UNIVERSIDADE FEDERAL DO PARANÁ

FERNANDA GUILHERME DO PRADO

NEW STRATEGY TO ENHANCE THE BIOSYNTHESIS OF BIOACTIVE COMPOUNDS PRODUCED IN *RHIZOPUS* FERMENTED SOYBEAN: RECOVERY OF BIOACTIVE COMPOUNDS BY CONVENTIONAL ORGANIC SOLVENTS, SUPERCRITICAL CO_2 AND EVALUATION OF THEIR POTENTIAL ACTIVITIES.

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Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Profº. Drº: Carlos Ricardo Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **FERNANDA GUILHERME DO PRADO** intitulada: **New strategy to enhance the byosynthesis of bioactive compounds produced in Rhizopus fermented soybean: Recovery of bioactive compounds by conventional organic solvents, supercritical CO2 and evaluation of their potential activities**, sob orientação do Prof. Dr. CARLOS RICARDO SOCCOL, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 06 de Julho de 2022.

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ATA Nº166

ATA DE SESSÃO PÚBLICA DE DEFESA DE DOUTORADO PARA A OBTENÇÃO DO GRAU DE DOUTORA EM ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

No dia seis de julho de dois mil e vinte e dois às 14:00 horas, na sala 03 (DEFESA FECHADA), Prédio de Engenharia de Bioprocessos e Biotecnologia, foram instaladas as atividades pertinentes ao rito de defesa de tese da doutoranda **FERNANDA GUILHERME DO PRADO**, intitulada**: New strategy to enhance the byosynthesis of bioactive compounds produced in Rhizopus fermented soybean: Recovery of bioactive compounds by conventional organic solvents, supercritical CO2 and evaluation of their potential activities**, sob orientação do Prof. Dr. CARLOS RICARDO SOCCOL. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: CARLOS RICARDO SOCCOL (UNIVERSIDADE FEDERAL DO PARANÁ), GUSTAVO HENRIQUE COUTO (UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ), MARIA GIOVANA BINDER PAGNONCELLI (UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ), LUIZ ALBERTO JUNIOR LETTI (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de doutora está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, CARLOS RICARDO SOCCOL, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 06 de Julho de 2022.

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Dedico essa tese à minha família,

em especial aos meus pais Irineu e Cleusa.

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Sobretudo, a Deus. Aprendi que a vida é um sopro! Sou grata à Ele todos os dias por esse privilégio, **a vida**. Especialmente por Ele ter me concedido uma nova vida, um novo livro branco nas mãos para que eu possa escrever novas histórias. Agradeço por me abençoar todos os dias durante toda essa caminhada e por todos os obstáculos dispostos no caminho, foram eles que me moldaram e me fizeram mais forte.

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E por fim, desejo exprimir os meus agradecimentos a todos aqueles que de alguma forma, permitiram que esta tese se concretizasse.

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“Não fui eu que lhe ordenei? Seja forte e corajoso! Não se apavore, nem se desanime, pois o Senhor, o seu Deus, estará com você por onde você andar.”

(Josué 1:9)

RESUMO

A busca por alternativas terapêuticas vem crescendo e ganhando destaque cada vez mais nos dias atuais. Uma das estratégias consiste no consumo de alimentos que são ricos em antioxidantes naturais e outros compostos bioativos que apresentam capacidade de prevenir ou reduzir processos patogênicos associados a doenças metabólicas. Devido à presença de muitos compostos com propriedades bioativas, incluindo antioxidantes, os alimentos fermentados são relacionados a uma série de efeitos benéficos para a saúde e surgem como uma alternativa promissora. A fermentação tem sido utilizada como uma forma potente de melhorar as propriedades da soja e dos seus componentes. Neste contexto, este estudo teve como objetivo em um primeira etapa avaliar o uso de diferentes substratos a fim de aumentar a produção de compostos bioativos através do bioprocessamento da soja por *Rhizopus oligosporus NRRL 2710,* e por seguinte comparar os rendimentos obtidos de diferentes métodos, nomeadamente Soxhlet e CO_2 + cosolvente supercrítico. A suplementação soja + arroz integral mostrou um aumento significativo na concentração de compostos fenólicos, expressa como equivalente á ácido gálico (GAE), (0,697 a 6,447 mg GAE g^{-1}) em comparação com o uso apenas da soja (1,792 a 3,10 mg GAE g^{-1}). Tal processo resultou em um produto rico em isoflavona e aglycone, contendo diferentes compostos antioxidantes, tais como ácido trans-cinâmico, ácido gálico, miricetina, quercetina e kaempferol. O substrato fermentado também mostrou grande potencial para inibir a enzima hialuronidase (actividade anti-inflamatória) e contra o crescimento de células tumorais CaCo-2 (actividade antitumoral). O Soxhlet foi utilizado como método de extracção de referência para efeitos de comparação; foi realizado com 7 solventes orgânicos incluindo n-hexano, éter de petróleo, acetato de etilo, acetona, etanol, metanol e água. Para a extracção de Soxhlet, os rendimentos mais altos e mais baixos obtidos foram 45,24% e 15,56% utilizando metanol e hexano, respectivamente. Além disso, foi alcançado um aumento de 18,62% no rendimento da extracção quando a soja fermentada foi seca antes das extracções. A extracção utilizando CO_2 supercrítico mais etanol como modificador estático ($scCO_2$ + EtOH) apresentou, a alta pressão (25 MPa) e temperatura (80°C), um teor de compostos fenólicos de 1391,9 µg GAE g^{-1} e eliminação radical de 0,17 g g g^{-1}, atingindo um rendimento de 42,87%. Todos os extractos mostraram actividade anti-inflamatória contra a enzima hialuronidase e os compostos bioactivos identificados por HPLC-DAD, incluindo ácido gálico, ácido transcinâmico, daidzeína e genisteína. Estes resultados mostraram que o produto obtido pode servir como um ingrediente de valor agregado para a industria alimenticia e farmaceutica e que as técnicas de extração utilizadas foram eficientes no rendimento do processo e os solventes de maior polaridade têm uma influência significativa. Foi também demonstrada a viabilidade técnica da $scCO_2$ + EtOH para obter um produto de alto valor agregado.

Palavras-chave: Soja fermentada. Atividade antioxidante. Compostos fenólicos. Extração por solventes orgânicos. Extração por fluidos supercríticos. Anti-inflamatório. Antitumoral.

ABSTRACT

The search for therapeutic alternatives has been growing and gaining prominence more and more these days. One of the strategies is the consumption of foods that are rich in natural antioxidants and other bioactive compounds that have the capacity to prevent or reduce pathogenic processes. Due to the presence of many compounds with bioactive properties, including antioxidants, fermented foods are related to a number of beneficial health effects and emerge as a promising alternative. Fermentation has been used as a potent way of improving the properties of soybean and their components. In this context, this study aimed in a first step to evaluate the use of different substrates in order to increase the production of bioactive compounds through the bioprocessing of soybean by Rhizopus oligosporus NRRL 2710, and next to compare the yields obtained from different methods, namely Soxhlet and CO_2 + supercritical solvent. The soy + brown rice supplementation showed a significant increase in the concentration of phenolic compounds, expressed as gallic acid equivalent (GAE), (0.697 to 6.447 mg GAE g^{-1}) compared to using soy alone (1.792 to 3.10 mg GAE g^{-1}). Such a process resulted in a product rich in isoflavone and aglycone, containing different antioxidant compounds such as trans-cinnamic acid, gallic acid, myricetin, quercetin and kaempferol. Soxhlet was used as a reference extraction method for comparison purposes; it was performed with 7 organic solvents including n-hexane, petroleum ether, ethyl acetate, acetone, ethanol, methanol, and water. For Soxhlet extraction, the highest and lowest yields obtained were 45.24% and 15.56% using methanol and hexane, respectively. In addition, an increase of 18.62% in extraction yield was achieved when fermented soybeans were dried before extractions. Extraction using supercritical CO_2 plus ethanol as a static modifier ($scCO_2$ + EtOH) showed, at high pressure (25 MPa) and temperature (80°C), a phenolic compound content of 1391.9 µg GAE g^{-1} and radical elimination of 0.17 g g g^{-1}, achieving a yield of 42.87%. All extracts showed anti-inflammatory activity against hyaluronidase enzyme and bioactive compounds identified by HPLC-DAD, including gallic acid, transcinnamic acid, daidzein and genistein. These results showed that the product obtained can serve as a value-added ingredient for the food and pharmaceutical industries and that the extraction techniques used were efficient in yielding the process and the higher polarity solvents have a significant influence. The technical feasibility of $scCO_2$ + EtOH to obtain a high value-added product was also demonstrated.

Keywords: Fermented soybean. Antioxidant activity. Phenolic compounds. Extraction by organic solvents. Supercritical fluid extraction. Anti-inflammatory. Antitumoral.

FIGURE LIST

TABLE LIST

SUMMARY

1 INTRODUCTION

Soybean (*Glycine max L.)* and its derivatives are present in the human diet all over the world, being one of the cheapest and most abundant sources of vegetable protein consumed. It is one of the world's main *commodities* and accounts for 60% of global oilseed production. World production in the 2021/2022 harvest was estimated by the United States Department of Agriculture at 385.5 million tons. It is projected an increase by 21.5 million tons over the past crop (CONAB, 2022; USDA, 2022a; ZHOU *et al.*, 2022).

Soy is thought to be the most abundant source of phenolic compounds in the human diet, being an important class of phytochemical antioxidants and have been widely used by the food and pharmaceutical industries (LI *et al.*, 2020; WANG *et al.*, 2016). In the human diet soy is the richest source of isoflavones (phytoestrogenic compounds with estrogenic activity), and these are divided into two basic phytochemical categories: glycosides and aglycones (JASKULSKI *et al.*, 2017; LI *et al.*, 2017; MOORE; FRANKS; FOX, 2017).

Fermented soy products have phenolic compounds available in the aglycone form and consequently have higher bioactive activities than *"in natura"* soybeans. This because aglycone forms are characterized by being more quickly absorbed by the organism and have higher estrogenic and antioxidant potential (KAYANO *et al.*, 2012; PRADO *et al.*, 2021).

Several studies in the literature mention fermented soy products and derived bioactive compounds, however, information that present the comparative effects of extraction methods and operating conditions is vague (PRADO *et al.*, 2021; SANJA *et al.*, 2018). Traditional extraction methods have been used to recover many bioactive compounds from plant materials. However, these methods require large amounts of organic solvents, which is not interesting because they end up becoming costly and environmentally harmful processes, resulting from the use of aggressive and toxic chemicals (ALESSANDRA *et al.*, 2019).

Bioactive components of interest, such as phenolic compounds, carotenoids, alpha-acids, methylxanthines, and vitamins are present in plant sources. Phenolics consist of flavonoids, isoflavones, tannins, and other related ones (ALVAREZ *et al.*, 2019; GRAJEDA-IGLESIAS *et al.*, 2016). In addition to the high protein content, other bioactive and remarkably rich components such as isoflavones, anthocyanins, and saponins are present in soy. These compounds are known to have antioxidant capacity that is related to different health benefits (GIORDANO *et al.*, 2015; MANI; MING, 2017).

Antioxidants are among the main health components that consumers look for in food products because of their benefits in preventing many diseases caused by cellular oxidative processes and reactive oxygen species (ROS), such as aging and chronic diseases like diabetes and cancer (Prior et al., 1998; Melo et al., 2006; Mukhopadhyay, 2006).

In the context, methods that encompass the principles of "green chemistry" are an alternative that reduces or eliminate extractive waste (ALVAREZ *et al.*, 2019). Supercritical carbon dioxide ($scCO_2$) appears as a modern method for the extraction of natural compounds with high added value (Attard et al., 2018). A considerable advantage of this method is the manipulation of temperature and pressure that adjust the properties of supercritical fluids, such as density, with the aim of increasing the selectivity of the solvent (AHMED *et al.*, 2017).

The use of the co-solvent is a useful strategy to enhance the solvent power, increase the extraction of polyphenols, and improve the selectivity regarding the extraction yield of compounds from plant matrices (Santos et al., 2017). This is because the solubility of long-chain molecules and high polar compounds in supercritical CO_2 is limited and this disadvantage can be solved using a polar co-solvent (SOLTANI; MAZLOUMI, 2017).

There are few reports on extractions of fermented soybeans by filamentous fungi and evaluation of the biological activities of the extracts obtained from the resulting material. Therefore, this study evaluated the extracts obtained by different extraction techniques - solid-liquid, Soxhlet, with different organic solvents, and extraction with CO_2 + supercritical ethanol ($scCO_2$ + EtOH). The antioxidant activity (AA), total phenolic content (TPC), phenolic compounds (by HPLC), fatty acid profile (FA), β-glucosidase activity, anti-inflammatory activity, antitumor activity against CaCo-2 and MCF-10 cells and oxidative stability were evaluated in order to verify the technical feasibility of the process and the recovery of molecules with high bioactive potential for the pharmaceutical and food industries.

2 OBJECTIVES

2.1 GENERAL OBJECTIVES

This work aimed at the production and quantification of bioactive compounds by fermentative process from soybean fermented by filamentous fungi and evaluation of biological activities, as well as evaluating the fermented product and potential for use in pharmaceutical and food industries.

2.2 SPECIFIC OBJECTIVES

- To verify the increase in antioxidant activity of the fermented after soybean supplementation with brown rice
- To evaluate the concentration of total phenolic compounds during the fermentation process kinetics

- To evaluate the changes in isoflavone profiles (genistein and daidzein) during fermentation
- Evaluate the increased anti-inflammatory and antitumor potential after the fermentation process
- Evaluate the different yields of Soxhlet extraction using organic solvents with different polarities
- Verify the influence of the drying process on the extraction yields in both extraction methods used, Soxhlet and $scCO_2$ + EtOH
- Check the extraction efficiency using $scCO_2$ + EtOH at different pressure and temperature

3 LITERATURE REVIEW

3.1 SOYBEAN

Soybean (*Glycine max*) is one of the most widely grown oilseed crops in the world and one of the cheapest and most abundant sources of vegetable protein consumed as a food and dietary supplement (JIA *et al.*, 2020). The soybean has in its composition about 40% protein, 20% lipids, 35% carbohydrates, 5% minerals, and 10% moisture, in addition to other compounds such as fatty acids, vitamins, flavonoids, isoflavones, phenolic acids, and saponins (MEDIC; ATKINSON; HURBURGH, 2014; REDDY; DUKE, 2015).

In the context between global supply and demand for food, soy stands out for being a highly nutritious grain capable of providing a range of by-products for human consumption, in addition to attributing an important role in the production of animal protein (chicken, pork, and beef) (DE LIMA *et al.*, 2018). Of the world oilseed production, 60% corresponds to soy and, of this total, only 6% is consumed in the form of grains, whole grains, and fermented products. The remaining 94% is processed industrially, being transformed into oil for human consumption, production of biodiesel, and development of chemicals, food, and cosmetics (18-20% of this total). The remainder crushed is usually transformed into protein-rich bran used for animal feed (PESSOA *et al.*, 2019).

The world soybean production in the 2019/2020 harvest was estimated by the U.S. Department of Agriculture (USDA) at 337.298 million tons, in a total world area occupied by the cultivation of 122.647 million hectares, corresponding to the average productivity of 2.750 kg/ha (United States Department of Agriculture USDA, 2022b). The largest suppliers are Brazil, the United States and Argentina (Table 1) (CONAB, 2022; United States Department of AgricultureUSDA, 2022b).

TABLE 1. PRODUCTION, PRODUCTIVENESS AND TOTAL PLANTED AREA IN HECTARES OF SOYBEAN IN 2019/2020.

	Area (million ha)	**Production (million ton)**	**Productiviness (kg/ha)**
Brazil	36.95	124.844	3.379
United States	30.33	96.676	3.187
Argentina	17.1	50.0	2.92
World	122.647	337.298	2.750

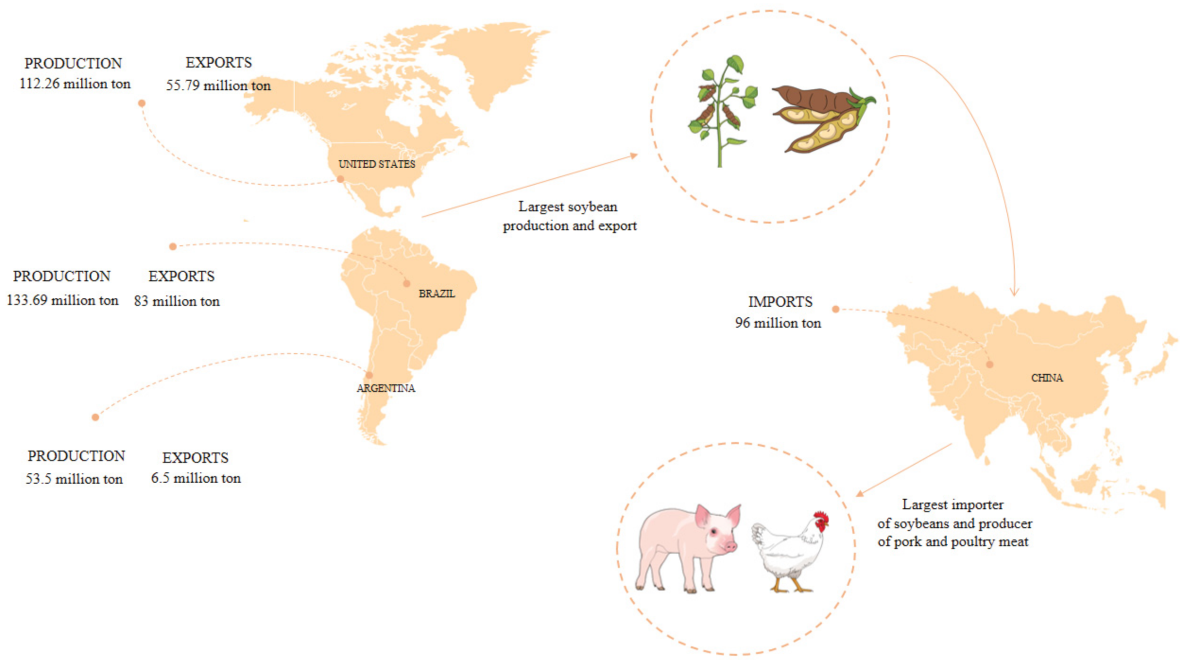
SOURCE: (CONAB, 2022; United States Department of AgricultureUSDA, 2022b)

USDA projects the 2021/22 world soybean crop at 372.56 million tons. Brazil remains the world's largest producer of soy, however the forecast was cut from 144 million, in the previous bulletin, to 139 million tons. Brazilian ending stocks of soy were also reduced from 28.25 to 23.55 million tons but estimates for Brazilian soy exports were maintained at 94 million tons. Argentina's production was also projected to fall by 46.5 million tons, a cut of 3 million from the previous report. For the US, the USDA brought a slight increase from 120.45 to 120.7 million tons (USDA, 2022a).

Asian countries are characterized by high grain consumption. In recent years, due to increased demand and domestic supply, China has become the largest importer of soybeans, accounting for more than 60% of world soy trading. This is because Chinese production accounts for only 20% of domestic demand and a large amount of soy is needed to supply pork feed. Compared to other crops, the application of soy resources is potentially greater, taking the efficiency of land use as follows: a soybean yield of 3000 kg/ha becomes a product to produce 343 kg of beef, 600 kg of swine, or 1200 kg of birds (GARRETT; RAUSCH, 2016; WU *et al.*, 2020).

In the 2020/2021 harvest, there is an estimate for Chinese imports of 96 million tons. Brazil also assumes the position of largest grain exporter, with more than 60% of the harvest destined for this purpose. Motivated by the high Chinese demand and the percentage, Brazilian exports are expected to reach 83 million tons. The global export forecast is 312.80 million tons, of which 55.8 million tons are from the United States and only 6.5 million tons from Argentina (Figure 2) (United States Department of AgricultureUSDA, 2022b).

FIGURE 1 - SOYBEAN PRODUCTION AND EXPORT OF LARGEST PRODUCERS AND ESTIMATES FOR IMPORTS IN THE 2020/2021 HARVEST.



SOURCE: The author (2022).

Plant genotype, location, climate, water, and maturity group are responsible for the quality characteristics of soy, e.g., protein, oil, fatty acids, soluble sugars, and isoflavones. The grain consists of 8% husks, 90% cotyledons, and 2% hypocotyls. The protein composition of the soybean grain on a dry basis is on average 40% (Table 2). The storage of proteins occurs in the intermediate layers of the grain, cotyledons, and hypocotyls. In the shell, the outer layer, there are greater amounts of carbohydrates and cellulosic material (ASSEFA *et al.*, 2019; PERKINS, 1995).

TABLE 1 - NUTRITIONAL COMPOSITION OF SOYBEAN

Components	**% grain**	**Chemical Composition (% dry weight)**			
		Proteins	**Lipids**	**Carbohydrates**	**Others**
Husks	8	9	1	86	4.3
Hypocotyls	2	41	11	43	4.4
Cotyledons	90	43	23	29	5.0
Total	100	40	20	35	5.0

* Minerals, vitamins, phytates and isoflavones.

SOURCE: The author (2022).

In addition to the high protein content, the soybean contains other bioactive and remarkably rich components, such as isoflavones, anthocyanins, and saponins. They are compounds known to have antioxidant capacity that is related to different health benefits (GIORDANO *et al.*, 2015; MANI; MING, 2017).

3.2 NUTRITIONAL CHANGES IN FERMENTED SOYBEAN PRODUCTS

Fermentation enriches the nutritional value of foods by increasing the content of vitamins, essential amino acids, or fatty acids, allowing detoxification and removal of anti-nutritional factors. In addition to proteins and isoflavones, soybean is made up of numerous other functional and nutritional substances, increased in the fermentation process through microbial biotransformations Microorganisms with abilities to produce specific hydrolytic enzymes, such as protease, amylase, and β-glucosidase, play a fundamental role in increasing functional properties (KUMAR *et al.*, 2016; XU; DU; XU, 2015; ZHENG *et al.*, 2017).

The modification of isoflavones occurs through β-glucose enzymes, which degrade cellulose, hydrolyze the β-D-cellulose terminal non-reducing glucoside bond, and, consequently, release β-D-glucose. Thus, the amount of glycosides decreases by hydration by β-glucosidase, increasing the amount of isoflavones aglycones (FLORINDO *et al.*, 2018; GUADAMURO *et al.*, 2017). After ingesting the aglycone isoflavones, such as genistein and daidzein, they are absorbed by the blood vessels, hydrolyzed in the small and large intestine by intestinal hydrolytic enzymes and microbial glycosidases through deglycosylation, increasing their bioactive potential (RAIMONDI *et al.*, 2009).

Some final characteristics of the fermented product, as well as the changes that occur during soy fermentation, are related to the type of microorganism used. In tempeh, for example, several amylases, lipases, and proteases are produced by fungi of the genus *Rhizopus spp*. These hydrolyze macronutrients into simpler, water-soluble compounds, resulting in the production of vitamins, phytochemicals, and antioxidant constituents (AHMAD *et al.*, 2015). The increased antioxidant effect of tempeh can still be attributed to the increased levels of polyphenols released by cell wall degradation by enzymes secreted by *Rhizopus* fungi during preparation in the boiling stage and through the course of fermentation (KULIGOWSKI; PAWŁOWSKA; JASIŃSKA-KULIGOWSKA, 2017).

Due to the metabolic activity of starter cultures, the levels of vitamin B complexes are also increased during fermentation. In tempeh *Rhizopus* and the bacteria, *K. pneumoniae* and *C. freundii* are the main producers of vitamin B_{12}. In natto, *Bacillus* is the agent responsible for the increase of vitamin K2 (KAMAO *et al.*, 2007; MO *et al.*, 2013). The functionality of these vitamins is well known, being essential bioactive substances that act in the coordination of the nervous system and the development of the brain, some studies, still confirm the significant increase in gamma-aminobutyric acid (GABA) in fermented soy products, responsible for the regulation of the central nervous system (XU; CAI; XU, 2017).

Microbial proteolytic enzymes involved in fermentation processes hydrolyze the protein content into peptides. The length of the chain and the composition and sequence of amino acids interfere with the biological activity of the peptide and, during enzymatic hydrolysis in fermentation and digestion, inactive bioactive peptides are released. Furthermore, the bioconversion of high molecular weight proteins into minors increases the solubility (CHAN; LIU, 2016; YOUNG *et al.*, 2011). One of the main biochemical changes that occur during fermentation is the hydrolysis of proteins by microbial proteases and the enrichment of nutritional effects depends on this reaction (MEINLSCHMIDT *et al.*, 2015).

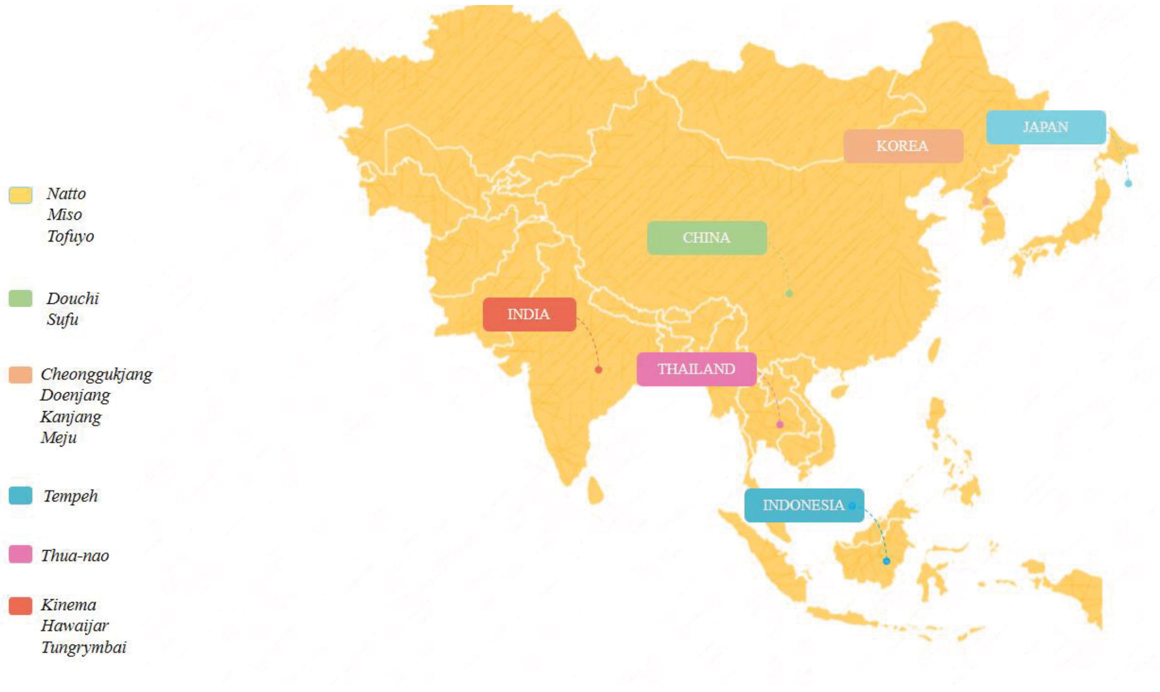
Angiotensin-converting enzyme (ACE) inhibitory peptides are generated by proteolytic degradation of glycinin and β-conglycinin, which consists of protein fractions from soybean. This enzyme acts in the conversion of angiotensin I into angiotensin II and inactivation of the bradykinin vasodilator, raising blood pressure and the risk of cardiovascular disease. Hydrophobic amino acids (Try, Phe, Trp, Ala, Ile, Val, and Met) or positively charged amino acids (Arg and Lys) show greater affinity with ACE. There are three classifications for ACE-inhibiting peptides, being (1) true inhibitor, unaffected by gastrointestinal digestion, (2) substrate, converted into other peptides with less activity in gastrointestinal digestion, and (3) prodrug, converted to true inhibitors by gastrointestinal digestion (HE *et al.*, 2012; RAI; SANJUKTA; JEYARAM, 2017). Still, several other nutritional changes are reported as a consequence of the soybean fermentation process, such as the increase in total soluble iron, the level of folic acid, the composition of tocopherol, with the levels of beta-, gamma- and delta-tocopherol being increased (MANI; MING, 2017).

In summary, many metabolic activities and biotransformations occurred during the soybean fermentation process. There are several beneficial health effects of the final fermented product, and its consumption is related to a series of bioactivities that will be mentioned below.

3.3 FERMENTED SOYBEAN PRODUCTS

The production and consumption of fermented soy are widespread in Asian countries. The main soybean products include *natto*, *miso*, *tofuyo* (Japan), *douchi*, *sufu* (China), *cheonggukjang*, *doenjang*, *kanjang*, and *meju* (Korea), *tempeh* (Indonesia), *thua-nao* (Thailand), *kinema*, *hawaijar*, *tungrymbai* (India) (Figure 3). In addition to other widely consumed products, such as sauce, pasta, and soybean milk (Production of bioactive peptides during soybean fermentation and their potential health bene fi tsSANJUKTA; RAI, 2016). Records of some production methods were found in *Cheminyoshul*, a Chinese manuscript dating back to 530-550 dC, and others in the Korean manuscript *Samkuksaki*, dating from the 1392s, pointing to the consumption of fermented soybeans since the 12th century (MAH, 2015).

FIGURE 2 - SOY FERMENTED PRODUCTS IN ASIAN COUNTRIES



SOURCE: The author (2022).

The large consumption of soybeans in Asian countries is related to the widespread adoption of grain cultivation. The variety of appropriate climates and geographic regions resulted in highly sizable crops, making soybean a staple in the region. With insufficient meat consumption, these fermented foods played a vital role as a source of protein in the Asian diet (PARK; LEE; MAH, 2019; WU *et al.*, 2004).

In ancient times, the basic idea behind soybean fermentation was the preservation of food. In the current perspective, the research is interested and directed to the application of fermentation to improve the bioactive components of soybean, responsible for health benefits, and reduce anti-nutritional factors (DIFO *et al.*, 2014; RAI; KUMARASWAMY, 2015).

The difference between fermented soybeans is based on several parameters, but mainly due to the microorganism used in the process. Thus, fermented soybean products are different in terms of aroma, texture, and therapeutic and nutraceutical values. Some fermentations occur only with bacteria, others using only filamentous fungi, and, in many cases, both these microbial groups are used in the fermentation. Some products are fermented only with Bacillus (*natto, kinema, chungkookjang*); some are fermented with fungi *Aspergillus oryzae, Mucor* spp. *Rhizopus* spp. and *Fusarium* spp. (*douchi, tempeh, miso, tofu)* and in some cases both microorganisms are used, as in the case of *doenjang*, where the bacteria involved in this process would be *B. subtilis* and fungi include *Rhizopus* spp., *Mucor* spp., *Geotrichum* spp., and *Aspergillus* spp. (CAO *et al.*, 2009; JUNG; LEE; JEON, 2014; SANJUKTA; RAI, 2016; STEINKRAUS *et al.*, 1959; YASUDA, 2011).

Soybean protein and isoflavones are the main functional constituents of fermented soybean foods. Soybean consists of one of the plant sources with the highest abundance of isoflavones. Because their chemical structure is similar and has an affinity for estrogen receptors, these compounds are usually called phytoestrogens (DAI *et al.*, 2019; ZHU *et al.*, 2020).

The native forms of isoflavones have their bioavailability compromised because they are usually combined with sugars that minimize their absorption through the human intestinal tract. Isoflavones are categorized into two groups: glycosides and aglycone. The beneficial and functional effects of isoflavones on health are conferred to their aglycone forms, which are absorbed more quickly by the body. In unfermented soybean, the presence of aglycone isoflavones is 2–3% of the total composition, this content being mainly corresponded to β-glucoside isoflavones (CAO *et al.*, 2019; HANDA *et al.*, 2019; ZHANG; YANG; HUANG, 2019).

Thus, the biotransformation of glycosidic forms into aglycones through fermentation is a desirable process to increase and produce more biologically active forms. In fermented soy products, the aglycone values vary from 40 to 100%. The conversion of the glycoside into isoflavone aglycones occurs through the action of β-glycosidase produced by microorganisms during the fermentation process. In addition to a higher absorption rate, aglycone forms have greater antioxidant activity than glycosidic forms. This explains the fact that the consumption of fermented soybean products in Asian countries is associated with the reduction of chronic diseases since the consumption of natural antioxidants is efficient in reducing the harmful impacts of reactive oxygen species (ROS) and in adjusting the body's antioxidant load (CHEN, M. *et al.*, 2014; PRADO *et al.*, 2021; XIAO *et al.*, 2015).

Soybean proteins, they have many enzyme inhibitors, such as proteinase and trypsin inhibitors, which make them less digestible. During fermentation, proteolytic enzymes generated by microbial populations hydrolyze proteins into peptides and free amino acids responsible for antioxidant activity and increasing the digestibility of soybean protein (MAI; TECHNOLOGY; RAI, 2011; SANJUKTA *et al.*, 2015).

In addition to proteins and isoflavones, soybean is made up of numerous other functional and nutritional substances, such as fatty acids, vitamins, peptides, minerals, flavonoids, phenolic acids, and saponins (MAN *et al.*, 2011).

Natto consists of popular and traditional food in Japan that has been consumed since the 17th century and produced by fermenting soybeans cooked with strains of *Bacillus subtilis* var natto. It is known to have large amounts of peptides because, in the fermentation process, proteins are cleaved by extracellular proteases produced by the *Bacillus* strain, which increases the free amino acid content by 10% to 30%. Studies report that the proteins derived from this food consist of at least seventeen different amino acids, including glutamic acid, glutamine, aspartic acid, leucine, proline, serine, lysine, methionine, threonine, glycine, isoleucine, tyrosine, phenylalanine, histidine, arginine, alanine and valine (MANABE, 2011; SATO *et al.*, 2018). The consumption of natto has been shown to have an anti-aging effect, prolonging life expectancy, due to the metabolites found in natto extracts, for example, the enzyme nattokinase. Often, reduced life expectancy is caused by oxidative stress, and the relatively high antioxidant activities of natto are mentioned (INOMATA; MIYAKAWA; AIHARA, 2018).

Miso is a fermented soybean paste, prepared from steamed soy, salt, and koji - cooked cereal or soy malted with *Aspergillus oryzae*. It is a traditional Japanese spice used to add flavor to soups and dishes consumed at breakfast by most Japanese families for over 1000 years. The process of maturation of the Miso takes from three to twenty-four months and involves several microorganisms, such as molds, yeasts and, lactic acid bacteria, which act by hydrolyzing the components of soybean (INOUE *et al.*, 2016; WANG, S. *et al.*, 2019). There are different types of miso, which vary according to local traditions and available ingredients; and this food can be classified according to the koji used: 1) rice miso, made by adding rice koji to soy; 2) barley miso with the addition of barley koji to soy and soy miso, made only with soybean. It is reported that during the miso ripening process, the peptides formed are made up of 3-20 amino acids and still include amino acids such as glutamic acid, aspartic acid, and proline (OGASAWARA; YAMADA; EGI, 2006; RATNANINGRUM *et al.*, 2018).

Tofu is a Japanese fermented soy curd similar to cream cheese a characteristic that results from the ripening or maturation process by proteases, carbohydrases and other catabolic enzymes found in red koji (*Monascus fungus*) or koji yellow (*Aspergillus oryzae*), used in the preparation of this food together with tofu (vegetable cheese based on soybean) (NURYANI *et al.*, 2018; YASUDA, 2011). Its functional properties were investigated and associated with the presence of bioactive peptides. Thus, tofu came to be seen not only as a nutritional accompaniment but becoming a valuable source of protein (KUBA *et al.*, 2003; YASUDA; TACHIBANA; KUBA-MIYARA, 2012).

Douchi is a popular product consumed for at least 2.000 years by the Chinese, as a source of protein and flavoring ingredient. The preparation of the douchi is carried out in two stages: pre-fermentation, which consists of an aerobic process using several microorganisms as the starter culture (for example, *Aspergillus oryzae*, *Zygosaccharomyces rouxii*, *Lactobacillus plantarum* e *Bacillus subtilis*) and takes 12-15 days; and post-fermentation, where the addition and mixing of salt and other spices is carried out and left for 9 months in anaerobic fermentation, a process where the development of the special nutrients and flavor of the douchi occurs (CHEN, T. *et al.*, 2014; HE *et al.*, 2016). In recent years, douchi has attracted attention as a functional food source. Some studies have revealed the benefits of this food to health, including antioxidants, antihypertensive activity and even lowering blood pressure (TAN *et al.*, 2019; WANG *et al.*, 2008).

Mentioned as “Chinese cheese” due to its texture, sufu is a traditional fermented soy product that has been used as a flavor enhancer and appetizer. There are different types of sufu, which are produced by various processes in different locations in China through microbial fermentation, and based on the types of starter culture; the sufu can be classified into fungi fermented sufu (inoculated with *Actinomucor, Mucor, or Rhizopus*), sufu fermented by bacteria (inoculated with *Bacillus* or *Micrococcus*) and others (naturally inoculated) (HUANG *et al.*, 2018; YANG; TAN; KAN, 2021).

3.4 POTENTIAL BENEFICIAL HEALTH EFFECTS OF SOYBEAN FERMENTED PRODUCT

3.4.1 Antioxidant effect

Many normal reactions in the body form by-products such as free radicals, which are species with unpaired electrons. If the antioxidant defense systems are not efficient, there is an increase in tissue damage and oxidative stress, associated with cell apoptosis and the appearance of several chronic diseases (SANJUKTA *et al.*, 2015).

Due to the beneficial effects of the prevention of these diseases caused by cellular oxidative processes and reactive oxygen species, antioxidants, essential to prevent the formation and suppress the activities of reactive nitrogen and oxygen species, become the main compounds with benefits for health to be included in the diet (DASTMALCHI *et al.*, 2020).

Several fermented soybean foods have bioactive components, such as polyphenols, phenolic acid, saponins, sterol, and flavonoids, that protect against oxidative damage, with flavonoids and phenolics being fundamental compounds responsible for antioxidant activity (SHUKLA *et al.*, 2016).

Phytoestrogenic compounds and phenolic compounds are an important class of phytochemical antioxidants. In soybean and soybean products, aglycone forms are characterized by having greater estrogenic and antioxidant potential (WANG, D. *et al.*, 2019).

3.4.2 Anticancer effect

Environmental factors, especially diet, are considered to play a key role in carcinogenesis. The incidence of cancer in the Asian population is relatively low, and Asians traditionally consume large amounts of soy-based foods, which are rich in isoflavones (KIMURA, 2012). One of the first studies that linked cancer risk reduction and the consumption of fermented soy-based foods took place in Singapore in 1991, reporting that a soy-rich diet resulted in less breast cancer development in women in pre-menopause (LEE *et al.*, 1991).

Soybean isoflavones are believed to have the potential to reduce cancer risk through their antioxidant activity and estrogen-like structure. Genistein presents Estrogen binding affinity compared to estradiol (estrogen steroid hormone) receptors ER-α and ER-β of 4% and 87%, respectively. As a result, it binds to these receptors and plays an important role in preventing hormone-related cancers (KHOSRAVI; RAZAVI, 2021). In addition, genistein is a known tyrosine kinase inhibitor and acts by preventing topoisomerase and angiogenesis. Through these functions, their effect is evident in the cascades of proliferation signals. Some bioactive peptides from soybean can also prevent the growth of tumor cells, such as lunasin and saponins, repelling the formation of the cell membrane and promoting cell apoptosis (CALVELLO *et al.*, 2016; SINGH; YADAV; VIJ, 2019).

Several soybean products are mentioned for their anti-cancer potential. In Korea, fungi and *Bacillus* sp. are used in the fermentation process in Meju, a dry soybean block, which is used to produce other products such as Kanjang, Doenjang, and Gochuchang. The anti-cancer potential of Doenjang is associated with compounds, such as trypsin inhibitor, isoflavones, vitamin E and an unsaturated fatty acid that contributes to the biological effect. Still, Doenjang extracts invigorate glutathione S-transferase and increase the vitalization of natural killer cells (SHIN; JEONG, 2015).

Mostly, the anticancer effects of fermented soy products are associated with isoflavones. Some studies have shown that methylation-mediated epigenetic gene silencing can be reversed. Genistein is said to have a broad-spectrum anticancer effect on cancers of the breast, prostate, esophagus, pancreas, stomach, and colon, and metacarcinoma, lymphoma, and neuroblastoma. It also acts as a positive regulator of the mRNA expression of several tumor suppressor genes, counteracting the function of growth-stimulating factors and inhibiting cell malignancy. Therefore, the consumption and inclusion of fermented soybean foods have stood out as a new therapy for the treatment of tumors (BILIR *et al.*, 2017; SUNDARAM *et al.*, 2018).

3.4.3 Anti-obesity and Antidiabetic effect

Physiologically, obesity consists of an imbalance between energy intake and consumption that indicates an excessive accumulation of fat in the tissue and is considered a major health problem that is advancing significantly worldwide. Obesity is generally correlated with diabetes and metabolic syndromes leading to hyperinsulinemia and dyslipidemia (HSIAO; HO; PAN, 2020).

As a result, interest in combating obesity and overweight is growing. Several studies show that a change in the diet prevents and alleviates a series of metabolic imbalances characterized by central obesity, dyslipidemia, and high fasting glucose. Some benefits are attributed to the physiologically active components of certain foods, therefore, they are used to prevent obesity and its complications (ROSAS-VILLEGAS *et al.*, 2017; WANG *et al.*, 2017).

The isoflavones daidzein and genistein found in high levels in fermented soybean foods are mentioned as having bioactivity, regulating the generation of lipids and thermogenesis *in vivo*. Through lipogenesis (synthesis of fatty acids and triglycerides), hyperlipidemia (high levels of fat particles in the blood), hyperglycemia (elevated blood glucose), and improved insulin resistance, aglycones and metabolites demonstrate their anti-obesity effects (GAMAN; STOIAN; ATANASIU, 2011). Some studies also associate these effects with the bioactive phytochemical content of fermented soybean foods as alpha-amylase and alpha-glucosidase inhibitors, protease inhibitors, hemagglutinin, and crude fibers, able to disturb normal metabolism and assist in the management of obesity and different metabolic disorders (SHARMA; BALUJA; PROFESSOR, 2015)

The consumption of isoflavones through soybean foods in the diet in HDL cholesterol and reduction of total cholesterol, LDL, and triglycerides. In addition to isoflavones, soy proteins, as well as peptides, are active ingredients that lower the levels of LDL cholesterol and triacylglycerols (SINGH; YADAV; VIJ, 2019).

There is an association between obesity and the transition from pre-menopause to post-menopause in women. This phase is correlated with the risk of several diseases due to the lack of hormonal regulation, including the accumulation of abdominal fat, hypertriglyceridemia, and high levels of low-density lipoprotein cholesterol (LDL-C), reduced high-density lipoprotein cholesterol (HDL-C), elevated blood pressure (BP) and impaired glucose tolerance/diabetes. Studies show that the consumption of fermented soybean foods rich in isoflavones has beneficial effects on the distribution of body fat and lipid profile in women during the menopause period, due to the structural similarity of these compounds with estrogen, their greater affinity for estrogen receptors and circulating concentration in the human body (HUANG *et al.*, 2016; SQUADRITO *et al.*, 2013).

Clinical and experimental studies indicate that the population with obesity and overweight is more vulnerable to type 2 diabetes mellitus (DM2), with obesity dramatically increasing the likelihood of DM2. Individuals with type 2 diabetes mellitus are at increased risk of cardiovascular disease, even with aggressive control of glucose, cholesterol, and blood pressure (GUO; LING, 2015; VIEIRA *et al.*, 2017).

Oxidative stress (OS) is closely associated with obesity and diabetes. Free fatty acids, which at high levels influence the production of reactive oxygen species (ROS) through mitochondrial electron transport chain complexes and enzymes in endothelial cells, decrease the bioactivity of nitric oxide, activate pro-inflammatory signaling pathways causing damage to cellular proteins and organelles. Damaged, more oxidizing mitochondrial enzymes enhance oxidative stress and cellular dysfunction. Chronic exposure to ROS negatively affects insulin signaling when stress pathways are activated. As a result, insulin resistance, glucose intolerance, β-cell, and mitochondrial dysfunctions are developed, advancing to a state of diabetes (PICARD *et al.*, 2013; RAINS; JAIN, 2011).

The consumption of foods rich in isoflavones is seen as a promising strategy in the treatment of diabetes and obesity. Genistein reduces the inflammatory state in obese people, decreases production, and neutralizes the effects of ROS, resulting in the relief of insulin resistance and, consequently, decreasing the risk of diabetes (BEHLOUL; WU, 2013). After ingestion, genistein enriches insulin resistance by increasing the production of insulin receptor substrate (IRS) 1, glucose transporter (GLUT) type 1, and N-terminal c-jun kinase, increasing the activity of superoxide dismutase, decreasing mitochondrial damage and lipid peroxidation. Daidzein, which is also found in soybeans, activates GLUT4 and IRS1 in adipocytes, adding insulin-stimulated glucose uptake (LEI *et al.*, 2011).

Spermidine is a naturally occurring polyamine present in all living cells noted to play an important role in cellular functions and is found in different concentrations in fermented soy foods such as *Chunjang (*1.4 –12.8 mg/kg), *Doubanjiang* (0.18 mg/kg), *Douchi* (74.92 mg/kg) and *Sufu* (1.3 – 32.87 mg/kg) (CHOKSOMNGAM *et al.*, 2021; PARK; LEE; MAH, 2019).

Increased spermidine flux is associated with increased glucose and lipid metabolism. Many *in vivo* studies reveal that spermidine overexpression protects against diet-induced obesity, and an epidemiological study shows that foods rich in polyamines, such as spermidine, are associated with a lower occurrence of cardiovascular disease (CVD), corroborating the theory that spermidine is beneficial in the treatment of obesity (EISENBERG *et al.*, 2009; KRAUS *et al.*, 2014; SODA; KANO; CHIBA, 2012).

3.4.4 Anti-inflammatory effect

Inflammation is a natural biological mechanism in the human body in which the immune system protects against tissue damage due to physical trauma, harmful chemicals, and microbial agents. A wide range of progressive diseases is related to inflammation, resulting in dysregulation of cell signaling, the exaggerated appearance of cytokines, abrogation of the barrier function to inflammatory cells, and oxidative damage of tissues and organs, resulting in systemic disorders. Thus, some studies suggest that the reduction or inhibition of chronic inflammatory mechanisms can prevent numerous diseases. Thus, a diet with anti-inflammatory components has beneficial biological activities (LEI *et al.*, 2018; YUSOF *et al.*, 2019).

In inflammation, macrophages can be activated by toll-like receptors (TLR) (e.g., lipopolysaccharide (LPS) and interferon-γ), which results in the production of several pro-inflammatory cytokine substances, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin 1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (MÜLLER *et al.*, 2017). Cytokines consist of soluble proteins that influence the behavior of other cells involved in cellular immunity and the inflammatory response. Thus, such pro-inflammatory substances contribute to the development of numerous metabolic disorders (GRAY; BLOCH, 2012; MURRAY; WYNN, 2011).

However, inflammation control appears as a promising therapeutic alternative to maintaining health and well-being. Foods that assist in this process should be considered. The use of fermented soy foods to control inflammatory processes is reported, and active metabolites, such as proteins, phenolic compounds, and antioxidants, are found and associated with anti-inflammatory effects (DIA; BRINGE; MEJIA, 2014).

In the inflammatory reaction, the macrophage produces nitric oxide (NO), which is usually detected as iNOS (inducible nitric oxide synthase). (LIAO *et al.*, 2010; SZLISZKA *et al.*, 2011). Isoflavones act as inhibitors of NO production and consequently cancel the production of IL-1β and TNF-α pro-inflammatory cytokines. After ingestion, these compounds inhibit the expression of COX-2, the production of pro-inflammatory cytokines and the activation of the nuclear transcription factor kappa-B (NF-κB). Thus, the expression of several genes during inflammatory responses is controlled and regulation of innate and adaptive immunity occurs. Isoflavones still affect the mechanisms of inflammation containing the inflammatory process through different intracellular signaling pathways triggered by AP-1, PPAR, Nrf2 and MAPKs (CHEN *et al.*, 2018; YUSOF *et al.*, 2019).

Epidemiological investigations show the associations between different soy foods and inflammatory markers, including highly sensitive C-reactive protein (hs-CRP), interleukin (IL) -6 and IL-18. The high levels of intake of these foods, including miso and soy sauce, are related to a reduction in the serum level of IL-6, a pro-inflammatory cytokine associated with several chronic diseases (ASSOCIATIONS BETWEEN INTAKE OF DIETARY FERMENTED SOY FOOD AND CONCENTRATIONS OF INFLAMMATORY MARKERS.PDF, [*s. d.*])

Above all, the anti-inflammatory effects of isoflavones are confirmed by the fact that they act in the elimination of reactive oxygen species (ROS), which are directly involved in inflammation

3.4.5 Preventive effect against cardiovascular disease

Some evidence reports the association between high soy consumption and the preventive effect against cardiovascular diseases (CVD), such as a lower risk of ischemic heart disease (IHD) or stroke. Soy protein and isoflavones are the constituents responsible for the lower risk of CVD, in addition to their beneficial effects such as lipid profile, arterial stiffness, blood pressure, and endothelial functions (NAGATA *et al.*, 2017).

Nattokinase (NK) is an enzyme contained in the sticky component of natto, cheese-like food made from soybeans fermented with *Bacillus* subtilis, which can dissolve thrombi and fibrin. Because it is considered stable in the gastrointestinal tract, NK becomes an appropriate agent for oral thrombolytic therapy (DABBAGH *et al.*, 2014).

It is because NK acts directly degrading fibrin or activates other fibrinolytic enzymes, such as pro-urokinase and tissue plasminogen activator (t-PA). NK also inactivates plasminogen activator inhibitor-1 (PAI-1) *in vitro*, the primary inhibitor of t-PA, resulting in the enhancement of fibrinolysis (TAKABAYASHI *et al.*, 2017; YATAGAI *et al.*, 2008).

The development of intravascular thrombi causes a variety of CVDs. Studies suggest that natto has broad thrombolytic efficacy and its ingestion has protective effects against CVD (KUROSAWA *et al.*, 2015).

3.4.6 Neuroprotective effect

The human brain is singularly vulnerable to oxidative damage and has high oxygen consumption, in addition to having a relatively high content of polyunsaturated fatty acids (PUFA), which are sensitive to oxidation. Otherwise, neurons are particularly sensitive to disturbances in the balance between antioxidants and the production of reactive oxygen species (ROS), since the levels of antioxidant defense in the brain are negligible. High content of active redox metals is found in the brain, which promotes the formation of ROS and is associated with the development of pathologies (KANG *et al.*, 2016).

Glial cells, such as astrocytes, oligodendrocytes, and microglia, are cell types present in the central nervous system that play a beneficial role in the viability and survival of neural cells. The latter is like macrophages found in the brain and plays an important role in defending the host and repairing tissues in the central nervous system

(CNS). In response to extracellular stimuli, microglia release pro-inflammatory mediators, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), reactive oxygen species (ROS), and pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α that works to restore CNS homeostasis by eliminating pathogens and infected cells (DANDONA; AJAY; SANDEEP, 2010; LOANE; BYRNES, 2010).

A possible therapeutic approach for the treatment of neurodegenerative diseases is to control microglial activation and reduce the number of pro-inflammatory factors since the overproduction of inflammatory mediators and cytokines causes chronic neuroinflammation, develops several neurodegenerative diseases, and can occasionally lead to neural cell death (DENG *et al.*, 2011).

Studies show that isoflavones are protective against neuronal cell death, elevate existing neuronal function, and boost neuronal regeneration. Thus, interest in the consumption of fermented soybean foods rich in isoflavones is growing due to their supposed beneficial effects, such as the ability of genistein to inhibit the apoptotic signaling cascade in neurons (DIAS *et al.*, 2012).

3.4.7 Anti-aging effect

Aging is seen as an inevitable, universal, multifactorial and, complex progressive decline in the physiological functions of all living beings, affecting the condition of relative stability and making it susceptible to age-related injuries and diseases (LARA; MICHAEL J.; MARGARET, 2016; MAGALHÃES; STEVENS; THORNTON, 2017).

Healthy aging is the surest way to extend your health. Thus, therapies that help achieve healthy aging become an efficient path to longevity in humans. In this search for the longevity of the body, antioxidant therapy has beneficial effects, pointing out the role of dietary antioxidants. The accumulation of oxidized molecules, such as lipid peroxides, proteins, and damaged DNA mediated by oxidative stress (OS), is the result of the aging process and the administration of antioxidants can prevent oxidation or exclude the production of free radicals, characterized by affecting the rate of aging (GAMAN; STOIAN; ATANASIU, 2011; LEONARDI, 2017; REVIEWS, 2016).

Traditionally, fermented soybean food products are mentioned as having anti-aging properties. These effects are associated with the isoflavones aglycone genistein and daidzein (PAN *et al.*, 2012). Studies suggest the anti-aging effect of Tempeh in the pre and postmenopausal with the maintenance of the quality of the uterus, the improvement of skin quality, and bone strength (KHOSRAVI; RAZAVI, 2021; SAPBAMRER; VISAVARUNGROJ; SUTTAJIT, 2013). It is because isoflavones act in the replacement of estrogen, improving the quality of life of postmenopausal women (DAS *et al.*, 2020).

Some research shows that neuroinflammation is related to low-grade systemic inflammation, common in aging. The cascade of neuroinflammation also correlated with systemic inflammation is one of the most widely accepted suspicions regarding Alzheimer's disease (AD), one of the main common forms of age-related dementia. Antioxidant and anti-inflammatory nutrients are mentioned as agents that help to reduce or delay the development of AD (SHIRLEY STEFFANY MUÑOZ; SANDRA MARIA LIMA, 2018).

Neuroinflammation in the brain can be reduced by the isoflavones present in fermented soy foods, known to have antioxidant activity. A promoter of proinflammatory activity, IL-1β, is decreased while a potent anti-inflammatory cytokine, IL-10, is increased. Also, the intake of isoflavones increases cognitive capacity and prevents oxidative damage in neurons. In AD neurodegeneration, the most damaging sequel is memory loss, with the first implicit mechanism being the deficit of cholinergic neurons, where the transmission of information is canceled due to the lack of neurotransmitters such as acetylcholine (ACh). Research suggests that isoflavones reverse amnesia by increasing acetylcholine and reducing levels of acetylcholinesterase (AHMAD *et al.*, 2014; CHAN *et al.*, 2018; OVERK *et al.*, 2010).

After dietary supplementation with Chungkookjang, a fermented soybean paste from Korea cultivated by *Bacillus* sp., components, such as the isoflavonoids daidzein and genistein, elevated the activity of superoxide dismutase, an important free radical scavenging enzyme (JEONG *et al.*, 2021). It has also been reported that, by improving memory functions and other neurological indications, gerbils induced by stroke are improved (KIM *et al.*, 2021). These, express cerebral ischemia after transitory artery occlusion, and afterward, have global neural cell death due to the addition of oxidative stress, and neuroinflammation (PARK *et al.*, 2011; YEON *et al.*, 2020).

Oxidative stress is the cause of reduced life expectancy. Some findings suggest that Natto significantly prolonged the life of nematodes, increased resilience to oxidative stress and postponed the accumulation of lipofuscin, a characteristic of aging cells. This is cited for its anti-aging effect, relying on actions such as preventing heart attacks, stroke, osteoporosis, bowel disease, and improved cognitive function, especially with age (IBE *et al.*, 2013; NAGATA *et al.*, 2017).

The anti-aging effect of fermented soy foods can also be attributed to the high concentration of spermidine, bioactive polyamines found in high levels in foods such as natto and tempeh. Such a compound has several important functional and regulatory properties related to the physiology of cell aging, such as reversing memory loss, improving the blood lipid profile, and reducing cardiovascular risks, inducing autophagy in damaged cells (SAGARA *et al.*, 2017).

3.4 SARS-COV-2 AND THE POTENTIAL APPLICATION OF FERMENTED SOY PRODUCTS IN THE PREVENTION

A new strain of Coronavirus not previously identified in humans was reported in Wuhan, China in December 2019, being identified as a beta type of Coronavirus ß-CoV Group 2B. A total of seven human coronaviruses (HCoVs) have been identified before: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-COV - responsible for severe acute respiratory syndrome, MERS-COV - responsible for the Middle East respiratory syndrome, and recently the new coronavirus, called SARS-CoV-2 - responsible for causing the new severe respiratory inflammatory disease, COVID-19 (OPAS, 2021; SANTACROCE *et al.*, 2021).

As of early May 2022, a total of 514.918.067 confirmed cases and 6.240.940 deaths caused by COVID-19 have been reported to the World Health Organization (WHO) (WHO, 2021). SARS-CoV-2 enters the body using angiotensin-converting enzyme 2 (ACE2) and transmembrane protease, serine 2 (TMPRSS2), as target receptors to infect the cells. After affecting the epithelial cells of the human respiratory tract, the rapid replication of the virus leads to a storm of pro-inflammatory cytokines and chemokines. This hyperinflammatory state causes oxidative stress leading to damage to the alveolar and endothelial cells of the lung and chronic lung inflammation (MAHMUDPOUR *et al.*, 2020; ZENG *et al.*, 2020).

Given the pandemic scenario, numerous researches investigate risk factors, clinical manifestations, and possible preventive and therapeutic actions. Health conditions such as obesity, diabetes, previous morbidities with risk of immunodeficiency, and chronic cardiovascular, renal, and respiratory diseases are also investigated for association and relationship to the high severity of COVID-19 (GOUDA *et al.*, 2021). Among the complications caused by the disease are ARDS, septic shock, coagulation dysfunction, metabolic acidosis, cardiac arrhythmia, kidney damage, liver dysfunction, heart failure, or secondary infection (COSTELA-RUIZ *et al.*, 2020).

A therapeutic strategy for the control of SARS-CoV-2 consists of identifying anti-inflammatory agents to act on the reduction of uncontrolled inflammation in patients and the receptors for the ACE-2 enzyme since it is widely expressed by epithelial cells of the lung, kidney, heart, blood vessels, and intestine (HUANG *et al.*, 2020). Flavonoid-derived bioactive compounds, such as isoflavones, are mentioned for their significant health benefits such as antibacterial, antioxidant, anticancer, anti-inflammatory, and immunomodulatory bioactivity (MUCHTARIDI *et al.*, 2020).

There are few studies related to dietary habits as a risk factor for COVID-19 instability. However, some differences in diet have been hypothesized to play a potential role in disease and fatality rate variability (CDC, 2020). Based on mechanistic and clinical data, vitamins and folate, polysaccharides and dietary fiber, lipids, peptides, and natural polyphenols are known to be necessary for the body's immune system against viruses (CALDER *et al.*, 2020).

For example, certain countries, such as Bulgaria, Greece, Romania, and Turkey, where there is high consumption of some types of fermented foods (cabbage and milk), are associated with lower mortality rates. The possible protective effects of antioxidants and angiotensin-converting enzyme (ACE) inhibiting peptides present in fermented foods may justify this hypothesis (BOUSQUET *et al.*, 2020; MOHSENI *et al.*, 2021).

Adem et al. (ADEM *et al.*, 2020) performed a molecular docking study to identify the ability of 80 flavonoid compounds to bind to the 3-chymotrypsin-like protease (3CLpro), a known enzyme important for SARS-CoV replication. Other polyphenols and flavonoids, such as daidzein and genistein (found in fermented soy products), have been proposed as potential inhibitors of the main SARS-CoV-2 protease (PENDYALAA; PATRASA, 2020).

As already mentioned, bioactive peptides with therapeutic properties, including antihypertensive antioxidant, antitumor, and antidiabetic, are present in fermented soy products. Fermented soy peptides have previously demonstrated activity against several viruses, including the SARS-CoV responsible for the SARS outbreak in 2003 (CHOURASIA *et al.*, 2020). In the soybean fermentation process, the proteolytic degradation of the soy protein fractions (glycine and β-conglycinin) generates the ACE inhibitory peptides (KUBA *et al.*, 2005). Studies have already mentioned the identification of ACE inhibitory and antihypertensive peptides in *Natto* and, also, two ACE inhibitory peptides isolated from *tofu* (SACHIE *et al.*, 2009; UBA *et al.*, 2003). Other foods, such as douchi (fermented by *A. egyptiacus*) and *sufu* (a soybean fermented by the fungus), have peptides with ACE inhibitory activity (MA; CHENG; YIN, 2013; ZHANG *et al.*, 2006).

Oba et al. (OBA *et al.*, 2021) conducted a study with *Natto* to investigate the antiviral activities of this food against SARS-CoV-2. The results showed that Natto extract fully inhibited severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in the cells. The protease activities of *Natto* extract were able to proteolytically degrade the British variant of the spike protein (receptor binding domain; RBD) of SARS-CoV-2, resulting in the inhibition of viral infections in cells.

A study conducted by Chourasia et al. (CHOURASIA *et al.*, 2020) from soy cheese fermented with *Lactobacillus delbrueckii* WS4, identified and selected peptides for antiviral activity in silico. A total of twenty-three peptide sequences were examined for binding affinity to critical residues of the SARS-CoV-2 RBD protein and important catalytic residues of the SARS-CoV-2 pro-enzyme 3CL using molecular docking. The authors also showed in molecular docking studies of the selected peptides that they revealed a potential peptide "KFVPKQPNMIL". This peptide showed a strong affinity for significant amino acid residues, for host cells (RBD) of the SARS-CoV-2 peak S1 glycoprotein that are responsible for binding the virus to the human ACE2 protein receptor and also an affinity for the important viral proteolytic enzyme 3CLpro. for viral replication.

Therefore, it can be concluded that fermented soy cheese could be explored as a prophylactic food for SARS-CoV-2 and related viruses. Furthermore, the multi-target inhibitor peptide, which effectively inhibited both viral proteins, could be used for in vitro and in vivo functions against SARS-CoV-2.

3.6 BIOACTIVE COMPOUNDS IN SOYBEAN FERMENTED PRODUCTS

Soy has long been consumed as a health food and fermented soybean products are important components of traditional diets in Asian countries. The benefits of fermented soy are attributed to its phytochemical content and bioactive compounds, which confer numerous benefits to human health (CUI *et al.*, 2020).

Many compounds are responsible for the bioactive properties of fermented foods mentioned in several studies. Isoflavones are compounds found in tempeh, which act as antioxidants and are also related to many chronic diseases (AMIN *et al.*, 2020). Surya and Romulo showed that tempeh extracts protect HepG2 cells (human liver cancer cell line) against induced oxidative stress by reducing ROS generation, and eventually cell death (SURYA; ROMULO, 2020).

Chungkookjang has compounds such as isoflavone aglycones, peptides, and dietary fiber, and is rich in poly-γ-glutamic acid (γ-PGA). Consumption of this food can act on memory impairment induced by Alzheimer's disease and cerebral ischemia, so it prevents and alleviates neural cell survival, thus improving brain insulin sensitivity and neuroinflammation (JEONG *et al.*, 2021).

To investigate the hypothesis that chungkookjang consumption improves sensitivity and insulin secretion capacity an animal model study reminiscent of the characteristics of type 2 diabetes in Asians was conducted. A high concentration of daidzein was observed and related to the anti-diabetic properties of chungkookjang, capable of improving glucose regulation by potentiating insulin secretion and reducing insulin resistance (JEONG *et al.*, 2020).

Recently, research in Japan has shown that higher consumption of natto and miso is associated with a lower risk of mortality (KATAGIRI *et al.*, 2020). These foods are sources of bioactive compounds such as nattokinase, bacillopeptidase F, vitamin K2, dipicolinic acid, γ-polyglutamic acid, isoflavones, vanillic acid and syringic acid, which have health-promoting effects (CAO *et al.*, 2019).

Nattokinase is shown to be responsible for anti-thrombotic and anti-coalugative activities. The anti-thrombotic effect of NK can be used for the treatment of cardiovascular diseases, and such a compound also acts on amyloid degradation related to Alzheimer's disease and on the suppression of atherosclerosis, heart attack, and stroke in sick patients (CHAN *et al.*, 2021; REN *et al.*, 2017). The health benefits of miso are associated with the presence of isoflavones, such as 8-OH-daidzein, 8-OH-genistein, 6-OH-daidzein, which have strong antioxidant activity, this is related to a series of beneficial effects on human health (HIROTA *et al.*, 2004).

Doenjang is an important food consumed in Korea, such food shows strong activities against several carcinogens/mutagenic agents, such as aflatoxin B1. Park et al showed through studies that genistein and linoleic acid present in doenjang extracts have strong antimutagenic activities, being more effective among the other bioactive compounds found in this food, such as β-sitosterol, soy saponin, α-tocopherol, genistein and phytic acid (PARK *et al.*, 2003).

Fermented soy foods are composed of molecules, vitamins, and peptides that are found in greater availability after the fermentation process, which point to being a potential source of numerous health benefits. The bioactive compounds found in fermented soy products are associated with the microorganism used in the process as well as the traditional practice used in each region.

Several bioactive compounds are found in different fermented soy products, these and their health benefits are illustrated in Table 3.

TABLE 2 - BIOACTIVE COMPOUNDS OF DIFFERENT FERMENTED SOY PRODUCTS AND THEIR HEALTH BENEFITS.

Soybean products	**Bioactive compounds**	**Health benefits**	**References**
Tempeh	Isoflavone aglycone	Antioxidant properties	(ROMULO; SURYA, 2021)
Tempeh	Isoflavone aglycone	Protection of HepG2 cells from oxidative stress	(SURYA; ROMULO, 2020)
Tempeh	Genistein	Immunomodulatory Function	(AOKI *et al.*, 2020)
Tempeh	Trans-cinnamic acid	Antioxidant properties	(PRADO *et al.*, 2021)

Chungkookjang	Poly-γ-glutamic acid (γ-PGA)	Prevention of memory loss from Alzheimer's and cerebral ischemia	(JEONG *et al.*, 2021)
Chungkookjang	Daidzein	Anti-diabetic property	(JEONG *et al.*, 2020)
Chungkookjang	Poly-γ-glutamic acid	Anti-obesity effect	(CHOI *et al.*, 2016)
Chungkookjang	Bacillomycin D and surfactin	Antimicrobial activity	(LEE *et al.*, 2016)
Natto	Nattokinase (NK)	Anti-thrombotic and anti-coagulant activities	(CHAN *et al.*, 2021)
Natto	Vitamin K2	Reducing osteoporotic fracture risk	(KOJIMA *et al.*, 2019)
Natto	Bacillopeptidase F	Anti-thrombotic and blood pressure-lowering	(HITOSUGI; HAMADA; MISAKA, 2015)
Natto	Nattokinase (NK)	Fibrinolytic activity	(PAGNONCELLI *et al.*, 2017)
Miso	Isoflavones aglycones	Anti-tumoral activity	(ABE *et al.*, 2021)
Miso	Isoflavones aglycones	Protective effects against stroke	(WATANABE *et al.*, 2018)
Miso	Isoflavones aglycones	Sympathetic nerve activity	(ITO, 2020)
Doenjang	Linoleic acid and Genistein	Antimutagenic activity	(PARK *et al.*, 2003)
Doenjang	Genistein	Antimutagenic and anticancer activities	(JUNG; PARK, 2006)
Doenjang	Genistein	Anti-obesity effects	(CHA *et al.*, 2012)
Kinema	Poly-γ-glutamic acid (γ-PGA)	Suppression of postprandial hyperglycemia	
Kinema	Isoflavones aglycones	Antioxidant properties	(SANJUKTA; 2016)
Kinema	Group B saponins	Prevention of dietary hypercholesterolemia	(OMIZU *et al.*, 2011)
Douchi	β-glucosidase and protease	Antioxidant activity	(YANG *et al.*, 2019)
Sufu	Isoflavones aglycones	Enhancement of the physiological function	(LI-JUN *et al.*, 2004)

SOURCE: The author (2022).

3.8 RECENT PATENTS AND INNOVATIONS ON BIOACTIVE COMPOUNDS IN SOYBEAN FERMENTED PRODUCT

As presented throughout this review, the bioactive compounds present in fermented soy products are related to several beneficial activities for human health and well-being. Scientific studies confirm this potential, and to complement this view, a patent search was conducted showing recent advances and innovations in the use of such compounds.

The patent search was conducted on the Derwent Innovations Index patent database, on March 25, 2022, performing one search for each bioactive compound in the field “Title”, namely *Linoleic acid*, *Daidzein*, *Genistein*, *Isoflavone aglycone*, *Nattokinase*, *Cinnamic acid*, and *Vitamin K2*, using the wildcard * to retrieve all documents containing the defined word roots. The keywords were combined with the International Patent Classification (IPC) A61P (Specific therapeutic activity of chemical compounds or medicinal preparations) using the Boolean operator AND. The IPCs A61P 003/04 (Anorexiants; Antiobesity agents), A61P 003/10 (Hyperglycaemia, e.g. antidiabetics), A61P 009/12 (Antihypertensives) and A61P 037/04 (Immunostimulants) were used to refine the search when necessary (WIPO, 2022). The time interval was the last five years, 2018 to 2022.

After analyzing the documents by reading the titles and abstracts, 334 documents were classified and analyzed using Microsoft Excel software. Along these years it is possible to observe an increase in the number of registered patent documents related to the development and innovation of products made from substances obtained in the soybean fermentation process. The apparent decrease in the number of documents in 2022 is attributed to the date of search (March 2022) and to the secrecy period of usually 18 months before publication. China (CN) and Japan (JP) were the countries that filed the most patents (Figure 4). China accounted for 169 registered patents, representing 50.6% of the total analyzed, while Japan registered 42 patents (12.57%).

The substances generally most abundant in the fermented soybean product were Nattokinase, Genistein and Cinnamic acid because they are widely used in pharmaceutic formulations, for instance, in the prevention and treatment of chronic diseases. This was observed in terms of technology, because the highest number of documents was found for Nattokinase (79), followed by Genistein (69) and Cinnamic Acid (66).

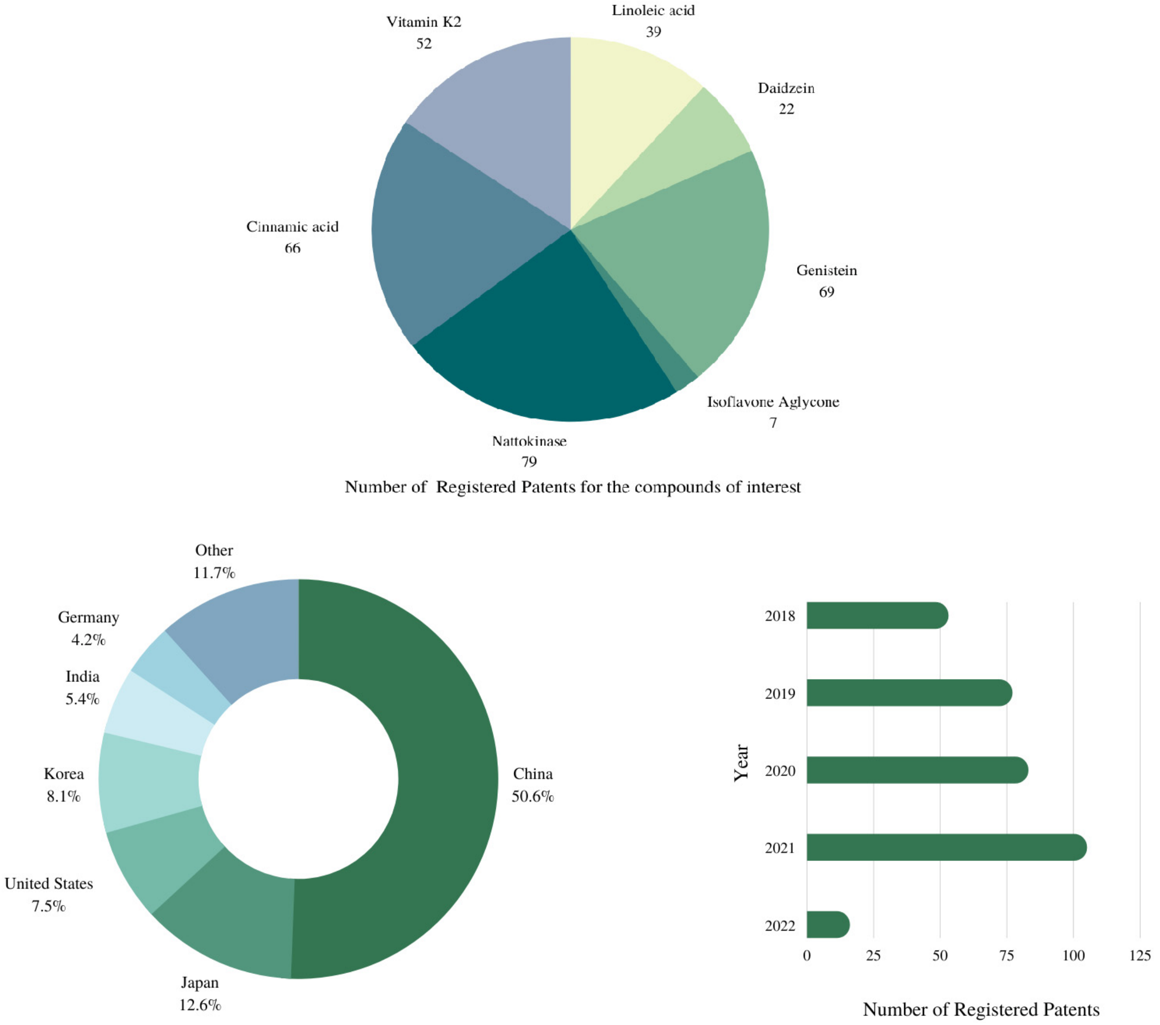


FIGURE 3 - NUMBER OF REGISTERED PATENTS FOR THE COMPOUNDS FOR DE INTEREST, COUNTRIES AND YEARS OF PUBLICATIONS.

SOURCE: The author (2022).

The main company (assignee) that contributed to these patent filings was HUGHES BIOTECHNOLOGY CO. LTD®, Taiwan, with 11.59% patent documents. This company is specialized in the development and manufacture of plant-based nutraceuticals, focusing on the discovery and development of dietary ingredients that are based on the most current science, and reformulating existing ingredients to increase their potency. Other important assignees were DONGGUAN ANHAO PHARM CO. LTD®, China, with 10.6% documents, a skin health management company that integrates product technology research and development, sales, and service, and KOBAYASHI PHARMACEUTICAL CO. LTD®, Japan, with 7.5% documents, that develops ideas for pharmaceuticals and various other applications in daily life, such as dental hygiene skincare, and nutritional supplementation

4 MATERIAL AND METHODS

In this section, a description of the materials and methods used in this work is presented. In the following topics, the materials and methods used in the fermentation process, in performing the product extractions and in analyzing the activities and biological potential are mentioned.

4.1 MICROORGANISM

Fermentation process was performed using a fungus of the *Rhizopus oligosporus* NRRL 2710, belonging for ARS Culture Collection of the Northern Regional Research Laboratory (NRRL, USA). The strains were maintained in Potato Dextrose Agar (PDA) slants at 4 ºC.

4.2 SUBSTRATE TREATMENT

The substrates used in this study (soybean and brown rice) were pre-soaked in deionized water containing 5% v/v of acetic acid. After 12 h, the substrate was thermally treated under flowing steam at 121 °C for 15 min. The excess water was removed, and the substrate cooled to the inoculation temperature at 30 ºC.

4.3 FERMENTED SOYBEAN

The substrate thermally treated was inoculated with 10^7 spores g^{-1} of a dry substrate. The experiments were carried out in perforated trays at 30 ºC for 72 h. After fermentation, the samples were crushed in a kitchen blender (Turbo Blender) for 5 min. A fraction was stored in a freezer, at -18 °C ± 2 °C for use in the extraction experiments (here named wet sample). Another fraction was dried an in oven at 50 °C until constant weight (here named dry sample). The samples were stored in a freezer at 4 °C until the use. The moisture of the raw material was measured using infrared moisture balance (Bel Engineering). All analyzes were performed in triplicate.

4.4 PREPARATION OF ETHANOL EXTRACTS

Alcoholic extraction of fermented substrates and control (non-fermented soybean) was performed according to the method described by (TYUG; PRASAD; ISMAIL, 2010). Dry powder was extracted with 70% (v/v) for one hour in a shaking at 120 rpm. The extracts were collected and centrifuged at 3000xg for 15 min. Supernatants were stored at -18°C for further analysis.

4.5 SOXHLET EXTRACTION

Fermented soybeans were submitted to the Soxhlet extraction following the methodology described in AOCS procedures (AOCS, 1998), using a mass of solids to solvent volume of 1:40 (m/v) ratio. Different solvents, with different polarities, were tested to evaluate the extraction yield, as well as the extraction efficiency of phenolic compounds. Tests were carried out in triplicate for each solvent, for 6 hours of extraction. All solvents used in this study were of analytical grade. At the end of extraction, the solvent was removed with a rotary evaporator (RV 10 digital, Ika, Wilmington, USA) and then held in an oven in 50°C for 24 hours to remove residual solvent. The extract amounts were weighed in an analytical balance to calculate the extraction yield (wt%), as follows:

$$Extracion\ yield\ (\%) = \left(\frac{mass\ of\ extrated\ oil\ (g)}{mass\ of\ sample\ (g)}\right)\ x\ 100 \qquad (1)$$

Where the mass of the sample was expressed on a dry basis, taking into account the moisture content in all samples used before the extraction.

4.6 SUPERCRITICAL EXTRACTION

The supercritical extraction process was performed in a home-made laboratory scale extraction unit (batch extractor with 80 cm^3 internal volume, L = 0.16 m, ϕ = 2.52 x 10^{-2} m) as described in (CORREA *et al.*, 2016). Basically, the high-pressure extractor is connected to an ultrathermostated bath and a needle valve is used for the flow control at the outlet stream of the extractor. The system also consists of a high-pressure syringe pump (ISCO, model 500D, Lincoln, NE 68504, USA), pressure and temperature sensors and transducers.

The extractions were performed using a liquid solvent modifier in a semi-batch fixed bed extraction process, using supercritical CO_2 as the solvent and ethanol as liquid solvent, named as scCO_2 + EtOH. The experimental procedure consisted of soaking the raw material in the solvent ethanol using a 1:1 (w/w) ratio kept the mixture static for 60 min. The mixture was loaded into the extraction vessel, and CO_2 laden until the pressure reached the extraction condition. Then, mixture containing the raw material (fermented solids) with ethanol and CO_2 was kept in a confinement period (static extraction step) for 60 min to assure the thermal and mechanical equilibration before one start the dynamic extraction period.

scCO_2+EtOH extractions were performed based on a 2^2 factorial experimental design with a center point, aiming to evaluate the effects of temperature from 40 to 80°C and pressure from to 15 a 25 MPa on the extraction yield and the quality of the extract. The compressed solvent (supercritical CO_2) was pumped at a constant flow rate of 2.0 ± 0.3 cm^3 min^{-1}.

The extracts were collected in amber glass vessels and its weight determined at each extraction time intervals of 5 min.

4.7 ANALYTICAL PROCEDURES

4.7.1 Measurement of DPPH radical-scavenging activity

The antioxidant activity of the extracts was assessed based on the radical scavenging effect of the stable DPPH-free radical activity (1,1-diphenyl-2-

picryhydarzyl) (Sigma-Aldrich, USA), as described by (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). A methanol solution of DPPH (0.004% w/v) was prepared. One milliliter of methanolic DPPH solution was added to 250 μL sample extracts at different concentrations (50; 100; 150; 200; 250 μL μL^{-1}). After incubation in the dark at ambient temperature for 30 min, the absorbance was measured at 517 nm (Spectrum band, UV-VIS). Ethanol solution without the sample was considered as a control. IC_{50} value (index representing the concentration of the antioxidant which can reduce 50% of free radicals) was obtained using a standard curve. The inhibition rate (%) was calculated to the following equation:

$$Inibição\ (\%) = \left(\frac{A_{controle} - A_{extrato}}{A_{controle}}\right) x\ 100 \qquad (2)$$

Where:

$A_{control}$ = DPPH absorbance (diluted with ethanol solution)

$A_{extract}$ =DPPH + sample extract absorbance (diluted with ethanol solution)

Percent antioxidant activity was plotted against log absorbance values, generates a straight line to calculate the half-inhibitory concentration (IC_{50}) in g g^{-1}. The anti-free radical activity (expressed as IC_{50}) is defined as the amount of antioxidant substances needed to reduce 50% of the initial concentration of DPPH. The higher the power anti-free radical, the most effective antioxidant and consequently lower the IC_{50} value (BRAND-WILLIAMS; CUVELIER; BERSET, 1995). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, USA) was used as standard, prepared in concentrations from 1 to 100 μm of trolox mL^{-1}. The results comparing the equivalence of the antioxidant potential of the extracts with the standard potential were expressed.

4.7.2 Determination of total phenolic content

Total phenolic content of the extracts was determined by the Folin-Ciocalteu method (SWAIN; HILLS, 1959) with modifications. This test is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. After oxidation, the absorbance of the green-blue complex formed was measured at 760 nm. In a test tube, 0.1 mL of the sample was mixed with 0.5 mL of Folin-Ciocalteau reagent (10% v/v) and 0.4 mL of sodium carbonate (7.5% w/v). Samples were shaken for 1 h protected from light (reaction time). Ethanol was used as blank. The samples were analyzed in triplicate. A standard curve prepared using gallic acid monohydrate was used to calculate the total phenol content, which was expressed as gallic acid equivalent in μg g^{-1} of the extract (Singleton, 1965).

4.8 DETERMINATION OF Β-GLUCOSIDASE ACTIVITY.

β-glucosidase activity of ethanol extracts (0, 24, 48 and 72 h) was assayed by monitoring the release of p-nitrophenol (pNP) using p-Nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate, according to the method described by Zhang et al., 2012. A total of 20 μl of appropriately diluted enzyme solution and 280 μl of 1.785 mM pNPG in 100 mM acetate buffer pH 4.8 were incubated for 15 min at 50 °C. The reaction was quenched by adding 400 μl of glycine buffer, and the amount of ρnitrophenol released during the reaction was determined with a spectrophotometer at 430 nm. A ρ-nitrophenol calibration curve was previously prepared to calculate the enzyme activity. One unit of β-glucosidase activity was defined as the amount of enzyme equivalent to the release of 1 μmol of p-nitrophenol per minute.

4.9 DETERMINATION OF FATTY ACIDS PROFILE

Fatty acid profile of fermented soybean oil was analyzed using a Shimadzu chromatograph (GC 2010 *Plus*), a capillary column (SH-Rtx-Wax (Shimadzu): 30 m x 0.32 mm x 0.25 µm), flame ionization detector (FID) and split injection mode (1:10). The injector and detector temperatures were 240 °C and 250 °C, respectively. The oven temperature was programmed to start at 100 °C during 5 min, followed by an increase up to 240 °C at a rate of 4 °C min^{-1} and maintained in this temperature for 5 min. The carrier gas was Helium at 32.5 cm^3 min^{-1}. The samples were prepared according official method (AOCS, 1998) to convert triacylglycerol and free fatty acid of samples into fatty acid methyl esters (FAMEs). FAMEs were identified by comparison with retention times of the standard mixture FAMEs (Supelco, MIX FAME 37, St. Louis, MO 63103, USA). The quantification of fatty acid was conducted by normalization area procedure. Results were expressed as percentage of each individual fatty acid present in the sample.

4.10 0IDENTIFICATION AND QUANTIFICATION OF ANTIOXIDANT COMPOUNDS BY RP-HPLC-DAD/UV

The ethanol extracts (0, 48 and 72 h) were filtered with Spritzen syringe filter of 0.22 µm (Millipore, Bedford, MA). The filtrate was used to quantify the polyphenolic compounds by High-performance liquid chromatography with UV–vis detection (HPLC-DAD/UV–vis). HPLC analysis was performed according to (Haminiuk et al., 2014), with a reversed phase Acclaim® 120 column, C18 5µm 120 Å (4.6 mm×250 mm). The column was maintained at 40 °C and the detection was recorded at 280, 300 and 320 nm. Polyphenolics and flavonols were detected in the range of 210 and 320 nm. The mobile phase was acidified water with phosphoric acid 1 % and methanol. The solvent gradient was as follows: 0–15 % B for 2 min, 15–25 % B for 5 min, 25–30 % B for 10 min, 30–35 % B for 15 min, 35–50 % B for 25 min, 50–60 % B for 30 min, 60–80 % B for 35 min, 80–100 % B for 45 min and 100–5 % B for 60 min. Flow rate: 1 mL min^{-1}. Standards used: for phenolic acids (gallic acid, chlorogenic acid, trans-cinnamic acid, caffeic acid, p-coumaric acid, ferulic acid) and for flavonols (rutin, myricetin, quercetin and kaempferol). Stock solutions of all standards were prepared in methanol and the calibration curves were obtained from triplicate injections of at least five concentrations and identification based on the retention times of the standards (Granato et al., 2011) in chromatogram at 280 nm. Retention times (min) for the standards at the conditions used were: gallic acid: 6.83 min; caffeic acid: 32 min; *p*-cumaric acid: 18.54 min; ferrulic acid: 20.07 min; rutin: 25.03 min; myricetin: 28.22 min; trans-cynamic acid: 32.40 min; kaempferol: 37.08 min.

4.11 HIGH – PERFORMANCE LIQUID CROMATOGRAPHY OF PHENOL CONTENTS (HPLC)

The phenolic content of the extracts was analyzed in high performance liquid cromatography using an Agilent Technology 1200 Series system, coupled to a diode array detector (DAD) at wavelengths 235, 260, 275, 280, 290, 311, 357, 370 nm and a scanning from 190 nm to 600 nm. A Zorbax Elipse XDB – C 18 (4.6 x 150 mm, 5 – micron) column was used at 0.7 mL min-1 flow. The mobile phase utilized was methanol and acetic acid 2.5% (50:50 v/v). Chlorogenic acid, caffeic acid, ferulic acid, tocopherol, genistein, daidzein, transcinnamic acid, catechin, rutin, p-coumaric acid, gallic acid, resveratrol and epicatechin (SIGMA Aldrich, EUA) was used as standards. To obtain the calibration curve, all standard reagents (SIGMA Aldrich, EUA) were solved in mobile phase and used at 1, 2, 5, 8 and 10 ppm. The injection volume was 10 µL and the run time was 36 min. The resulting chromatograms values were graphed, and linear equation was used to calculate the phenolic compounds contents of the samples. The samples were microfiltered trough a hydrophilic membrane GV (Durapore) made of polyvinylidene difluoride (PVDF), with a pore size of 0.22 µm. The results were showed in µg g^{-1} of the phenolics compounds identified.

4.12 EVALUATION OF ANTI-INFLAMMATORY ACTIVITY

In order to evaluate the anti-inflammatory activity with inhibition of the hyaluronidase enzyme, an *in vitro* method was used according to (STROMINGER; LELOIR; REISSIG, 1955). The anti-inflammatory potential was measured at concentrations of 0.5 to 2.5 µg mL^{-1}. For the sample analysis, was placed 50 µL of extract in different concentration and 0.5 mL of potassium salt of hyaluronic acid (Sigma-Aldrich, St. Louis, MO, USA) (1.2 mg hyaluronic acid per mL of 0.1 M acetate buffer, pH 3.6, containing 0.15 M NaCl) in a reaction tube. The control tube consisted of the same reagent of test tubes without ExPP. All tube were incubated for 5 min at 37 °C and after that 50 µL of the enzyme hyaluronidase (350 units of the enzyme hyaluronidase type IV-S from bovine *testes*, Sigma-Aldrich, St. Louis, MO, USA - dissolved in the same buffer substrate at concentration 6.5 mg/mL) was added, and incubated at 37 °C for 40 min. The reaction was stopped by adding 10 mL of 4 N sodium hydroxide solution and immediately placing 0.1 mL of 0.8 M potassium tetraborate into the reaction mixture and incubating it in a boiling bath for 3 min. After the incubation time, we added 3 mL of 4-dimethylaminobenzaldehyde (DMAB) (10% solution in glacial acetic acid containing 12.5% 10 N hydrochloric acid) to the reacted mixture and incubated the solution at 37 °C for 20 min. Next, we measured the samples in a spectrophotometer (SP 2000 UV Spectrum) at 585 nm. DMSO was used as positive control due to its ability to completely inhibit de hyaluronidase enzyme. Propolis, a natural anti-inflammatory agent, was also included as a positive control. The results were expressed as the ability to inhibit the hyaluronidase enzyme, in percentage.

4.13 CYTOTOXICITY ASSESSMENT FOR TUMOR (CACO-2) AND NORMAL (MCF-10) CELLS BY COLORIMETRIC ASSAY MTT (1- (4,5-DIMETHYLTHIAZOL-2-YL) -2,5-DIPHENYL TETRAZOLIUM)

The cell lines CaCo-2, which is Colorectal Cancer, and MCF-10 A, which is a non-tumoral breast epithelial cell, were used to perform the assays with ethanol extracts from *R. oligosporus* NRRL 2710 fermentation of SB + BR. The ethanol extracts were first lyophilized for subsequent resuspension. Both cell lines were purchased FROM the Cell Bank of Rio de Janeiro, Brazil, passage 33. The lines were cultured in a 96-well plate in DMEM F12 medium with 1% penicillin/streptomycin for 24 h for cell adhesion. CaCo-2 cells were plated at 5 x 10^4 cells per well and MCF 10A cells at the concentration of 3 x 10^4 cells per well. After the 24 h incubation period, the medium was monitored by DMEM F12 with 10% penicillin/streptomycin and supplemented with 10% fetal bovine for both species. Plates were maintained in an incubator at 37 ºC, 95% air atmosphere and 5% CO_2 for 48 h.

Cell viability was analyzed by colorimetric assay MTT (1- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium), described by (MOSMANN, 1983). The MTT solution

(Sigma-Aldrich) was prepared at the concentration of 5 mg ml^{-1} and added to the culture medium, where the final concentration in the well was 0.5 mg ml^{-1}. After 3 h of incubation at 37°C, 95% air atmosphere and 5% CO_2, the blue crystals formed were solubilized in DMSO (Panreac®), considered the most effective solvent to be capable of dissolve the formazan crystals easily. The reading was performed in a spectrophotometer (Biotek) at 595 nm.

4.14 DETERMINATION OF OXIDATIVE STABILITY

The thermogravimetric curves were obtained on a Perkin-Elmer thermogravimetric analyzer model TGA 4000 model. The temperature range was 30 °C to 800 °C and the heating rate was 10 °C min^{-1}. A dynamic atmosphere of synthetic air was used at a flow rate of 50 mL min^{-1}. Ceramic pots and initial sample masses ranging from about 8 to 10 mg were used in the apparatus.

4.15 STATISTICAL ANALYSIS

Analysis results were submitted to the statistical variance analysis and the post-test was chosen according to the normality of the data obtained such as Tukey Post-Test for data which follow normality. The program PRISMA® (GraphPad Prism 5 for Windows, version 5.4) was used. The averages of data are presented in graphs and the standard deviation shown by the error bars.

5 RESULTS AND DISCUSSION

In this section, the experimental results of *Rhizopus spp*. fermentation using different substrates as well as the effect of different extraction methods are presented. First, the influence of using different substrates on the alcoholic extract fermentation process is presented and the optimal point of bioactive compound production is evaluated by kinetics. The anti-inflammatory and anti-tumor potential of these extracts is also shown.The results of these studies are published in the journal Biointerface Research in Applied Chemistry (Topics 5.1 – 5.6).

Then, the experimental results are presented when the extraction methods applied were Soxhlet and supercritical CO_2 and the results when ethanol was used as a co-solvent in the process ($scCO_2$ + EtOH). These researches focus on these extractions with the aim of making a comparison between the results obtained and finding the best solvent for a given matrix. The results of this study are being published (Topics 5.6 – 5.11).

5.1 SUBSTRATE SELECTION FOR ANTIOXIDANT BIOSYNTHESIS

In Asian countries, the genus *Rhizopus spp.* is mentioned as one of the economically important molds due to its role as an inoculum source for the preparation of a traditional soy-based food, tempeh (Hesseltine, 1983; Astuti et al., 2000; Hartanti et al., 2015).

In preliminary studies *Rhizopus spp.* strains were grown on soybeans supplemented with different cereals (brown rice, wheat, corn and oat), aiming to achieve higher antioxidant activity. All supplementation processes were efficient in increasing the antioxidant power after 72h. Therefore, the best results ($p<0.05$) were achieved when soybean (SB) was supplemented with brown rice (BR), resulting in the greatest antioxidant potential for *R. oligosporus* NRRL 2710 (IC_{50} 14.3 mg mL^{-1}) (Table 4) (PRADO *et al.*, 2021).

TABLE 3. IC_{50} VALUES (MG ML^{-1}) DURING FERMENTATION IN SB ETHANOL EXTRACTS AND GRAIN MIXTURES AT THE STARTED POINT (T_0) AND AFTER 72 HOURS OF FERMENTATION

		IC_{50} (mg mL^{-1})	**Phenolic compounds (mg GAE g^{-1})**
SB	T_0	105.01 ± 0.62^{aA}	1.792
	72 h	20.69 ± 0.27^{aA}	3.10
SB + BR	**T_0**	**374.5 ± 0.61^{dA}**	0.697
	72 h	**14.3 ± 0.13^{aA}**	6.447

* Means followed by the same letter did not differ statistically ($p > 0.05$).
SOURCE: The author (2022).

In the literature is vague a data on the actual increase in scavenger activities due to the fermentation process. (HUANG; LAI; CHOU, 2011) found 19.1 mg mL^{-1} scavenging activity of fermented soybean + rice by *Aspergillus oryzae* after 16 days for sufu production, but the increment of the values in the course of the fermentation was not reported. The data of the present study clearly show the real increment in scavenging activities promoted by the fermentation process.

Also, soy supplementation with brown rice increased the concentration of phenolic compounds (0.697 to 6.447 mg GAE g^{-1}) significantly compared with the only use of soybean (1.792 to 3.10 mg GAE g^{-1}), using *R. oligosporus* NRRL 2710 (Table 4) (PRADO *et al.*, 2021). For this reason, the SB supplemented with BR and fermented by are selected for further studies (published data).

5.2 IDENTIFICATION AND QUANTIFICATION OF POLIPHENOLIC COMPOUNDS

The identification and respective concentrations of polyphenolic compounds synthetized by R. oligosporus NRRL 2710 under SSF of SB and SB+BR were shown in table 5, as quantified by RP- HPLC-DAD/UV. Compounds such as gallic acid, ferrulic acid, rutin, miricetin, caffeic acid, trans-cinnamic acid, quercetin, kaempferol, siringic acid and p-cumaric acid were detected. These compounds have been previously found in SSF by different fungi species (ALEJANDRA *et al.*, 2019; HIROTA *et al.*, 2005; OLMOS; GARRO, 2020; ZHANG *et al.*, 2012).

TABLE 4. IDENTIFICATION AND QUANTIFICATION IN GRAM DRY BASES (MG GDB^{-1}) OF POLYPHENOLIC AT THE ETHANOLIC EXTRACTS DURING SOLID STATE FERMENTATION OF SOYBEAN MIXTURE WITH BR SYNTHESIZED BY *R. OLIGOSPORUS* NRRL 2710.

Substrates	**Concentrations (µg mL^{-1})**	**Fermentation time (h)**	
		0	72
SB	Gallic acid	0.2	0.0
	Ferrulic acid	0.3	0.3
	Rutin	29.3	8.7
	Miricetin	9.8	7.4
	Caffeic acid	0.0	0.0
	Trans-cinnamic acid	0.0	124.7
	Quercetin	0.0	0.2
	Kaempferol	0.0	0.1
SB + BR	Gallic acid	0.0	0.1
	Ferrulic acid	0.1	0.2
	Rutin	11.8	2.9
	Miricetin	6.5	7.4
	Caffeic acid	0.0	0.0
	Trans-cinnamic acid	19.8	103.3
	Quercetin	0.0	0.0
	Kaempferol	0.0	0.1
	Siringic acid	4.0	0.0
	p-cumaric acid	0.1	0.0

SOURCE: The author (2022).

Trans-cinnamic acid was the compound present in higher amounts, reaching 124.7 µg mL^{-1} (3,197 mg gdb^{-1}) and 103.3 µg mL^{-1} (2,649 mg gdb^{-1}) in SB and SB+BR fermented after 72 h, respectively. There are few reports on cinnamic acid production by fermentation process (NODA *et al.*, 2011). The cinammic acid or trans-3-

phenylacrylic acid is biosynthesized by the enzyme phenylalanine ammonia lyase (PAL), which converts the aminoacid phenilalanine in cinnamic acid and is an important precursor of the biosynthesis of other important groups of polyphenolic compounds in the microrganism methabolism pathway. Cinnamic acid has been studied as an anti-tumoral compound, which induces tumor cell differentiation by modulating the expression of genes implicated in tumor metastasis and immunogenicity in cultured human melanoma cells. This work indicates a great potential for producing this compound due to the high accumulation in the fermented substrate. The separation and concentration of cinammic acid from SB +BR fermented by *R. oligosporus* NRRL 2710 represents a great perspective for application in food and supplement products.

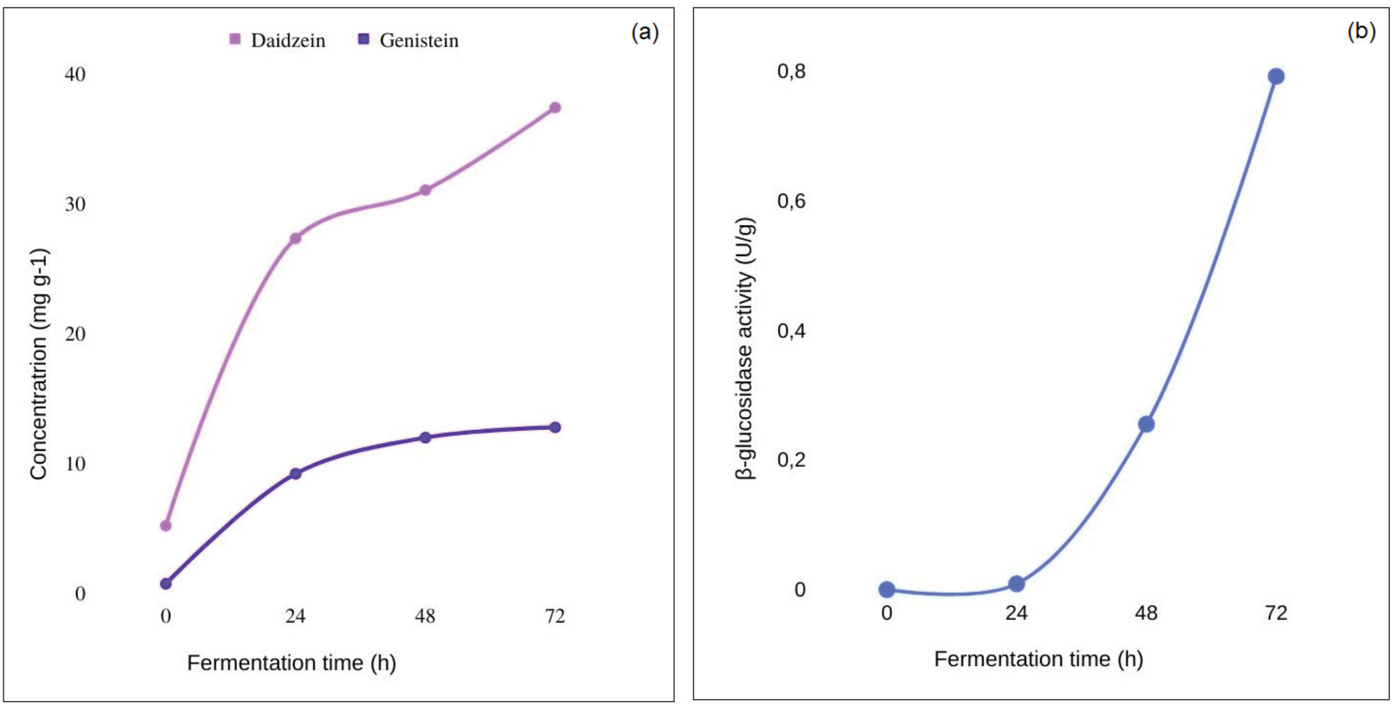
5.3 CHANGES IN THE ISOFLAVONE PROFILE DURING FERMENTATION

The isoflavones consist of a subclass of flavonoids with powerful antioxidant activity and linked to cancer prevention (BENEDETTI *et al.*, 2015). Soybeans is a known source of these compouns and isoflavones in the glycoside form (daidzin and genistin) are more present than aglycone form (daidzein and genistein). Fermentation of soybeans promotes the conversion of glycoside into aglycone isoflavones through the action of β-glucosidase produced by microorganism (DA SILVA; CELEGHINI; CHANG, 2011; MARAZZA *et al.*, 2012). All isoflavones are absorbed into the mucosa of the small intestine. However, the aglycone form is absorbed at a greater rate and has higher antioxidant activity than the glycoside form (HONG; MANDAL; LIM, 2012). In this study, the biotransformation of isoflavones during fermenttaion of SB+BR by *Rhizopus oligosporus* NRRL 2710 was studied using high-performance liquid Chromatography (HPLC) and β-glucosidase activity using spectrophotometry (Figure

5a). The concentrations of gaidzein and genistein (aglycone form) in the final fermented product (18.65 ± 0.08 mg g^{-1} and 12.68 ± 0.05 mg g^{-1}, respectively) were 3.75 and 20.10 fold higer, respectively, compared to non-fermented soybeans.

Simultaneously with the accumulation of aglycone isoflavones, β-glucosidase increased significantly after 24 h of fermentation (ranging from 0.01 to 0.81 U/g at the end of the process) (Figure 5b), being thus responsible for catalyzing aglycone isoflavones from glycine form. The values found in this study are significantly higher than those observed by (LEE *et al.*, 2019) (0.24 mg g^{-1} and 0.043 mg g^{-1} for daidzein and genistein, respectively) after 72 h of soybean fermentation using *Tricholoma matsutake*, and by (SANTOS *et al.*, 2018) (1.084 mg g^{-1} and 1.810 mg g^{-1} of daidzein and genistein, respectively) after 72 h of okara fermentation with Saccharomyces *cerevisiae.*

FIGURE 4 - B-GLUCOSIDASE ACTIVITY (U/G) AND ISOFLAVONES AGLYCONE CONTENT (MG G^{-1}) (DAIDZEIN AND GENISTEIN) DURING SOYBEANS PLUS BROWN RICE FERMENTATION



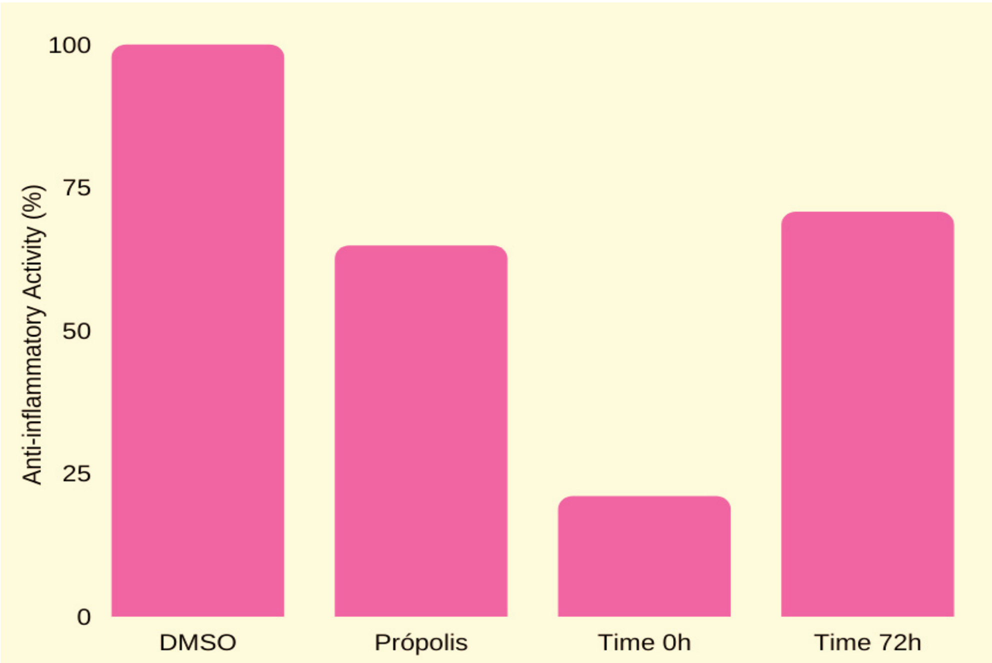
SOURCE: The autor (2022).

5.4 ANTI-INFLAMMATORY ACTIVITY

The anti-inflammatory activity of the *R. oligosporus* NRRL 2710 ethanolic extract was assessed by hyaluronidase inhibitory activity, these enzyme acts on the extracellular matrix of the tissues, increasing the permeability and facilitating the access of pathogens involved in inflammatory processes (EL-SAFORY; FAZARY; LEE, 2010; GIRISH; KEMPARAJU, 2007; SGARIGLIA *et al.*, 2013).

The results obtained shown the fermentation process increased hyaluronidase inhibitory activity 3.36 times when compared to unfermented soybeans (70.75% and 21.03% inhibition capacity for fermented and unfermented soybeans, respectively). In addition, showed significant higher activity than propolis, a commercial anti-inflammatory product(Figure 6).

FIGURE 5. ANTI-INFLAMMATORY ACTIVITY OF ALCOHOLIC EXTRACT OF THE *R. OLIGOSPORUS* NRRL 2710 CULTIVATED IN SOYBEAN SUPPLEMENTED WITH BROWN RICE BY 72 H.

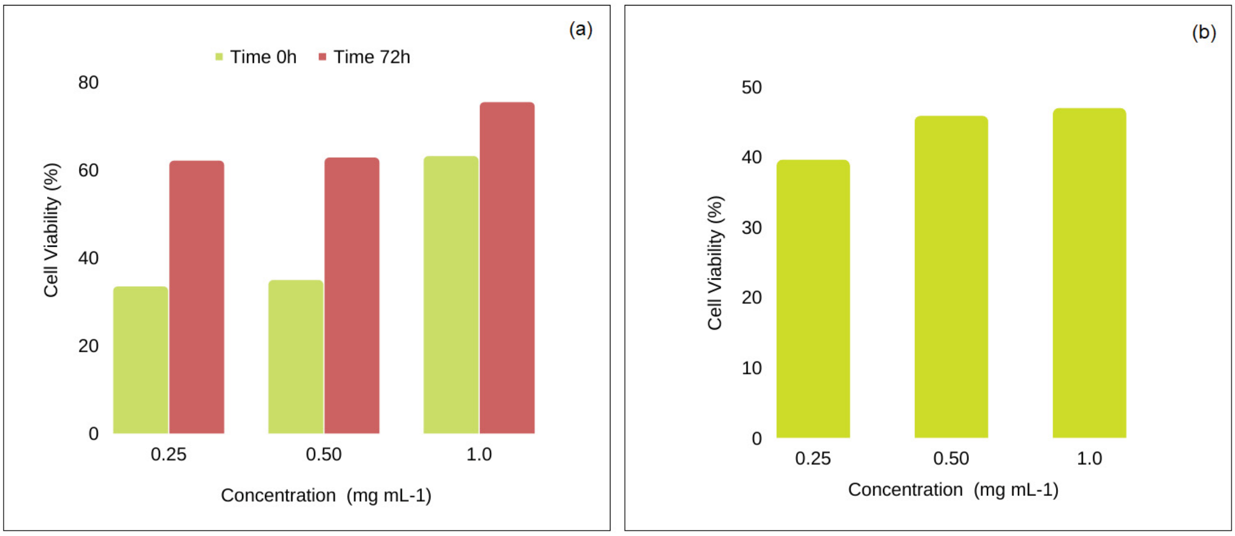


*DMSO: positive control capable of completely inhibiting the enzyme hyaluronidase (HIA); Propolis: commercial anti-inflammatory
SOURCE: The autor (2022).

5.5 CYTOTOXICITY ASSESSMENT FOR TUMOR (CACO-2) AND NORMAL (MCF-10) CELLS

The decrease of viable cells at levels below 50% demonstratred by the values of cytotoxicity assessment for both unfermented soybeans (0h) and fermented substrate (72 h) showed cytotoxicity for normal cells (MCF-10). According to statistical analysis, these extracts presented no significant difference at the 95% level ($p < 0.05$). Considering this results, a cell viability study was carried out with CaCo-2 cancer cell to evaluate the cytotoxicity of the 72h *R. oligosporus* NRRL 2710 fermented ethanolic extract at different concentrations (Figure 7b). The extracts presented percentage of cellular viability between 39.53 and 46.89%.

FIGURE 6. CYTOTOXICITY ASSESSMENT OF ALCOHOLIC EXTRACT OF THE R. OLIGOSPORUS NRRL 2710 CULTIVATED IN SOYBEAN SUPPLEMENTED WITH BROWN RICE AGAINST (a) NORMAL CELLS (MCF-10) AND (b) CACO-2 TUMOR CELLS.



SOURCE: The autor (2022).

The inhibitory concentration values (IC_{50}) were 0.27 mg mL^{-1} for CaCo-2 cells and 0.79 mg mL^{-1} for normal MCF-10 cells, indicating a higher cytotoxic effect against carcinogenic cell. A lower percentage of cancer cell viability was observed at lower concentrations, showing that high concentrations are not required to attain cancer cell death. According to the statistical analysis, the extracts at different processing times showed a significant difference at the 95% level ($p < 0.05$), suggesting that the *R. oligosporus* NRRL 2710 SB+BR fermentation process was efficient; the lower value of cell viability of the cancer cells was obtained after 72 h of fermentation (39.53%).

Several studies show the inverse association between the consumption of soy products and the risk of degenerative diseases, such as cancer (SÁNCHEZ-CHINO *et al.*, 2015; ZHU *et al.*, 2015). The chemopreventive effects of soy are attributed to its bioactive molecules, such as isoflavones, which interact with the metabolic pathways responsible for controlling cell growth, proliferation and differentiation (DE MEJIA; DIA, 2010; DIA; DE MEJIA, 2011; MONTALES *et al.*, 2015; TSAI *et al.*, 2010).

5.6 SOXHLET EXTRACTION

The identification and quantification of bioactive compounds in natural sources can be performed by extraction with solvents of different polarities.

In this study, the results from soxhlet extraction with different organic solvents showed an increase in yield as the polarity of the solvent increased. The highest extraction yields were 44.10% and 45.24%, for ethanol and methanol, respectively, suggesting that the compounds present in the matrix studied have intermediate to high polarity (Table 6). Statistical data show that these values did not differ significantly ($p > 0.05$).

TABLE 5 - RESULTS OF EXTRACTION YIELD (%) OF THE SOXHLET EXTRACTION PROCESS WITH FERMENTED SOYBEAN DRIED AND WHOLE (WITHOUT DRYING) SAMPLES USING DIFFERENT SOLVENTS.

Run	Solvents	T (°C)*	Polarity	Extraction Yield (wt%)	
				Dry sample	**Wet sample**
S1	Hexane	68.0	0.0	15.56±4.02[c]	-
S2	Petroleum ether	34.61	0.1	29.05±0.51[a]	-
S3	Ethyl acetate	77.5	4.3	32.04±2.14[ab]	-
S4	Acetone	56.0	5.4	34.07±0.68[ab]	19.76±1.05[ab]
S5	Ethanol	78.5	5.2	44.10±1.99[b]	25.48±4.25[bc]
S6	Methanol	64.7	6.6	45.24±3.70[b]	32.84±3.70[c]
S7	Water	100.0	10.2	23.66±4.32[a]	14.20±2.82[a]

* Boiling point of solvents. Means followed by the same letter do not differ statistically ($p > 0.05$)

SOURCE: The autor (2022).

The moisture levels in the raw material influence the extraction kinetics and yield since water is an interfering factor in the penetration of the solvent into the solid matrix and in the diffusion of the oil. Extractions with solvents from medium to high polarity were performed using dry sample; the results in Table 1 show yields of 25.48% and 32.84% for ethanol and methanol, respectively. It was observed that the drying process allowed a significant increase of up to 18.62% in extraction yield using ethanol. Previous studies also reported the optimization of extraction with lower moisture content, stating that the drying process facilitates the contact between the solvent and the solute to be extracted, resulting in higher yields (PIGHINELLI *et al.*, 2009; SANTOS *et al.*, 2013; TANGO; CARVALHO; SOARES, 2004).

The results in Table 6 show yields of 25.48% and 32.84% for ethanol and methanol, respectively. It was observed that the drying process allowed a significant increase of up to 18.62% in extraction yield using ethanol. Previous studies also reported the optimization of extraction with lower moisture content, stating that the drying process facilitates the contact between the solvent and the solute to be extracted, resulting in higher yields (PIGHINELLI *et al.*, 2009; SANTOS *et al.*, 2013; TANGO; CARVALHO; SOARES, 2004).

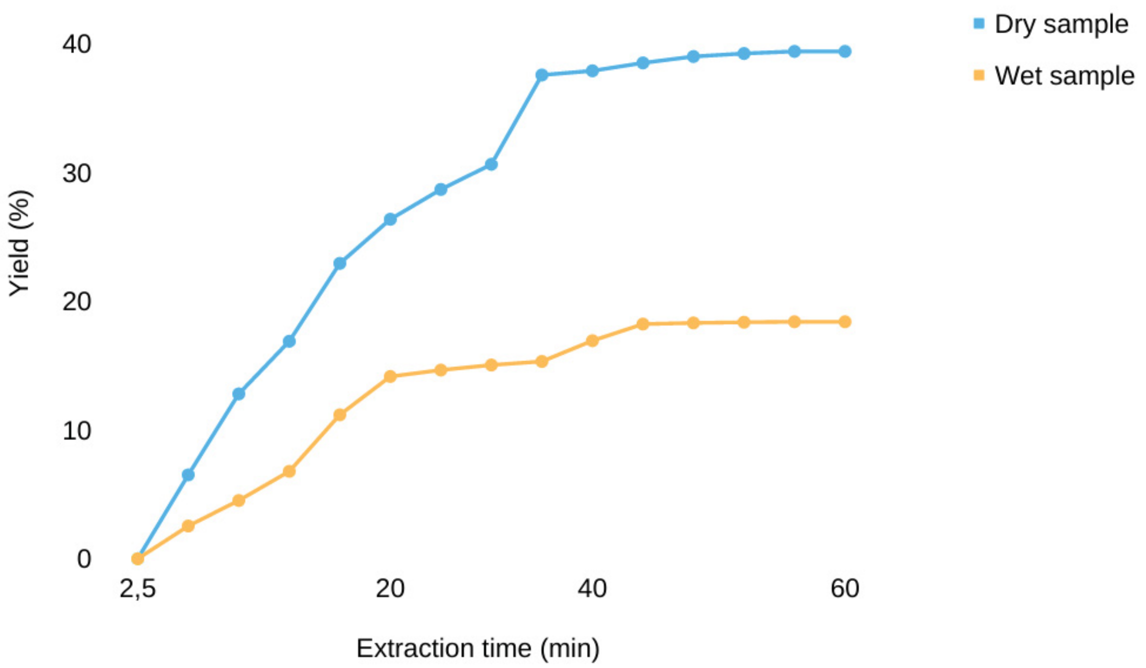
Water as a solvent in soxhlet extraction results in a higher yield. Although, the use of the high boiling temperature can lead to the degradation of bioactive compounds, such as polyphenols (BAHRIN *et al.*, 2018). Ethanol, besides providing greater extraction among the organic solvents due to its high polarity, also comprises low cost, lower toxicity and risks to human health and the environment. This way ethanol becomes more desirable than methanol for applications and was, therefore, selected as the liquid solvent for further extraction procedures.

5.7 EXTRACTION BY COMPRESSED SOLVENTS

For comparative purposes of the extraction techniques, supercritical CO_2 extractions were performed. Initially, an experiment was performed using supercritical CO_2 at 80 °C and 25 MPa, in which the extraction yield obtained was extremely low (<1%). Therefore, we chose to perform all additional extractions using the combined solvents $scCO_2$ + ethanol. For to promote and improve the contact between the solvent and the matrix before the compressed solvent extractions a preliminary pretreatment of the feedstock with ethanol was performed. After some tests, it was defined that 60-minute of contact was a suitable condition for this pretreatment. Raw material-ethanol contact can alter the matrix by swelling, thereby facilitating the analytes transport from interstitial pores to the surface and then to the bulk phase (TIM *et al.*, 1998).

A preliminary test using the described conditions was performed with dry and wet samples to evaluate the influence of humidity on $scCO_2$ + EtOH extraction. This same behavior was observed in the results obtained by Soxhlet extraction, in this case the drying process of the samples positively influenced the extraction yield resulting in an increase of 2.14 times in the procedure performed with dried samples.

FIGURE 7 - KINETICS OF DRY AND WET EXTRACTION OF FERMENTED SOYBEANS WITH CO_2 + ETOH AT A CONSTANT TEMPERATURE OF 80 °C AND PRESSURE OF 25 MPA EXPRESSED



SOURCE: The autor (2022).

A factorial design 2^2 with a central point was performed to verify the influence of the process parameters, such as pressure and temperature. The results obtained are shown in Table 7.

TABLE 6. EXPERIMENTAL CONDITIONS AND EXTRACTION YIELD USING $SCCO_2$ + ETOH AS SOLVENT CONSIDERING DRIED FERMENTED SOYBEAN RAW MATERIAL.

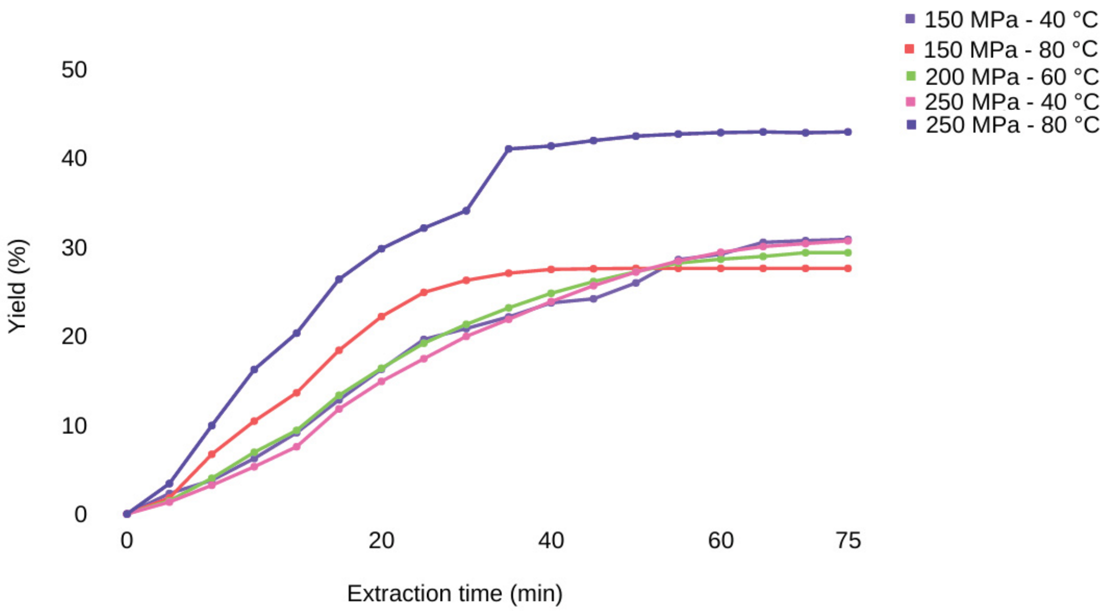
Run (M_{RM})	Solvent	P (MPa)	T (°C)	Time (min)	Extraction yield (wt%)
1	CO_2+ EtOH	15	40	75	30.81
2	CO_2+ EtOH	15	80	75	27.56
3	CO_2+ EtOH	25	40	75	30.65
4	CO_2+ EtOH	25	80	75	42.87
5	CO_2+ EtOH	20	60	75	29.32±1.78

SOURCE: The autor (2022).

All $scCO_2$ + ethanol experiments were performed using static extraction time, called confinement period, a 60-minute. The highest extraction yield (42.87 wt%) was obtained at the highest condition of pressure and temperature, 25 MPa and 80 °C. The comparison of runs 2 and 4 showed that changing the pressure, at 80°C, increased the yield to 15 percentual points (p.p.). Varying the temperature at a fixed pressure of 25 MPa, an increase in yield by 12 p.p. was observed. In general, the addition of ethanol as polarity modifier to $scCO_2$ showed to be an efficient approach to lead the extraction yields to levels compared to extraction in Soxhlet (Table 6).

The general extraction curves are provided and discussed in Figure 9 to better understanding the extraction using $scCO_2$ + EtOH. The operational parameters studied affect significantlyd the yields obytained. The parameters pressure and temperature had a positive effect on the yield because, at higher levels (80 °C and 25 MPa), they allowed higher yield values. The values obtained at different conditions presented a significant difference ($p > 0.05$).

FIGURE 8 - OVERALL EXTRACTION CURVES FOR DRIED FERMENTED SOYBEAN USING $SCCO_2$ + ETOH AS SOLVENT.



SOURCE: The autor (2022).

The lowest yield (27.56%) can be observed when using the lowest pressure (15 MPa) and highest temperature (80 °C). Pressure is the parameter that positively influences the yield of $scCO_2$ so that, its increasing reflects on the increase of density, viscosity and solvation power of CO_2 (AHANGARI; SARGOLZAEI, 2013; AKGÜN *et al.*, 2014). Therefore, the lower yield is probably a reflection of the low solubility of the extract since the lower CO_2 density results in low mass transfer rates during extraction (JOKIĆ *et al.*, 2011; MESOMO *et al.*, 2012).

The overall extraction curves in Figure 9, reveal that up to 40 minutes there were high initial extraction rates and a slope of the curve in the subsequent minutes. This indicates that there was first a period of dynamic extraction rate, where the outer surface of the particles is covered with the easily accessible and available solute. Then, the $scCO_2$ stops operating as a carrier and starts operating as an extraction agent and the mass transfer occurs by diffusion within the solute particles, resulting in the extraction rate dropping (JUCHEN *et al.*, 2019).

Among the methods mentioned, the Soxhlet extraction using ethanol and methanol as solvents showed a higher yield (44.10 and 45.24%, respectively) compared to $scCO_2$, where the maximum yield was obtained under different extraction conditions was 42.87%. However, $scCO_2$ extraction offers an advantage less time consuming than Soxhlet extraction.

5.8 FATTY ACIDS PROFILE

Table 8 shows the fatty acid (FA) profiles for the fermented soybean oil samples obtained from the different methods studied. The FA profiles have presented similarities, despite the different extraction conditions and solvents used.

TABLE 7 - FATTY ACID COMPOSITION OF FERMENTED SOYBEAN EXTRACTS OBTAINED FOR SOXHLET EXTRACTION USING DIFFERENT SOLVENTS AND $SCCO_2$+ETOH UNDER DIFFERENT CONDITIONS.

Run	Extraction condition	Composition (%)						
	Soxhlet	**Palmitic (C16:0)**	**Stearic (C18:0)**	**Oleic (C18:1)**	**α-Linolenic (C18:3)**	**Linoleic (C18:2)**	**Gondoic (C20:1)**	**Tricosanoic (C23:0)**
S1	Hexane	11.35^d	4.33^b	31.86^e	5.47^c	46.32^f	-	0.67^a
S2	Petroleum ehter	12.11^d	4.49^b	30.75^e	5.49^c	46.04^f	0.51^a	0.61^a
S3	Ethyl acetate	11.53^d	4.17^b	30.94^e	5.62^c	46.52^f	0.59^a	0.62^a
S4	Acetone	11.58^d	4.24^b	30.83^e	5.59^c	46.66^f	0.45^a	0.64^a
S5	Ethanol	13.27^d	4.88^b	29.96^e	5.23^c	45.22^f	0.62^a	0.81^a
S6	Methanol	12.37^d	4.50^b	30.21^e	5.64^c	46.14^f	0.45^a	0.69^a
S7	Water	14.68^a	-	33.41^b	-	51.92^c	-	-
	$scCO_2$+EtOH							
1	15 MPa:40°C	11.27^d	4.57^c	37.34^e	4.27^b	41.45^f	0.51^a	0.59^a
2	15 MPa:80°C	11.28^d	4.69^c	37.24^e	4.17^b	41.38^f	0.59^a	0.64^a
3	25 MPa:40°C	10.98^d	4.55^c	37.25^e	4.26^b	41.93^f	0.50^a	0.54^a
4	25 MPa:80°C	11.31^d	4.63^c	37.17^e	4.23^b	41.42^f	0.55^a	0.68^a
5	20 MPa:60°C	11.36^d	4.71^c	37.13^e	4.27^b	41.37^f	0.56^a	0.61^a

Different superscripts indicate a significant difference ($p<0.05$).

SOURCE: The autor (2022).

The results showed the main fatty acids present in all oil samples were linoleic acid and oleic acid. Ranged from 45.22 to 51.92%, and 29.96 to 33.41% respectively, in the extracts obtained by Soxhlet. The content of unsaturated fatty acids (C18:3, C18:1 and C18:2) account for about 82.70 of total fatty acids present in fermented soybean oil, such values suggest these as an attractive option in the functional food market.

Comparing the fatty acid profile of the oils obtained in this study with the fatty acid profile of the soybean oil reported *in nature*, an increase of 2.31% in oleic acid content and 7.7% in unsaturated fatty acid content is observed. Therefore, it is not possible to state that the fermentative process had a positive influence on the composition of FA.

In a study by (MAN *et al.*, 2013), the fatty acid profile of soybean oil *in natura* of the analyzes performed by Soxhlet with *n*-hexane as solvent showed a predominant value of linoleic acid (range 49.56–55.83%), followed by oleic acid (21.77–26.55%) and palmitic acid (10.50–11.82%). In addition, the content of unsaturated fatty acids was approximately 75%. A comparison between soybean of different cultivars, tegument colors and years of harvest showed that the results in the fatty acid content did not present significant differences.

In a study that aimed to analyze fluctuations in the composition of soybean fatty acids over two years of storage at room temperature, the fatty acid profile obtained ranged from 49.2–56.8% for linoleic acid, 19.7–23.1% for oleic acid and 9.4–12.4% for palmitic acid, still presenting a total average content of unsaturated fatty acids> 75% (LEE; CHO, 2012).

(TIAN *et al.*, 2017) performed a comparative analysis of fatty acid composition between unfermented and fermented soybean extracts extracted by SC-CO_2. While the oleic acid content decreased from 22.35% to 18.59% after the fermentation process, the levels of palmitic acid and α-linolenic acid showed an increase of 12.13% to 15.41% and 7.30% to 8.43%, respectively. Comparing this mentioned study with the present work, it is not possible to state that the fermentation process and the addition of ethanol as a co-solvent are factors that affect the fatty acid profile of the extracts.

Fatty acids are key components of phospholipids in cell membranes playing important roles in various cellular functions, including metabolism and immune responses (Magnan, Levin & Luquet, 2015). The inclusion of oleic acid in the diet has been indicated because of its protective effects against cardiovascular and neurodegenerative diseases. Linoleic acid, characteristic of soybeans and present in most of the oils obtained, has gained prominence in recent decades due to its biological and physiological benefits, including anti-carcinogenesis (MORAES *et al.*, 2017), anti-obesity (OLSON *et al.*, 2017), antidiabetic (YUCE *et al.*, 2016) as well as bone formation promoting properties (KIM; PARK; PARK, 2014).

5.9 TOTAL PHENOLIC CONTENT (TPC) AND ANTIOXIDANT POTENTIAL

Table 9 presents the total phenolic content (TPC) and antioxidant potential for both extraction techniques.

In Soxhlet extraction, the values ranged from 132.10 ± 0.01 µg GAE g^{-1} to 1305.6 ± 0.03 µg GAE g^{-1}. The best results were obtained from fermented soybeans using solvents with higher polarity, as ethanol and water, 962.9 µg GAE g^{-1} ± 0.02 and 1305.6 µg GAE g^{-1} ± 0.03, respectively. The results highlight a significant difference ($p<0.05$). This shows that the polarity of the solvent is a determining factor for obtaining phenolic compounds in the extract, pointing out that higher polarity extracts a greater amount of polar compounds.

The quantification of total phenolic compounds contained in the extracts obtained by $scCO_2$ + EtOH ranged from 1058.2 ± 0.01 µg GAE g^{-1} to 1391.9 ± 0.09 µg GAE g^{-1} (Table 4), with statistical difference ($p> 0.05$).

High pressure and the temperature was the most efficient condition in the extraction of such compounds (1391.9 µg GAE g^{-1}), revealing the pressure had a positive influence on the extraction since a higher content of total phenolics is observed in the extracts of runs 3 and 4 (25MPa).

The obtained TPC values reveal the extracts as beneficial for human health since, according to the regulation established by EU no. 432/2012, the limit of 0.25 mg phenolics per g of oil is set to declare benefits (SQUEO *et al.*, 2019).

TABLE 8 - RESULTS OF PHENOLIC COMPOUNDS (µG GAE G^{-1}), ANTIOXIDANT ACTIVITY EXPRESSED BY INHIBITORY CONCENTRATION (IC_{50}), RADICAL SCAVENGING ACTIVITY BY DPPH (%) AND CAPACITY EQUIVALENT TO THE RADICAL TROLOX (µM TROLOX/G) OF THE SOXHLET AND $SCCO_2$ + ETOH EXTRACTION

Run	Extraction condition	Total Phenolic compounds (µg GAE g^{-1})	Antioxidant Activity		
	Soxhlet		**IC_{50} (g g^{-1})**	**Inibition DPPH (%)**	**µm Trolox/g**
S1	Hexane	132.1±0.02^a	2.91±0.47^a	18.25±0.99^a	*
S2	Petroleum ehter	423.8±0.02^b	1.75±1.65^e	29.68±0.74^b	*
S3	Ethyl acetate	581.7±0.11^d	2.93±0.84^a	17.53±0.20^a	37.48±0.01^a
S4	Acetone	904.7±0.03^e	0.79±0.27^c	61.28±0.51^f	616.0±0.01^d
S5	Ethanol	962.9±0.023^f	1.57±1.20^d	38.97±0.83^c	200.44±0.02^b
S6	Methanol	446.0±0.06^c	1.82±0.76^f	43.12±0.92^d	303.40±0.03^c
S7	Water	1305.6±0.03^g	0.55±0.84^b	81.27±0.77^g	962.66±0.02^e
	scCO2+EtOH				
1	15 MPa:40°C	1221.6±0.075^a	0.28±0.73^c	89.66±0.74^b	976.74±0.01^d
2	15 MPa:80°C	1158.2±0.01^{ab}	0.34±1.25^d	84.60±0.29^a	848.59±0.02^a
3	25 MPa:40°C	1249.0±0.01^a	0.18±2.31^b	93.62±0.37^c	903.41±0.01^c
4	25 MPa:80°C	1391.9±0.09^c	0.17±0.79^a	94.09±0.32^c	984.89±0.01^e
5	20 MPa:60°C	1058.9±0.02^a	0.34±1.20^d	89.94±0.24^b	884.89±0.01^b

Different superscripts indicate a significant difference ($p<0.05$).

* The values found were outside the confidence interval studied, so they are not shown.

SOURCE: The autor (2022).

According to the values reported in Table 9, in Soxhlet extraction, values vary from 2.93 ± 0.47 g g^{-1} to 0.55 ± 0.84 g g^{-1}. The extract with the highest antioxidant potential (water), showed a DPPH radical inhibition potential of 81.27% and 962.66 µm Trolox g^{-1}. This behavior showed that the solvents of intermediate and high polarity used in the extractions favored the solubilization of compounds with antioxidant activity, detectable by the DPPH method.

The extracts obtained at high-temperature conditions (80 °C) and pressure (25 MPa) in $scCO_2$ + EtOH showed highest antioxidant activity, corresponding to an IC_{50} of 0.17 g g^{-1} and 984 µm Trolox g^{-1}, which were able to inhibit 94.09% of the DPPH radical, significantly higher than other treatments ($p < 0.05$), confirming the correlation between the phenolic compounds and the antioxidant activity, already reported by other authors (VEBER *et al.*, 2015). Analyzing these results, it can be stated that no tendency was observed in the antioxidant activity concerning the pressure or temperature for any of the extracts obtained.

In terms of comparation among the different extractions techniques, $scCO_2$ + EtOH demontred to be a viable approach, allowing the extraction of higher quality extracts when compared to those obtained by Soxhlet extraction with an organic solvent. Comparing the extracts obtained from fermented soybean using $scCO_2$ + EtOH, the antioxidant potential was 3.23 times higher than the extracts obtained by Soxhlet. Given these results, further analysis in this study was focused on the extracts obtained by sCO2 + EtOH.

5.10 HIGH PERFORMANCE LIQUID CROMATOGRAPHY OF PHENOL CONTENTS (HPLC)

The chromatographic analysis revealed the presence of four phenolic compounds, identified in all the extracts testeds, being gallic acid (Benzyl 7-hydroxy-2,2-diphenyl-1,3-benzodioxole-5-carboxylate), transcinamic acid ((E)-3-phenylprop-2-enoic acid), genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one) and daidzein (7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one), with variations in retention time (Rt) (Table 10).

TABLE 9 - RESULTS OF IDENTIFICATION OF PHENOLIC CONTENT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ON THE EXTRACTS OBTAINED BY SCO_2 + ETOH.

	Phenolics compounds identified ($\mu g\ g^{-1}$)

Different superscripts indicate a significant difference ($p<0.05$).

Run (M_{RM})	P (MPa)	T (°C)	Rt (min)	Gallic acid	Rt (min)	Transcinnamic acid	Rt (min)	Genistein	Rt (min)	Daidzein
1	15	40	3.46	416.3[a]	25.46	518.3[b]	26.06	593.1[a]	24.59	606.1[c]
2	15	80	3.42	449.5[b]	25.37	521.4[c]	25.94	625.3[d]	24.50	605.0[c]
3	25	40	3.42	414.5[a]	25.36	507.0[a]	25.94	596.5[b]	24.50	577.3[a]
4	25	80	3.42	457.2[c]	25.38	518.1[b]	25.95	621.7[c]	24.50	603.9[c]
5	20	60	3.42	417.7[a]	25.38	518.7[b]	25.95	597.6[b]	24.51	565.9[b]

SOURCE: The autor (2022).

Phenolic acids are non-flavonoid compounds that can be divided by their chemical structure (Sanja, Nikoli, Lukovi, Jovanovi, & Stefanovi, 2018). The chromatographic analysis revealed the presence of benzoic acid derivatives, such as gallic acid, and derivatives of cinnamic acid, such as transcinnamic acid. The values of gallic acid ranged from 414.5 to 457.2 μg g^{-1} among the extracts obtained by different conditions, and transcinnamic acid ranged from 518.1 to 521.4 μg g-^{1}.

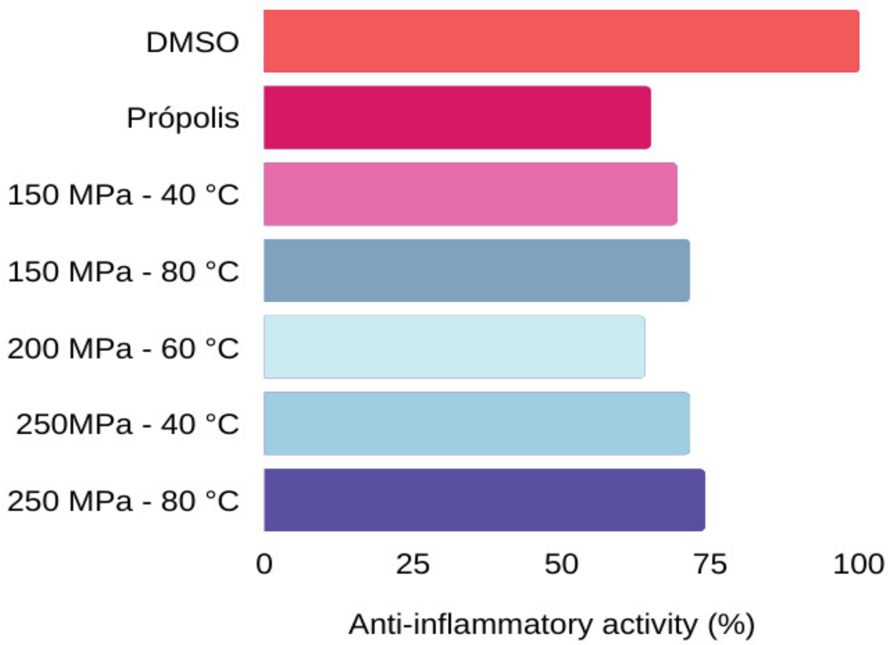
The presence of aglicone isoflavones, such as daidzein and genistein, has also been observed. The values obtained vary from 565.9 to 606.1 μg g^{-1} of daidzein and 593.1 to 625.3 μg g^{-1} of genistein. (Kim et al., 2016) a study of the presence of bioactive compounds in soybean reported daidzein values of 77.18 μg g^{-1} and genistein 332.02 μg g^{-1}. (Santos et al., 2018) in studies using fermented soybean by-product (okara) by *Saccharomyces cerevisiae* also reported the presence of aglycone isoflavones. The presence of these compounds is expected since the fermentation of grains by microorganisms that produce β-glucosidase, such as *Rhizopus spp.*, promotes the biotransformation of glycosidic isoflavones.

5.11 EVALUATION OF ANTI-INFLAMMATORY ACTIVITY IN VITRO

The anti-inflammatory potential of the extracts obtained by the $scCO_2$ + EtOH method under different conditions was evaluated by the inhibitory activity of the hyaluronidase enzyme (HIA), this is directly involved in inflammatory processes. HIA acts on the extracellular matrix of the tissues, increasing permeability and facilitating the access of pathogens (GIRISH; KEMPARAJU, 2007).

The extracts showed potential for inhibition of the hyaluronidase enzyme in the range of 63.83% to 74.0%. These results were compared with the dimethyl sulfoxide control (DMSO), since it has a complete HIA inhibition capacity and with the propolis control, a commercial anti-inflammatory. The samples showed a significant difference ($p < 0.05$) (Figure 10). Comparing than the tested commercial control, which was able to inhibit 64.85%, the extracts obtained by $scCO_2$ + EtOH showed 9.15% greater anti-inflammatory potential. It is possible to analyze that, as with the previous results, the extract with the highest inhibitory capacity of the hyalurinidase enzyme was obtained at conditions of high pressure and temperature (25 MPa and 80 °C).

FIGURE 9 - DETERMINATION OF THE ANTI-INFLAMMATORY ACTIVITY OF THE SAMPLES EXTRACTED BY $SCCO_2$ + ETOH UNDER DIFFERENT CONDITIONS OF TEMPERATURE AND



DMSO: positive control capable of completely inhibiting the enzyme hyaluronidase (HIA).
Propolis: commercial anti-inflammatory.

SOURCE: The autor (2022).

In a early study, mentioned in item 5.4, investigated the anti-inflammatory activity of fermented and unfermented soybean extracts. The results showed that the alcoholic extract obtained in the 72hour fermentation process showed a maximum inhibition capacity of 70.75%, corresponding to an increase in activity of 3.36 times compared to the "in nature" extract (21.03%) (PRADO *et al.*, 2021). The extracts obtained by $scCO_2$ + EtOH have presented higer anti-inflammatory activity in 4%.

The anti-inflammatory potential of the tested extracts may be associated with the presence of total phenolic compounds in them. Several studies have demonstrated that plant-derived polyphenols, in particular, flavonoids have potential anti-inflammatory activity in vitro and in vivo (DOUBLE-BLIND *et al.*, 2015). Isoflavones, found mainly in soybean, are especially studied because they have acted as an anti-inflammatory agent due to the fact that genistein negatively regulates the signal transduction events induced by cytokines in the cells of the immune system (VERDRENGH *et al.*, 2003).

6 CONCLUSIONS

It can be concluded that in the interest of preventive and therapeutic strategies the future of fermented foods is quite optimistic. Natural antioxidants consist of one of the main components with beneficial effects in the prevention of many diseases caused by cellular oxidative processes, and reactive oxygen species. Oxidative stress (OS) is intimately related to a series of chronic diseases and metabolic imbalance. In this context, interest arises in fermented soy foods, which have bioactive compounds such as flavonoids, isoflavone, peptides, and soy proteins. Soy aglucone isoflavones are antioxidant compounds and their activities are associated with the ability to eliminate reactive oxygen species (ROS). This study reported that soybean supplementation with brown rice is an important strategy to improve the biosynthesis of antioxidant compounds by SSF and the fermentation process was effective in the accumulation of myricetin, gallic acid, ferrulic acid, kaempferol and increased accumulation of trans-cinnamic acid. Fhis fermentation process point out to accumulation of cinnamic acid, which can be used as a novel, alternative way for production of this antitumor compound. The results presented showed that the use of solvents with greater polarity culminates in higher extraction yields of bioactive compounds with antioxidant power, rich in phenolic compounds. The extraction efficiency using $scCO_2$ + EtOH was related to greater pressure and fixed temperature (25 MPa and 80 °C). The total phenolic content and the antioxidant capacity of the extracts showed present an important correlation. The extracts obtained by the supercritical fluid showed anti-inflammatory activity with a high potential for inhibition of the hyaluronidase enzyme (74%). In addition, important bioactive compounds were detected, including gallic acid and transcinnamic acid. Extraction with $scCO_2$ + EtOH allowed a significant improvement in soybean extractions fermented with Rhizopus spp., demonstrating the technical feasibility of the process and allowing the recovery of molecules with high bioactive potential for the pharmaceutical and food industries.

6.1 RECOMMENDATIONS FOR FUTURE STUDIES

As a suggestion for further studies, investigation of the actual compounds responsible for the anti-inflammatory and anti-tumor activities is needed. So is the purification of these compounds of interest. A thorough investigation of the other compounds present in fermented soy foods is needed, so as to be clear about their relationship with the bioactive properties.

Also, further in vivo studies should be conducted for the specification of these biologically active principles.

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ATTACHMENT 1 – PUBLICATION RESULTS ARTICLE

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Fungal-Mediated Biotransformation of Soybean Supplemented with Different Cereal Grains into a Functional Compound with Antioxidant, Anti-Inflammatory and Antitumoral Activities

Fernanda Guilherme do Prado [1], Mitiyo Fukuda Miyaoka [1], Gilberto Vinícius de Melo Pereira [1], Maria Giovana Binder Pagnoncelli [2], Maria Rosa Machado Prado [3], Sandro José Ribeiro Bonatto [4], Michele Rigon Spier [5], Carlos Ricardo Soccol [1,*]

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