were evaluated for levels of depression before and after consumption of green tea during five weeks of the experiment. In the end, mean scores on the depression scale before and after the intervention significantly reduced. That is, green tea consumption significantly reduced the rate of depression in these participants.

Teng *et al.* (2017) evaluated the effects of gamma aminobutyric acid (GABA) in green tea on depression and found that this substance led to a reduction in depressive symptoms in mice, as GABA is the main inhibitory neurotransmitter in the brain and plays an important role on brain metabolism (HINTON; JOHNSTON, 2018), in addition to having beneficial effects on blood pressure, stress and anxiety, which may involve GABA actions in the central and peripheral nervous systems (HINTON; JOHNSTON, 2020). In Figure 4, the compounds present in matcha can be seen.

Figure 4. Bioactive compounds in matcha.



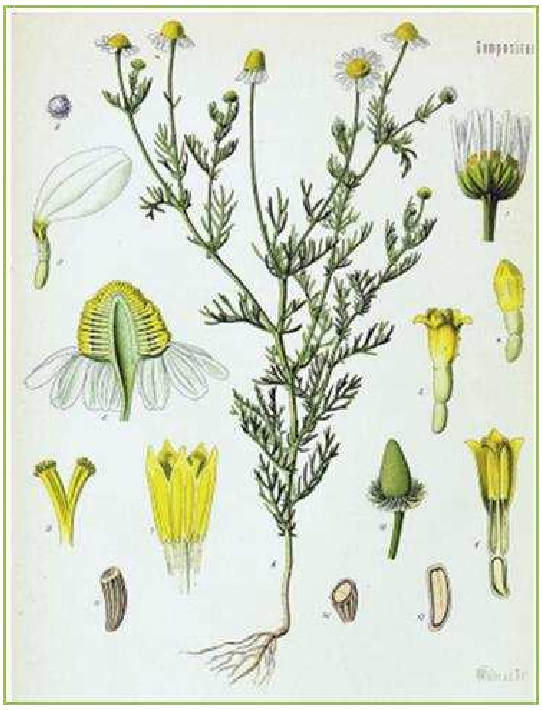
Source: Devkota *et al.* (2021).

Because of the content of antioxidants and other compounds such as L-theanine, caffeine and GABA present in this tea and because its use is associated with health and well-being, matcha tea has been gaining popularity (SCHRÖDER *et al.,* 2019) , being used and marketed not only as a beverage, but as a functional component of other beverages, snacks, chocolates, ice cream and many other products in Japan and abroad (KOCHMAN *et al.,* 2020; KURAUCHI *et al.,* 2019; DEVKOTA *et al*., 2021).

3.2.4.2. Chamomile (Matricaria chamomilla)

Among the medicinal plants known for their relaxing effect on the central nervous system, chamomile (*Matricaria chamomilla*) is one of the most remembered. *Matricaria recutita* L. (*Matricaria chamomilla*, *Chamomilla chamomilla*, *Chamomilla recutita*) is a medicinal herb belonging to the Asteraceae family, popularly known as German chamomile or chamomile (SHIVANANDA *et al.*, 2007; SRIVASTAVA; GUPTA, 2007; PACÍFICO *et al.*, 2018) and is native to Southern and Eastern Europe (SINGH *et al.*, 2011). Chamomile, in the form of teas and oils, is often used for its relaxing and calming effect (AMSTERDAN *et al.*, 2009). The general appearance of the *Matricaria chamomilla* plant can be seen in Figure 5.

Figure 5. Chamomile (*Matricaria chamomilla* L. *Asteraceae*).



Source: Franz Eugen Köhler (1887), in Köhler's Medizinal-Pflanzen.

Chamomile has eleven bioactive phenolic compounds, such as herniarin and umbelliferone (coumarin), chlorogenic acid and caffeic acid (phenylpropanoids), apigenin, apigenin-7-Oglucoside, luteolin and luteolino-7-O-glucoside (flavones), quercetin and rutin (flavonols) and naringenin (flavanone) (SINGH *et al.*, 2011).

Amsterdam *et al.* (2009) performed a controlled clinical study of chamomile extract in patients with mild to moderate Generalized Anxiety Disorder (GAD), where the results suggested that chamomile may have modest anxiolytic activity, but the authors justify that they could achieve a more anxiolytic activity. robusta if a higher dose of the extract had been used.

In a more recent study, Araújo *et al.* (2019) sought to identify the benefits of chamomile in the elderly, in view of its possible therapeutic activities from its use, and observed that chamomile can significantly improve the quality of sleep in the elderly, both in the form of tea (infusion of herb) or through its extract.

3.2.4.3. Lemon balm (Melissa officinalis)

Lemon balm (*Melissa officinalis*), from southern Europe and belongs to the Lamiaceae family (OMS, 2005) is a prominent medicinal plant. This herb is a good source of volatile oil, flavonoid glycosides and caffeic acid derivatives such as rosmarinic acid, which have antioxidant capacity (PETKOVA *et al.,* 2017). The *Melissa officinalis* plant is represented in Figure 6.

Figure 6. Representation of the general appearance of *Melissa officinalis*.

Source: Franz Eugen Köhler (1887), in Köhler's Medizinal-Pflanzen.

Buedo *et al.* (2015) observed that *Melissa officinalis* can be used to control insomnia and anxiety, and may be an alternative to the use of benzodiazepines, commonly prescribed anxiolytic drugs, such as diazepam and alprazolam.

In a clinical trial with children, Aldave *et al.* (2009) studied the effect of *Melissa officinalis* ethanolic extract in modifying the behavior of anxious children during a dental appointment and obtained a positive result, as well as Naseri *et al.* (2021), who evaluated the impact of *Melissa officinalis* L. hydroalcoholic extract on learning and memory in diabetic rats, attributing the positive antidiabetic effects to the content of pure flavonoids from the herb, such as rutin and apigenin.

In addition to anxiolytic properties, memory improvement and insomnia control, lemon balm extract has also shown to have antibacterial potential, being used for the treatment of herpes (AMIN *et al.,* 2014) and acne (CARVAJAL *et al.,* 2011), as well as being considered a factor that prevents the progression of Alzheimer's disease (NOGUCHI-SHINOHARA *et al*., 2020).

3.3. Functional beverages and global market trends

According to Ordinance No. 123, of May 13, 2021, of the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA), a composite drink is the drink obtained by mixing a vegetable ingredient, in the form of juices, pulps or vegetable extracts, together or separately , with a product of animal origin, with predominance in its composition of a product of vegetable origin, which may be in the form of a solid preparation or a liquid preparation, which must meet the standard of a ready-to-drink composite drink when dissolved or diluted in drinking water of according to the manufacturer's guidance.

Brazil's great biodiversity makes it possible to creatively exploit abundant raw materials in the country, such as sugarcane (*Saccharum officinarum*), for the development of new products, such as functional composite beverages. In addition to the variety of flavors, these foods can have considerable levels of bioactive compounds.

According to Tireki (2021), functional beverages are developed with the addition of positive ingredients such as vitamins and/or reduction or removal of negative ingredients such as sugar. In this sense, consumer trends have shifted from sugary formulations to functional drinks as proactive wellness and health actions, where the functional drinks market is the fastest growing among all functional food categories, estimating that functional drinks correspond to 40% of total consumer demand by 2025 (NAZIR *et al.,* 2019).

Tireki (2021) also points out that consumers will look for functional products that improve mood and stimulate more brain health in the future, relaxing drinks and drinks that help with stress relief are examples. That is, the consumer's interest in functional products should persist in the long term, making the market turn to this global consumption trend.

3.4. Behavioral studies using adult zebrafish (*Danio rerio*)

The zebrafish model gains increasingly popularity in behavioral research.

And one of the factors for this is that this small cyprinid provides a favorable compromise between neurobiological complexity and practical simplicity (SYED *et al*., 2023).

Zebrafish (*Danio rerio*) has a molecular mechanisms similar to those of humans. Furthermore, its well-developed neurological structure, such as the presence of neurotransmitter systems and neurochemical pathways, allows this animal to be widely used to study behaviors and disorders linked to the central nervous system (SYED *et al.,* 2023; STEWART *et al*., 2015). The neurochemistry of zebrafish *in vivo* test model makes it a useful model for testing drugs with anxiolytic or anxiogenic activity (CHOI *et al*., 2018).

In addiction, was demonstrated the ability of this animal to perform well in learning tasks and to acquire association memory between an unconditioned stimulus (the US, a reward or punishment) and a conditioned stimulus (the CS, a previously neutral cue) (GERLAI, 2020; GERLAI, 2016), making its use even more promising as an important tool in behavioral neuroscience, both for translational and basic research, (GERLAI, 2023).

As well as to its degree of similarity with the human species and its wide use, zebrafish are a simple, small and inexpensive vertebrate to maintain and study in the laboratory. For this reason, it is breaking the barriers of pharmacology and medicine, and is also being used to test foods in development.

Recently, the zebrafish model was successfully used to determine the neurological effects of food and beverage products and ingredients, such as food preservatives (LEONARD *et al.,* 2023; XU *et al.,* 2022). An another study did a phenotypic screening of novel probiotics with potential anti-neuroinflammation using zebrafish models (HE *et al*., 2023). Zebrafish was also used to test the action of the estrogenic and non-estrogenic effects of bisphenol A (BPA), a raw material often used to compose food packaging (YUAN *et al*., 2023).

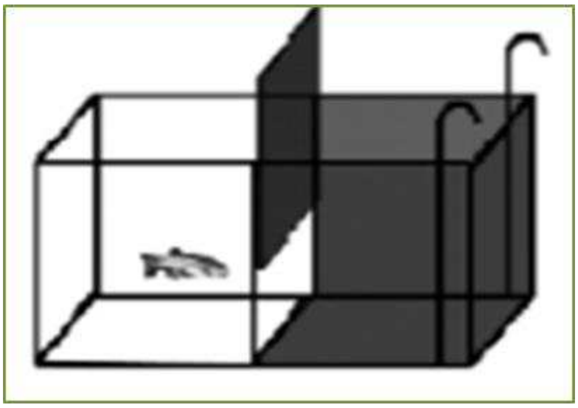
This shows that zebrafish can be an allied tool for the food industry, facilitating the development of functional products in a more assertive way, since the use of the zebrafish model allows predicting behavior and neurological action, among others, based on compatibility with the human organism.

3.4.1. Anxiolytic-like effect study in adult zebrafish

The anxiolytic-like effects of a food can be investigated through light/dark tests and the open field test.

According to Blaser & Penaloza (2011), the behavior of zebrafish in a light/dark preference test was first reported by Serra *et al.* (1999), who found that in an aquarium with two chambers, one with black walls and one with white walls, zebrafish exhibited a robust preference for the black compartment. The light/dark test consists of observing the time spent by the zebrafish in the light area of the aquarium, after 1 hour from ingestion of the product to be tested. Therefore, the longer the animal stays in the light zone, within a 5 minute period, the greater the anxiolytic effect tested, when compared between groups, including a control and a naive group (MELO *et al*., 2019). Figure 7 below illustrates how is the aquarium used in this test.

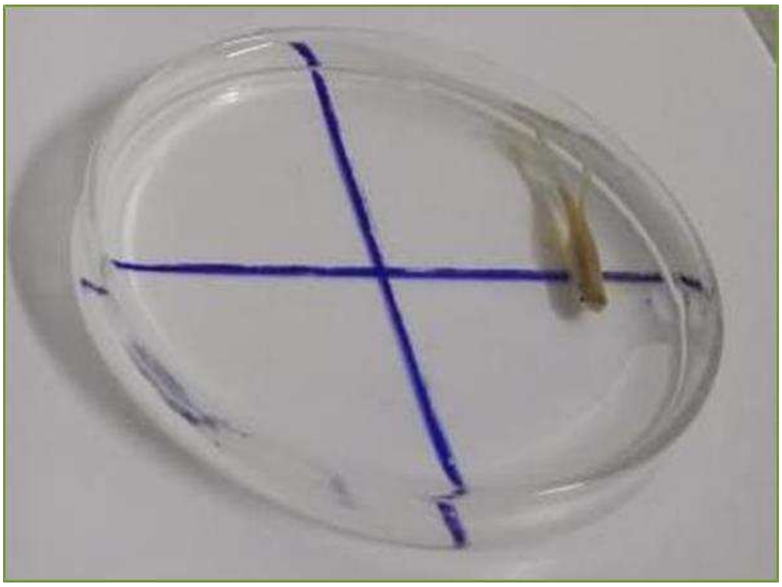
Figure 7. Aquarium used for the light/dark test.



Source: Melo *et al*., 2019.

The open field test evaluates whether or not there were changes in the animal's locomotion after ingesting the product. This test is based on the number of times the zebrafish crosses the lines within the field (Petri dish) and compares this data between groups, including a control and a naive group (MELO *et al.*, 2019). Figure 8 shows how is the Petri dish used in the experiment.

Figure 8. Petri dish used in the open field test.



Source: Author, 2023.

4 FINAL CONSIDERATIONS

The development of a functional beverage based on matcha tea and sugarcane juice results in an innovative product, since there are no options in the national market for compound beverages made simultaneously with these two bases, as well as exploring a raw material that is part of the national culture, such as sugarcane juice.

A product read to drink with an anxiolytic-like effect may serve the public looking for functional drinks, especially people with insomnia and damage caused by stress and anxiety, who are looking for a product with good nutritional characteristics, with the presence of bioactive compounds, mainly antioxidants, amino acids (such as L-theanine), among other constituents, which can improve cognitive functions, favoring the feeling of relaxation and well-being in its consumers.

The analyzes that prove the effect of the developed beverage make up the article presented in chapter II. According to the experiments, both the base formulation (composed only of sugarcane juice and matcha) and the final products created from it (adding lemon balm and/or chamomile), presented an anxiolytic-like effect in adult zebrafish, as well as presenting a high content of total phenolic compounds. And, when samples of the final products were analyzed by HPLC, 18 different types of polyphenols were detected, consequently, a high antioxidant potential was also found in all analyzed samples. The bioaccessibility of these phenolic compounds was also analyzed and the result for the final products was approximately 30%. This demonstrates that the developed beverage, in addition to having antioxidant and anxiolytic action, also has good bioaccessibility of the compounds responsible for its functionality, achieving the objective proposed for this study.

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APPENDIX A - PHENOLIC PROFILE, BIOACCESSIBILITY AND ANXIOLYTIC-LIKE EFFECT OF A BEVERAGE MADE WITH MATCHA AND SUGARCANE JUICE.

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ABSTRACT

The consumer demand for value-added functional foods that support brain health is now an important segment of the marketplace. In this work, functional beverages formulated with matcha, sugarcane juice, and medicinal plants chamomile and lemon balm were developed. The bioactivity (phenolic load and antioxidant capacity) and the anxiolytic-like effects were evaluated using adult zebrafish (*Danio rerio*) *in vivo* model regarding behavioral and toxicological analyses. Initially, three beverage bases with matcha and sugarcane juice in different volume ratios were tested. The prioritized beverage base BB 35/65 showed antioxidant activity of 188.95 ± 26.45 µM TE/mL and soluble solids of 10.2 ºBrix, as well as positive *in vivo* anxiolytic-like effects. Beverages BLB (70% BB 35/65, 30% lemon balm tea extract) and BCLB (70% BB 35/65, 15% lemon balm tea extract and 15% chamomile tea extract) showed high phenolic content (669.84 ± 19.47 mg GAE/L and 729.43 ± 41.49 mg GAE/L, respectively) and antioxidant activity (267.1± 35.67 and 269.0 ± 96.9 µM of Trolox/mL, respectively) which might play an important role on the observed desirable anxiolytic-like effects of both products. A complex phenolic profile was detected and myricetin and chlorogenic acid were the predominant phenolic compounds in both BLB and BCLB beverages. Similar polyphenol bioaccessibility (29.8% and 30.8% for BLB and BCLB, respectively) was reported. Taken altogether, the successful development of functional phytochemical-rich beverages with anxiolytic-like effects made with readily available plant-based materials was demonstrated in this study.

Keywords: plant-based; product development; mental health; lifestyle diseases.

1 INTRODUCTION

To prevent the spread of Severe Acute Respiratory Syndrome Coronavirus-2 (Sars-CoV-2, COVID-19), different forms of government-enforced measures disrupted people's lives worldwide. Physical distancing recommendations, school closures and stay-at-home orders reduced social contacts that aggravated mental health impairment and led to increased rates of depression and anxiety disorders (Kindred & Bates, 2023). For example, comparing the years 2021 and 2022, an average increase of 27.5% in the number of patients with symptoms such as sudden discomfort, recurrent and unpredictable feelings of danger, inability to relax, feelings of suffocation, chest pain, cognitive distortions, and intense death anxiety has been recently reported (CHIATTONE *et al.*, 2022).

Because of the current scenario, the consumer demand for foods that improve mood and support brain health is now a growing market (TIREKI, 2021). Plants have always been a prolific source of health-relevant molecules. Indeed, phytochemical-rich foods, including medicinal plants, have proved to be effective in mitigating stress, insomnia, anxiety and depression due to their reported antioxidant and anti-inflammatory effects (VASCONCELOS *et al.*, 2007; ARAÚJO *et al.*, 2020; NASERI *et al.* 2021; ESMAEILPOUR-BANDBONI *et al.*, 2021).

Matcha is a type of green tea originated in Japan (JAKUBCZYC, 2020) obtained from the leaves of the tea plant (*Camellia sinensis* (L.) Kuntze) and grown under specific conditions (DEVKOTA *et al.*, 2021) that enhance the content of bioactive compounds. The high content of chlorophyll and L-theanine (JAKUBCZYC, 2020), amino acids and phenolic compounds (KOLÁČKOVA *et al.*, 2020) of matcha have been linked to potential neuroprotective effects against neurological disorders and cognitive impairments (PERVIN *et al.*, 2019). It has also been demonstrated that abundant natural resources such as sugarcane juice and medicinal plants such as chamomile (*Matricaria chamomilla*) and lemon balm (*Melissa officinalis*) have brain-active compounds that support mental health. For example, octacosanol found in sugarcane juice promotes neurological, cognitive and memory properties (SCHRÖDER *et al.,* 2019; KAMBLE *et al.*, 2021), while the polysaccharide 4-O-methyl-glucuronoxylan from chamomile tea has shown sedative and anxiolytic activities (CHAVES *et al.,* 2020). Stojanovič *et al*. (2022) studied the gastrointestinal motility disorder associated with anxiety conditions and found that both lemon balm essential oil and citronellal inhibited ileum contractions in mice. In addiction, the study by Motahareh *et al*. (2022) showed that lemon balm extract reduces anxiety in pre-orthopedic surgery patients, and this anxiolytic effect has been attributed to rosmarinic acid (DANCHOUR, 2022).

Functional products can be designed as vehicles of health-promoting molecules in a convenient, and attractive format. In this regard, functional beverages constitute the fastest growing market segment among all functional food categories and are estimated to represent 40% of the functional market share by 2025 (NAZIR *et al.,* 2019). Therefore, in this study our goal was to develop a beverage with the good-for-your-brain-health concept in mind using matcha, sugarcane juice, and selected medicinal plants (chamomile and lemon balm) as ingredients. While there are few products available in the market, there is an evident need for novel, and reliable food alternatives with scientifically proved claim benefits that would potentially support consumers' well-being and mental health. Initially, beverage base formulations were developed and used to produce two beverage treatments that were characterized regarding their polyphenol content and profile and antioxidant activity. The anxiolytic-like effect of developed beverages was evaluated by zebrafish (*Danio rerio*) *in vivo* test model, by conducting toxicological studies and behavioral analyzes related to stress, fear, anxiety and depression (RINKWITZ *et al*., 2011; MELO *et al.,* 2019) with the objective of establishing phytochemical-rich beverage formulations with brain's health support effects. This study unveils the potentiality of readily available plant-based resources to develop a value-added food product aligned with the current needs of the trendy health-oriented market.

2 MATERIALS AND METHODS

2.1 Materials

Matcha tea powder (*Camellia sinensis* (L.) Kuntze), chamomile flowers (*Matricaria chamomilla*) and lemon balm leaves (*Melissa officinalis*) were purchased locally. The sugarcane (*Saccharum officinarum*) juice was obtained from sugarcane locally harvested (March 2022) and pressed (model B-722 Maqtron, Brazil) in a local sugar cane mill (Engenho Três Irmãos, Pindoretama, Ceará, Brazil). After collection, the sugarcane juice was immediately pasteurized (95ºC for 30 seconds) according to Andrade (2014) and then kept refrigerated at 5ºC until further use.

Reagents 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid; ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbox-ylicacid (Trolox®) were purchased from

Sigma Aldrich Co. (St. Louis, MO, USA). All chemicals used in this research were analytical grade.

2.2 Matcha, chamomile and lemon balm liquid tea preparation

Tea was prepared according to the manufacturer's instructions (Chás da Amazônia, Brazil). For matcha, 8.04 g were weighed and transferred to 1.2 L of hot water (100 ºC) followed by manual stirring. The mixture was left to rest for 10 minutes followed by filtration through a nylon filter (Ø 11 cm x 20 cm). For chamomile flowers, a similar protocol was used using 1.08 g of dehydrated material in 200 mL of hot water. For lemon balm, 1.05 g of dehydrated herb was used in 1 L of hot water and left to rest for 15 minutes before filtration.

2.3 Functional beverage production

Initially, the definition of a prioritized beverage base (BB) formulation (to be used for the formulation of functional beverages) was carried out in two steps. First, three formulations of beverage base (BB) were produced by blending matcha tea and sugarcane juice in different percentage volume ratios. The parameter total soluble solids content was chosen as the primary criteria to define the BB formulations considering the range of total soluble solids content of non-alcoholic beverages available in the market (5 and 11°Brix; Braga, 2019). Based on this, the formulations BB 50/50 (50% matcha and 50% sugarcane juice, v/v), BB 60/40 (60% matcha and 40% sugarcane juice, v/v), and BB 35/65 (35% matcha and 65% sugarcane juice, v/v) with 8.3, 7.5 and 10.2 ºBrix, respectively, were prepared.

Secondly, the selected BB formulations were evaluated regarding the mitigation of anxiety markers using zebrafish model. One prioritized beverage base was used to produce two experimental functional beverages developed by mixing a fixed volume ratio of 70% of the BB formulation and 30% of lemon balm tea (BLB) or 70% of BB formulation with 15% of lemon balm tea and 15% of chamomile tea (BCBL).

2.4 Total phenolic content (TPC) and antioxidant activity measured by 2,2′ -azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid) radical method (ABTS)

The TPC was determined according to the Folin-Ciocalteu method (Larrauri *et al.*, 1997). The results were expressed in mg of gallic acid equivalent (GAE)/L of sample.

The antioxidant capacity was determined by the ABTS radical scavenging method according to the methodology described by Benzie and Strain (1999) adapted by Rufino *et al.* (2010). Results were expressed as micromoles of Trolox equivalent per volume of sample (µM TE/L or µM TE/mL sample).

2.5 Phenolic profile by high-performance liquid chromatography (HPLC)

The phenolic profile of beverage samples was determined by HPLC using an Agilent HPLC system model 1260 Infinity LC (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary solvent pump (model G1311C), degasser, thermostatic column compartment (model G1316A) and autosampler (model G1329B), coupled to a Diode Array Detector (DAD) (model G1315D). The methodology was conducted according to Padilha *et al*. (2017). Results were expressed as mg of compound per mL of sample (mg/mL sample).

2.6 *In vitro* simulated gastrointestinal digestion

The bioaccessibility of phenolic compounds in the final beverage samples (BLB and BCLB) was determined using a simulated digestion model according to Miller *et al.* (1981). The *in vitro* digestion was carried out simulating the gastric and intestinal phases. To simulate gastric digestion, 20 g of beverage sample was placed in a 250 mL amber Erlenmeyer flask and the pH was adjusted to 2.0 with 2M HCl, followed by the addition of 3.2 mL of pepsin solution from porcine gastric mucosa (0.16 g /mL; P7000, Sigma Aldrich, St. Louis, MO, USA). The solutions were incubated in a thermoregulated bath (Banho Dubnoff, TE-053, Technal, Brazil) at 37 °C and shaken (130 rpm) for 2h. To simulate the intestinal phase, the digested mixture was adjusted to pH 7.5 with 0.5M NaOH. Samples were dialyzed using a tubular cellulosic membrane (with water and 0.5M $NaHCO_3$, pH 7.5, membrane vg. flat width 10 mm, 0.4 in.; Sigma Aldrich, St. Louis, MO, USA) and stirred (130 rpm) for 30 min at 37 ºC in a thermoregulated bath (Banho Dubnoff, TE-053, Technal, Brazil). Finally, 5 mL of bile-pancreatin solution (0.03 g/mL) (50 mL of 0.1 M $NaHCO_3$, 1.3 g bovine bile B8381, Sigma Aldrich, St. Louis, MO, USA), and 0.2 g pancreatin from porcine pancreas (P1625; Sigma Aldrich, St. Louis, MO, USA), were added to the samples and the flasks were placed in a thermoregulated bath (Banho Dubnoff, TE-053, Technal, Brazil) at 37 °C and shaken (130 rpm) for 2 h. The dialyzed solution was used to quantify TPC following the procedure described in section 2.2.4. The bioaccessibility of polyphenols was measured according to Briones-Labarca *et al.* (2011) and expressed in percentage by Equation 1:

Bioaccessibility (%) = 100 × (Ca/Cb) (Equation 1)

where Ca is the TPC (mg GAE/L) of samples after simulated digestion and Cb is the TPC (mg GAE/L) of samples before the simulated digestion.

2.7 Assessment of anxiety-like behavior in adult zebrafish

2.7.1 Animals

Adult wild zebrafish (*Danio rerio*), male and female (n=6/group), short-fin phenotype, 60–90 days with similar size (3.5 ± 0.5 cm) and weight (0.4 ± 0.1 g) were obtained from Agroquímica (Comércio de Produtos Veterinários LTDA, Fortaleza, Brazil). A first batch (120 fish) was used to evaluate the performance of three beverage base formulations. A second batch (150 fish) was further used to determine the anxiolytic-like effects of beverage formulations (BLB and BCBL).

The fish groups were acclimated for 24 h in a 10-liter glass tank (30 cm × 15 cm × 20 cm) containing dechlorinated tap water (ProtecPlus®). An air pump with submerged filter at 25 °C and pH 7 under near-normal circadian rhythm (14:10 h of light/dark cycles). The fish received food (Alcon Gold Spirulina Flakes®) *ad libitum* 24 h prior to the experiments. After the experiments, the animals were euthanized by immersion in icy water (2°C–4°C) for 10 min until loss of opercular movements (CONCEA, 2018). All experimental procedures were approved by the Ethics Committee on Animal Research of the Ceará State University (CEUA-UECE; #7210149/2016).

2.7.2 Experimental protocol

Animals were randomly selected to receive the treatment (beverage samples) orally (*p.o.*), using an automatic pipette (20 µL), as described by Magalhães *et al.* (2017). Subsequently, they were placed individually in glass beakers (250 mL) containing 150 mL of aquarium water for 60 min.

2.7.3 Treatments

Animals (n=6/group) were pretreated (orally - *p.o.*) with beverage base (BB), beverage formulations (BLB or BCBL), or vehicle (mineral water, control group). The naive group (n=6) did not receive any treatment.

2.7.4 Locomotor behavior (open field test)

Groups of animals (n=6/group) were pre-treated (20 µL; *p.o.*) with beverage base samples, final product formulations (BLB or BCBL) or vehicle (mineral water). After 60 min, the animals were placed individually in Petri dishes (⌀ 15 cm) divided into quadrants and the number of line crossings was recorded during 0 – 5 min. A naive group (n = 6) was included.

2.7.5 Light and dark test

The methodology described by Gebauer *et al.* (2011) was used to evaluate the anxiolytic-like effect of samples. For this, groups of animals (n=6/group) were pre-treated (20 µL; *p.o.*) with the beverage base samples, final product formulations (BLB or BCBL) or vehicle (mineral water). The test apparatus consisted of a half-dark, half-light tank (30 cm × 15 cm × 20 cm) and a 3-cm dechlorinated tap water column. After 1 h, the fish were placed individually in the light area of a glass aquarium, in which the time spent in the light area was recorded after a 5 minute-interval. The water level in the tank was reduced to 3 cm to increase anxiety, inducing the fish to remain in the dark zone. A naive group (n = 6) was included.

2.7.6 Acute toxicity test (96 h)

The animals (n=6/group) were treated as previously described (see 2.6.4 and 2.6.5). After treatment, the animals were left to rest to analyze the mortality rate and the number of dead fish in each group was recorded every 24 h and up to 96 h. The lethal concentration able to kill 50% of the animals (LC50) was determined using the mathematical Trimmed Spearman-Karber method with a 95% confidence interval (ARELLANO-AGUILAR, 2015; SILVA *et al.*, 2020).

2.8 Statistical analysis

Results were expressed as mean values ± standard error. After confirming the normality of distribution and data homogeneity, the differences between the groups were submitted to one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism v 5.0 software. The level of statistical significance was set at 5% ($p < 0.05$). The phenolic profile data obtained by HPLC was processed using OpenLAB CDS ChemStation Edition™ software (Agilent Technologies). Analyses were conducted in triplicate, unless noted.

3 RESULTS AND DISCUSSION

3.1 Selection of a prioritized beverage base

3.1.1 Antioxidant activity

Initially, the antioxidant activity of each raw material and the beverage bases were evaluated. Matcha tea showed a remarkable 540.6±75.23 µM trolox/mL (540,600 µM TE/L), while sugarcane juice showed 1.86±0.14 µM TE/mL (1,860.81 µM TE/L). The BB formulations had antioxidant activities of 269.13±37.75 (BB 50/50), 322.58±45.28 (BB 60/40) and 188±26.45 µM TE/mL (BB 35/65). As expected, results showed a clear direct relationship between the concentration of matcha and the final antioxidant activity of BB formulations. The remarkable antioxidant activity of matcha is justified by expressive amounts of catechins, epigallocatechin gallate (EGCG), amino acids (theanine, glutamic acid, gamma aminobutyric acid), methionine and threonine, besides other antioxidant compounds such as caffeine (DEVKOTA *et al.*, 2021; KOLÁČKOVA *et al.*, 2020) found in this plant-based resource.

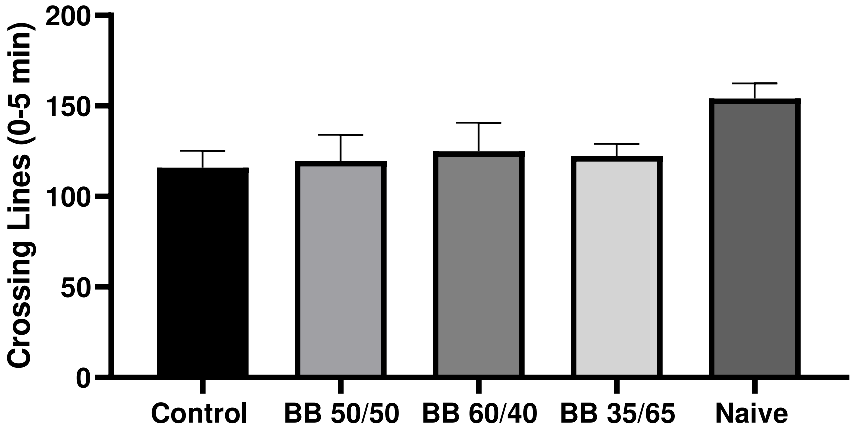
The antioxidant activity of sugarcane juice has been attributed to the presence of amino acids, acids (malic, succinic and aconitic) and antioxidant pigments (chlorophyll, saccharine and anthocyanin), in addition to octacosanol, a primary aliphatic alcohol of high molecular weight found in sugarcane juice (KAMBLE *et al.,* 2021; TAYLOR *et al.*, 2003) besides phenolic acids, flavonoids and other phenolic compounds (DUARTE-ALMEIDA *et al.*, 2006).

3.1.2 Assessment of anxiety-like behavior in adult zebrafish

In vivo tests were used to screen the beverage bases (BB 50/50, BB 60/40 and BB 35/65) in the first step of beverage development. For this, the performance of zebrafish submitted to the open field test, light and dark test and acute toxicity test were used as selection criteria. The neurochemistry of zebrafish (*Danio rerio*) *in vivo* test model makes it a useful model for testing drugs with anxiolytic or anxiogenic activity. Recently, the zebrafish model was successfully used to determine the neurological effects of food and beverage products and ingredients, such as food preservatives (LEONARD *et al.,* 2023; XU *et al.,* 2022).

For the open field test, there was no significant difference between groups ($p>0.05$), including the control group and the naive group. This indicates that the beverage bases exerted no effect on the locomotion behavior of the animals (Figure 1).

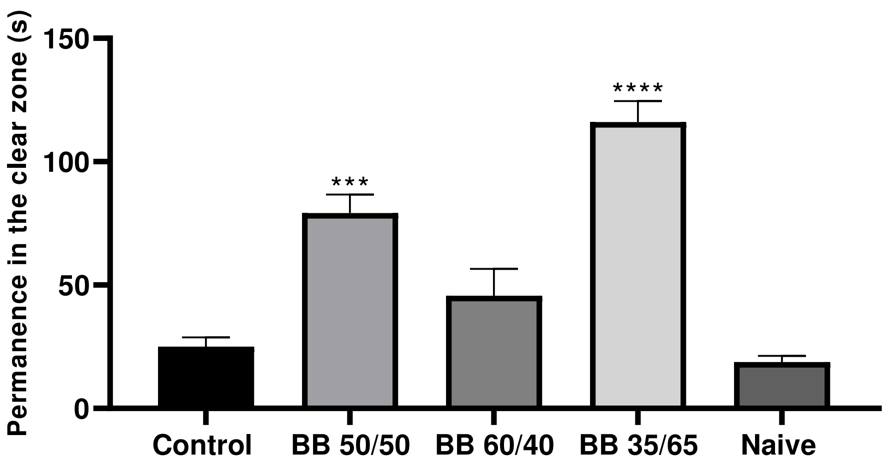
Figure 1. Effects of beverage base formulations on locomotor behavior of adult zebrafish.



Control: vehicle (mineral water 20 µL; *p.o.*). BB 50/50 (50% matcha, 50% sugarcane juice v/v, 20 µL; *p.o.*). BB 60/40 (60% matcha, 40% sugarcane juice v/v, 20 µL; *p.o.*). BB 35/65 (35% matcha, 65% sugarcane juice v/v, 20 µL; p.o.). Naive - untreated group. Results expressed as mean values ± standard error. Means are not statistically different by the Tukey test ($p < 0.05$).

The light and dark test showed that samples BB 50/50 and BB 35/65 presented anxiolytic-like effect (Figure 2) when compared to the control and naive groups, at a significance level of $p<0.001$ and $p<0.0001$, respectively.

Figure 2. Anxiolytic-like effect of beverage bases in adult zebrafish on light and dark test.



Control: vehicle (mineral water 20 µL; p.o.). BB 50/50 (50% matcha, 50% sugarcane juice v/v, 20 µL; p.o.). BB 60/40 (60% matcha, 40% sugarcane juice v/v, 20 µL; p.o.). BB 35/65 (35% matcha, 65% sugarcane juice v/v, 20 µL; p.o.). Naive - untreated group. Results are expressed as mean values ± SEM (n = 6/group). Analysis of variance followed by the Tukey test at 5% ($p<0.05$): **$p<0.01$; ***$p<0.001$ and ****$p<0.0001$ vs.

control (vehicle) and naïve.

Results show a direct relationship between increased amounts of sugarcane juice and the anxiolytic-like effect expressed as the time of permanence in the light zone (Figure 2). In fact, it has been demonstrated that octacosanol in sugarcane juice reduces stress and sleeplessness, being potentially used for treating stress-induced insomnia (KAUSHIK *et al.,* 2017). Besides, several other health-relevant attributes of octacosanol have been demonstrated (KAMBLE *et al.*, 2021) such as cholesterol reduction, antiplatelet properties, cytoprotective and ergogenic properties (TAYLOR *et al.*, 2003), as well as antinociceptive and anti-inflammatory (OLIVEIRA *et al.*, 2012).

Moreover, clinical studies have shown that consumption of green tea has a positive effect on relieving stress and reducing anxiety in individuals suffering from psychopathological symptoms (SAKURAI *et al.*, 2020; SCHOLEY *et al.*, 2012; GIESBRECHT *et al.*, 2010). Compared to green tea leaves, the antioxidant protective effect of matcha was significantly greater due to the increased amount of catechins (FUJIOKA *et al.*, 2016). Sakurai *et al.* (2020) carried out a clinical study with elderly individuals (60-84 years old) to assess the effect of matcha consumption on their cognitive capacity, memory, and impulsivity. The participants were subjected to a cognitive psychological function test at the beginning of the trial and after 12 weeks of daily intake of matcha tea and an improvement in cognitive function was observed in women. Bioactive molecules found in matcha have been pointed out as functional compounds with cognitive function activity. For example, EGCG (VIGNES *et al.,* 2006) and GABA play important roles in brain metabolism (HINTON; JOHNSTON, 2018), besides having beneficial effects on blood pressure, stress, and anxiety, partially attributed to GABA effects on nervous central and peripheral systems (HINTON; JOHNSTON, 2020).

Both beverage bases 50/50 and 35/65 showed positive anxiolytic-like effects on adult zebrafish (Figures 1 and 2). However, animals treated with BB 35/65 showed superior performance on the light and dark test compared to BB 50/50, by staying longer in the light zone of the tank (Figure 2). Because of this, BB 35/65 was chosen to formulate beverages BLB and BCLB that were further investigated in this study.

3.2 Characterization of functional beverages

3.2.1 Total phenolic content (TPC)

The TPC of the two final product formulations BLB and BCLB were evaluated. TPC results were 669.84 ± 19.47 mg GAE/L and 729.43 ± 41.49 mg GAE/L, respectively.

Our TPC results are higher than previously reported plant-based beverages. For example, Conceição *et al.* (2019) evaluated a beverage composed of rice, quinoa and cocoa and observed 12.39 mg GAE/L. When Silva *et al.* (2021) evaluated different types of beverages produced with ingredients such as oat, coconut, rice, cashew nut, TPC results ranged from only 0.91 to 12.39 mg GAE/L. On the other hand, Paludo (2017) found TPC values of 605.9 mg GAE/L and 785.2 mg GAE/L for mate tea kombucha and 938.5 mg GAE/L and 1087.4 mg GAE/L for green tea kombucha. Overall, we consider that both BLB and BCLB have substantial TPC, even compared to fermented tea-derived products like kombucha, expected to have high TPC because of the increased phenolic content induced by fermentation (PALUDO, 2017; JAYABALAN *et al*. 2008).

3.2.2 Phenolic compounds profile by HPLC

Eighteen phenolic compounds were identified in BLB and BCLB beverages including flavonoids (flavanols, flavonols and flavanones) and non-flavonoids compounds (stilbenes and phenolic acids) (Table 1). Epicatechin, epicatechin gallate, epigallocatechin gallate, procyanidin B1 and procyanidin B2 were detected in both beverages, but procyanidin A2 only in BCLB. Procyanidins, dimers, trimers and tetramers of monomers of flavan-3-ols, seem to have the glycosylation of (-)-epicatechins as a contributing factor for their biosynthesis and are found abundantly in the roots of *Camellia sinensis* (ZHANG *et al.,* 2017). When comparing previous literature on matcha phenolic composition, it is reasonable to infer that many of the detected phenolic compounds originated from matcha. Indeed, Devkota *et al.* (2021) has demonstrated that matcha is a rich source of flavonoids (catechin, epicatechin, epigallocatechin gallate, epicatechin gallate, epigallocatechin, kaempferol, rutin, myricetin, quercetin), phenolic acids (chlorogenic acid, caffeic acid, gallic acid, protocatechuic acid, p-hydroxybenzoic acid, ferulic acid, synaptic acid, ellagic acid), besides other compounds such as L-theanine and caffeine.

Table 1. Phenolic compounds profile of functional beverages BLB and BCLB.

Phenolic compounds (mg/mL)	**Experimental groups**	
	BLB	BCLB
FLAVANOLS		
Epicatechin	0.13 ± 0.03^{a}	0.12 ± 0.01^{a}
Epicatechin gallate	0.30 ± 0.03^{a}	0.43 ± 0.10^{b}
Epigallocatechin gallate	0.13 ± 0.01^{a}	0.15± 0.01^{a}
Procyanidin A2	ND	0.23 ± 0.001^{a}
Procyanidin B1	0.25 ± 0.01^{b}	0.29 ± 0.005^{a}
Procyanidin B2	0.26 ± 0.002^{b}	0.31 ± 0.001^{a}
FLAVONOLS		
Myricetin	7.63 ± 0.16^{b}	13.33 ± 0.31^{a}
Quercetin 3-Glucoside	1.37 ± 0.14^{b}	2.27 ± 0.22^{a}
Rutin	0.55 ± 0.03^{a}	0.69 ± 0.12^{b}
Kaempferol 3-glucoside	0.81 ± 0.14^{b}	1.50 ± 0.20^{a}
Isorhamnetin	0.05 ± 0.01^{b}	0.07 ± 0.02^{a}
STILBENES		
trans-Resveratrol	0.22 ±0.02^{b}	0.38 ± 0.03^{a}
Cis-Resveratrol	0.10 ± 0.001^{b}	0.27 ± 0.03^{a}
FLAVANONES		
Hesperidin	0.43 ±0.02^{b}	0.94 ± 0.12^{a}
PHENOLIC ACIDS		
Syringic acid	0.08 ±0.01^{a}	0.08 ± 0.01^{a}
Chlorogenic acid	6.05 ±0.27^{b}	7.88 ± 0.25^{a}
Caffeic acid	0.58 ± 0.05^{a}	0.66 ± 0.007^{a}
p-Coumaric acid	ND	0.07 ± 0.008^{a}

Legend: Beverages BLB (70% BB 35/65, 30% lemon balm tea extract and BCLB (70% BB 35/65, 15% lemon balm tea extract and 15% chamomile tea extract). Means in the same row followed by different superscript letter differ statistically by Tukey's test at 5% probability. Results are expressed as mean ± standard deviation. ND: not detected.

Moreover, myricetin and chlorogenic acid are the predominant phenolic compounds in both BLB and BCLB beverages (Table 1). BCLB is notably rich in myricetin, which suggests that the chamomile tea found in that formulation contributes significantly to this finding, as shown by Moraes-de-Souza (2007).

Ameida (2017) found high concentrations of chlorogenic acid in sugarcane stems, that is consistent with the results found here. As the major ingredient in the developed beverages, we hypothesize that an expressive content of chlorogenic acid originates from sugarcane juice. Sugarcane stem compounds are transferred into sugarcane juice, even after the filtration stage (KAMBLE *et al.,* 2021; OU *et al.*, 2012).

Matsubara and Rodriguez-Amaya (2006) evaluated the phenolic profile of teas consumed in Brazil and demonstrated substantial concentrations of quercetin, kaempferol and myricetin in bancha, green tea and black tea. Additionally, quercetin was detected in chamomile, boldo, strawberry and yerba mate teas. This agrees with the observed significantly higher quercetin 3-glucoside shown for BCLB compared to BLB (Table 1).

The health benefits attributed to phenolic compounds have been consistently attributed to their ability of neutralizing harmful oxidative radicals and mitigate cascade reactions involved in the development of diseases (FERREIRA *et al.*, 2021). It has been suggested that EGCG inhibits neuroinflammation by lowering the concentration of inflammatory mediators and their related degenerative pathways (DEVKOTA *et al.*, 2021).

3.2.3 Antioxidant activity

In addition to the sugarcane juice and matcha tea, the antioxidant activity of lemon balm and chamomile teas were also assessed. Chamomile tea showed 462.2 ± 56.60 µM TE/mL sample and lemon balm tea 256.4 ± 38.41 µM TE/mL sample. These results are higher than sweet potato leaves tea and commercial green tea, with 8.61 and 7.98 µM TE/mL sample, respectively (CERVANTES-SIERRA *et al.*, 2019). Moreover, the beverage base formulation BB 35/65 showed antioxidant activity of 188.95 µM TE/mL.

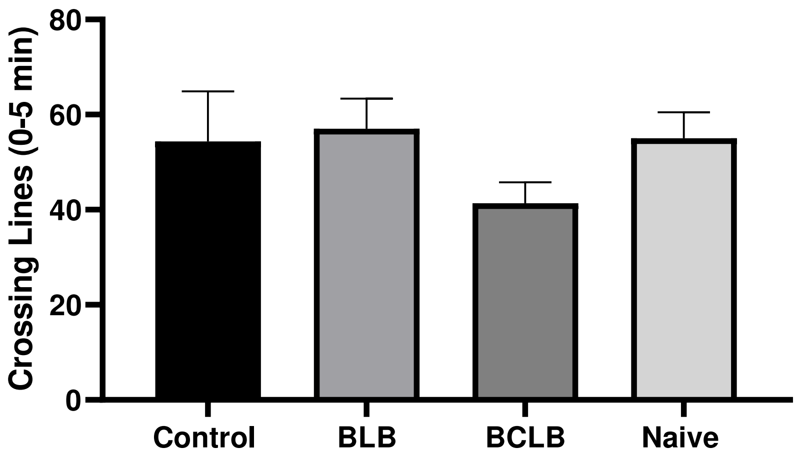
Beverages BLB and BCLB had similar antioxidant activity results of 267.1± 35.67 and 269.0 ± 96.9 µM of Trolox/mL, respectively. The antioxidant activity of beverages BLB and BCLB are higher than the beverage base 35/65 (188.95 ± 26.45 µM TE/mL) used to produced them. This increase can be attributed to the addition of lemon balm and chamomile teas to the base formulations to produce BLB and BCLB. Our results for BLB and BCLB are higher compared to the beverage produced with jaboticaba (*Myrciaria cauliflora*) and oat (*Avena sativa*) with 47.68 µM TE/mL by Reis (2022). Aris *et al*. (2023) evaluated the antioxidant activity of a powdered soy-based drink mix enriched with functional hydrolysates from swift (*Aerodramus fuciphagus*) and found antioxidant activity of 21.95 mg TE/g, 20.75 mg TE/g and 2.93 mg TE/g. Another study that evaluated the antioxidant activity of different vegetables to develop functional drink powders, the best result of the antioxidant potential was observed for beverages enriched with beet powder (17 mg TE/100 mL; Bochnak-Niedźwiecka *et al*., 2020). Rodrigues Silva *et al.* (2021) found 7333.2 µM of TE/L for green tea concentrated extract.

Several bioactive molecules found in matcha, sugarcane juice, lemon balm and chamomile contribute to the total antioxidant activity. In fact, the rich and diverse phenolic profile found for both beverages contributes to the potent antioxidant activity of the developed beverages. Overall, results indicate that both BLB and BCLB are excellent sources of antioxidant phenolics.

3.2.4 Assessment of anxiety-like behavior in adult zebrafish

Similarly to previous results found for beverage bases (Figure 1), no significant differences for the locomotor behavior were observed between treatments, considering all groups together - BLB and BCLB beverages, control and naive groups. This indicates that the beverage formulations were not able to affect the locomotion behavior of zebrafish animals (Fig. 3).

Figure 3. Effects of final product formulations on locomotor behavior of adult zebrafish.

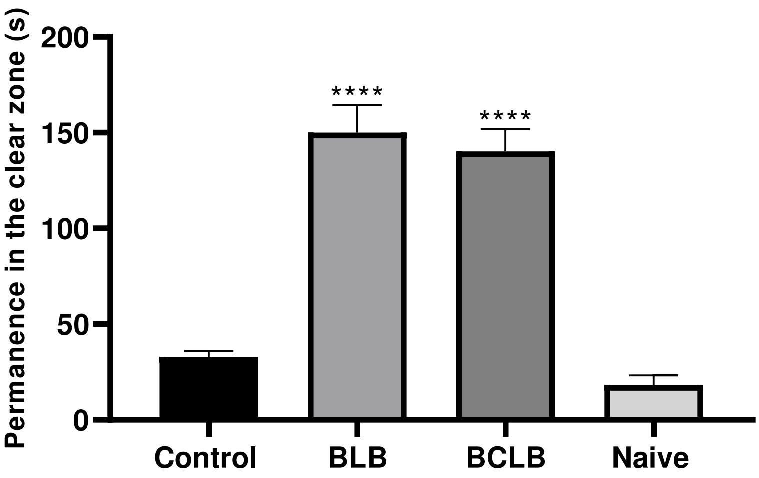


Control: vehicle (mineral water 20 µL; *p.o.*). BLB (70% BB 35/65, 30% lemon balm tea extract, 20 µL; *p.o.*). BCLB (70% BB 35/65, 15% lemon balm tea extract and 15% chamomile tea extract, 20 µL; *p.o.*). Naive - untreated group. Results are expressed as mean values ± standard errors of the mean. Means are not statistically different by the Tukey test ($p < 0.05$).

Because the developed beverages intend to provide well-being and promote relaxation and anxiolytic effects, the locomotion test results shown here are desirable and imply the preservation of locomotion patterns. Contrarily, drugs with mood and anxiety regulation claims, such as benzodiazepines, often negatively affect locomotion, and induce sedative effects and decrease cortical alert levels (CARDOSO *et al.*, 2021; SILVA, 2017), besides other collateral effects (CARVALHO, 2020).

For the light and dark test, BLB and BCLB showed potent anxiolytic-like effect in adult zebrafish. When compared to each other, similar effects were observed ($p<0.0001$), and both treatments induced significantly longer permanence in the light zone against the control and naive groups ($p<0.0001$; Figure 4). During the acute toxicity test, no adult zebrafish died within 96 h of analysis, and the lethality of 50% (LC50) of adult zebrafish could not be determined in that period. This finding demonstrates the absence of toxicity of the beverage formulations developed in this study.

Figure 4. Anxiolytic-like effect of final product formulations in adult zebrafish on light and dark test.



Control: vehicle (mineral water 20 µL; *p.o.*). BLB (70% BB 35/65, 30% lemon balm tea extract, 20 µL; *p.o.*). BCLB (70% BB 35/65, 15% lemon balm tea extract and 15% chamomile tea extract, 20 µL; *p.o.*). Naïve: untreated group. Results are expressed as mean values ± SEM (n = 6/group). Analysis of variance followed by the Tukey test at 5% ($p<0.05$): **$p<0.01$; ***$p<0.001$ and ****$p<0.0001$ vs. control (vehicle) and naïve.

Both medicinal plants, lemon balm and chamomile, have been reported as natural agents that help fight anxiety. Lemon balm, an ingredient of both formulations, can be used to control sleeplessness and anxiety, and may be an alternative to the use of commonly prescribed benzodiazepines anxiolytic drugs, such as diazepam and alprazolam (BUEDO *et al.*, 2015). Besides, it can be used to control nervous breakdowns, tachycardia, melancholy, hysteria and anxiety, in addition to helping with insomnia. Citral, one of the major constituents of lemon balm tea has been reported as having relaxing effects (SILVA *et al.*, 2021; MEIRA, 2012). Chamomile, an ingredient of BCLB formulation, had modest anxiolytic activity in patients with mild to moderate Generalized Anxiety Disorder (GAD; AMSTERDAN *et al.*, 2009). It has also significantly improved the quality of sleep of elderly individuals (ARAÚJO *et al.,* 2019), and these effects were attributed to chamomile flavonoids with calming and tranquilizing activity (ROCHA *et al.*, 2022). Taken altogether, our hypothesis is that the rich bioactive composition, potent antioxidant activity and diverse phenolic profile of both developed beverages plays an important role in the experimental anxiolytic-like effects observed in this study.

3.3 *In vitro* simulated gastrointestinal digestion (SGD)

Lila *et al*. (2022) define bioaccessibility as the proportion of a compound or phytochemical that is released during digestion from the food matrix into the aqueous phase in the lumen (pre-systemic metabolism) and becomes available for transport across jejunum and ileal membranes. For phenolic compounds to exert their benefits, efficient bioaccessibility and bioavailability are required. Because of this, several studies have been conducted to clarify polyphenol bioaccessibility in different food matrices (Lila *et al*., 2022). Thus, when developing a product rich in polyphenols, it is important to determine its polyphenol bioaccessibility, since the interaction between ingredients and processing methods are important factors that influence bioaccessibility, among others (Eran Nagar *et al*., 2020).

Table 2. Total polyphenol content (TPC) and bioaccessibility (%) of BLB and BCLB beverages before and after simulated *in vitro* gastrointestinal digestion.

Parameter	BBLB	BBCLB
TPC (mg AGE/L) before *in vitro* SGD	669.84 ± 19.47[a]	729.43 ± 41.49[a]
TPC (mg AGE/L) after *in vitro* SGD	199.78 ± 47.71[a]	225.01 ± 6.52[a]
Bioaccessibility (%)	29.8[a]	30.8[a]

Legend: Beverages BLB (70% BB 35/65, 30% lemon balm tea extract and BCLB (70% BB 35/65, 15% lemon balm tea extract and 15% chamomile tea extract). Means in the same row followed by same superscript letter don't differ statistically by Tukey's test ($p<0.05$). Results are expressed as mean ± standard deviation.

In this study, the bioaccessibility of polyphenols after the *in vitro* SGD was directly proportional to the TPC of the beverages (Table 2). Similar bioaccessibility (29.8% and 30.8%) were obtained for BLB and BCLB beverages, respectively. Results of studies that correlate the bioaccessibility of bioactive compounds in tea-based beverages or in sugarcane juice are scarce, however similar results were found by Rodrigues Silva *et al.* (2021) for encapsulated green tea extract (28.2%). Metabolites of tea polyphenols are transported in the circulatory system and distributed in a wide range of organs and tissues, in which they contribute to the health functions of tea (TANG *et al*., 2019). In another study, Stafussa *et al.* (2021) evaluated the bioaccessibility of phenolic compounds in different frozen purees of Brazilian fruits from different raw materials, such as açaí (*Euterpe oleracea*), acerola (*Malpighia emarginata*), guava (*Psidium guineenses*), cambuci (*Campomanesia phaea*), jabuticaba (*Myrciaria cauliflora*), murici (*Byrsonima verbascifolia*), panã (*Annona crassiflora*) and pitanga (*Eugenia uniflora*). The formulations with sugarcane juice, matcha, chamomile and lemon balm have more bioaccessible phenolics when compared to the lower bioaccessibility (from 1.49% jaboticaba to 17.18% araçá) registered for these frozen purees.

4. CONCLUSION

Here we demonstrated the successful development of functional beverages using phytochemical-rich ingredients matcha, sugarcane juice, chamomile, and lemon balm. The beverages showed antioxidant activity, besides no toxicity and positive anxiolytic-like effects were found in zebrafish *in vivo* model. A diversified and rich profile of phenolic compounds was observed, and we hypothesize that this attribute plays an important role on the observed health-relevant attributes. Taken altogether, here we show an efficient strategy to take advantage of readily available plant-based materials to produce beverage products directed to the ever-increasing health market.

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APPENDIX B – GRAPHICAL ABSTRACT