UNIVERSIDADE FEDERAL DO PARANÁ

DIANA CAROLINA SAAVEDRA PLAZAS

THE ANTIOXIDANT POWER EXTRACT OF *Ganoderma lucidum* SPORES: A POTENTIAL COADJUVANT FOR REPRODUCTIVE CELLS SURVIVAL

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Tese apresentada para o curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, linha de pesquisa em saúde humana e animal, setor de tecnologia, Universidade Federal do Paraná, como requisito parcial á obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

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I dedicate this work to my family, my partner who has always supported me and taught me the patience and persistence to work and achieve my goals.

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RESUMO

Ganoderma lucidum é um fungo usado na medicina tradicional chinesa. Este cogumelo é conhecido mundialmente pelos efeitos antioxidantes e terapêuticos contra várias doenças. A ação biológica tem sido relacionada na literatura com diferentes estruturas de fenóis, flavonóides, lipídios e carboidratos. Neste trabalho estudamos os efeitos de vários extratos aquosos preparados a partir de esporos de *G. lucidum* nas células reprodutivas (oócitos e espermatozoides). Para melhor compreensão o desenvolvimento deste trabalho foi baseado na proposição de um fluxograma de trabalho contendo 7 pasos. O passo 1 consiste duma revisão da literatura sobre *G. lucidum* mais especificamente abordando sua taxonomia, moléculas bioativas e seus componentes químicos gerais. No passo 2 é elaborado um extrato aquoso e avaliado a citotoxicidade dos esporos de *G. lucidum* em cultura *in vitro* de folículos pré-antrais. A sobrevivência celular não diferiu estatisticamente quando comparada com os grupos controle. O passo 3 do processo teve como objetivo realizar a caracterização dos extratos por diferentes metodologias de extração de substâncias antioxidantes dos esporos do fungo. As metodologias avaliadas foram: disruptor celular de ultrassom, micro-ondas, Soxhlet e maceração. O poder antioxidante foi avaliado por 1,1-diphenyl-2-picrylhydrazyl (DPPH) em uma curva de ácido ascórbico (μM AA). A melhor atividade antioxidante se obteve para a metodologia de micro-ondas, pois teve um poder antioxidante de 100,60 ± 0,01 μM AA, e a metodologia de maceração teve um poder de 67,57 ± 0,06 μM AA. No passo 4 foi realizada a otimização de metodologias de extração de substâncias bioativas. Os esporos de *G. lucidum* foram rompidos com esferas de aço / crômio de 1 mm de diâmetro durante 10 minutos de maceração (97,48% de rompimento). Após rompimento, foi feita uma extração aquosa por agitação (extrato BR) e uma extração adicional que incluiu exposição a micro-ondas antes da extração (extrato MBR1). A extração aquosa foi realizada à temperatura de 30 °C e agitação de 120 rotações por minuto (rpm) por 24 h. O extrato MBR1 apresentou o maior teor de polifenóis (2,21 ± 0,01 mg / 1 g de esporos de *Ganoderma*) e carboidratos com alto teor de glucose (77,73%). No passo 5 foi realizada maior purificação dos extratos BR e MBR1. A razão da escolha destes extratos foi pela amostra BR, pois a metodologia produz o maior conteúdo de proteinas na análise por dodecil-sulfato de sódio de poliacrilamida (SDS PAGE), e o MBR1 por possuir o maior conteúdo de carboidratos e a maior quantidade de mg extraídos dos esporos. O resíduo dos esporos foi exposto a diferentes temperaturas (60, 90 e 100 °C) e seguido de diálise (membrana de 12 - 14 kDa) obtendo-se as frações eluídas BR2 e MBR2 e as frações retidas BR1 e MBR1. Os extratos BR1, MBR1 e MBR2 apresentaram carboidratos do tipo glucana. A fração MBR2 foi a única a apresentar glucanas de baixo peso molecular α e a fração BR2 foi a única com maior conteúdo de bandas proteicas (análise feita pela SDS PAGE). No passo 6 do algoritmo foi realizada a avaliação da atividade biológica. O extrato aquoso de esporos de *G. lucidum* adicionado à cultura *in vitro* de folículos pré-antrais estimulou o crescimento folicular, pois aumentou o diâmetro folicular (38,36 ± 05,03 μm) e o diâmetro do oócito (24,60 ± 03,48 μm). Por outro lado, a adição da fração MBR2 no teste antioxidante em espermatozóides permitiu manter a viabilidade celular (19,20 ± 0,13 %). A atividade mais importante dos extratos de *G. lucidum* foi a ação antioxidante. Os extratos de

esporos ajudam manter o equilíbrio osmótico celular nos meios de cultura, porque seus carboidratos ajudam controlar os radicais livres e, seus micros - e macros-nutrientes (Na, K, Ca, Mn, Mg, P) podem fornecer maior apoio à viabilidade celular. Finalmente, o passo 7 descreve as aplicações em medicina, em que o extrato MBR2 com glucanas 1,4-α de baixo peso molecular podem proteger a membrana celular da peroxidação lipídica. No entanto, para confirmação destes resultados são necessários mais estudos para purificar e descrever este tipo de carboidratos. Em conclusão, os extratos de *G. lucidum* podem ser adicionados como adjuvantes aos meios de cultura celular porque seu poder antioxidante é tão eficaz como o ácido ascórbico.
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Palavras-chave: *Ganoderma lucidum,* espermatozoide, foliculo preantral, citotoxicidade, antioxidante.

ABSTRACT

Ganoderma lucidum is a fungus used in traditional Chinese medicine. This mushroom is known worldwide for its antioxidant and therapeutic effects against several diseases. The biological action has been related, according to the literature, with different structures of phenols, flavonoids, lipids and carbohydrates. In this work we studied the effects of several aqueous extracts prepared from fungal spores on reproductive cells (oocytes and sperm). To achieve the proposed goal an algorithm was here constructed and seven steps were worked. The step 1 a literature review about *G. lucidum* specially its taxonomy, the bioactive molecules and their general chemical components. In the step 2 we prepared aqueous extract and evaluated their cytotoxicity based in the biotechnique of manipulationn of oocytes enclosed in preantral follicles (MOEPF). Cell survival had no statistical differences when compared to the control groups showing that this extract lack of cytotoxicity. In the step 3 was made an extracts characterization using different extraction methodologies and the antioxidant fungus spores power. The best antioxidant value was exposure of the spores to microwaves with 100.60 ± 0.01 μM ascorbic acid (AA) and maceration with 67.57 ± 0.06 μM AA. In the step 4 was done the extraction methodologies otimization. The spores of *G. lucidum* were broken using 1 mm diameter Steel / chrome spheres during 10 min of maceration (97.48 % breakage). After the breakage was made an aqueous extraction by agitation (BR extract) and a further extraction that included exposure to microwaves before extraction (MBR1 extract). The aqueous extraction was carried out at a temperature of 30 °C and stirring at 120 rpm (revolutions per minute) for 24 h. The MBR1 extract presented the highest content of polyphenols (2.21 ± 0.01 mg / 1 g *Ganoderma* spores) and carbohydrates with high glucose content (77.73%). In the step 5 a new purification of the extracts BR and MBR1 was carried out. BR was chosen because it presented the largest number of protein bands by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis. MBR1 was chosen because it had the highest carbohydrate content and the highest amount of extract produced. The residue of the spores was exposed to different temperatures (60, 90 and 100 °C) and dialysis was carried out (membrane of 12 - 14 kDa) obtaining the eluted fractions BR2 and MBR2 and the retained fractions BR1 and MBR1. The extracts BR1, MBR1 and MBR2 presented glucan-type carbohydrates. The MBR2 fraction was the only one to present α low molecular weight glucans and the BR2 fraction was the only one with the highest content of protein bands (analysis done by SDS PAGE). The step 6 was the evaluation of biological activity. The aqueous extract of *G. lucidum* spores that was added to the in vitro culture of preantral follicles stimulated the follicular growth; because, it increased the follicular diameter (38.36 ± 05.03 μm) and oocyte diameter (24.60 ± 03.48 μm). On the other hand, the addition of the MBR2 fraction in the antioxidant test in spermatozoa allowed cell viability to be maintained (19.20 ± 0.13 %). The most important activity of the extracts of *G. lucidum* was the antioxidant action. Spore extracts were useful to maintain cell osmotic balance in culture media; because their carbohydrates help control free radicals and because their micro- and macronutrients (Na, K, Ca, Mn, Mg, P) can provide greater support for cell viability. Finally in the step 7, the MBR2 fraction was partially characterized and explained because its 1.4-α glucan has effects against lipid peroxidation of the cell membrane. However, more studies are needed to purify and

describe this type of carbohydrate. A general conclusion, *G. lucidum* extracts can be added as adjuvants to cell culture media because their antioxidant power is as effective as ascorbic acid.

Keywords: *Ganoderma lucidum,* espermatozoid, preanthral follicle, cytotoxicity, antioxidant.

RESUMEN

Ganoderma lucidum es un hongo utilizado en la medicina tradicional china. Este hongo es conocido en todo el mundo por sus efectos antioxidantes y terapéuticos contra varias enfermedades. La acción biológica se ha relacionado, según la literatura, con diferentes estructuras de fenoles, flavonoides, lípidos y carbohidratos. En este trabajo se estudiaron los efectos en las células reproductivas (ovocitos y espermatozoides) de varios extractos acuosos preparados a partir de esporas de *G. lucidum.* Para este objetivo se propuso un algoritmo y se trabajaron 7 pasos. En el paso 1 se hizó una revisión de la literatura sobre *G. lucidum*, donde se describe su taxonomía, las moléculas bioactivas y sus componentes químicos. En el paso 2 se preparó un extracto acuoso y se evaluó su citotoxicidad teniendo como base la biotecnología de manipulación de ovocitos incluidos en folículos preantrales (MOIFOPA). El resultado sobre la supervivencia celular no presento diferencias estadísticas cuando se comparó con los grupos control, lo que muestra que este extracto carece de citotoxicidad. En el paso 3 se desarrollaron diferentes metodologías de extracción del poder antioxidante de las esporas de *G. lucidum* y se evaluaron. El mejor valor antioxidante fue la exposición de las esporas a microondas con 100,60 ± 0,01 µM de ácido ascórbico (AA) y la maceración con 67,57 ± 0,06 µM de AA. En paso 4 se realizó la optimización de las metodologías de extracción. Las esporas de *G. lucidum* se rompieron utilizando esferas de acero/cromo de 1 mm de diámetro durante 10 minutos de maceración (97,48 % de rotura). Después del rompimiento se hizó una extracción acuosa por agitación (extracto de BR) y otra extracción que incluyó la exposición a microondas antes de la extracción (extracto de MBR1). Todas estas extracciones se realizaron a una temperatura de 30 °C se mantuvieron en agitación a 120 rpm (revoluciones por minuto) durante 24 h. El extracto MBR1 presentó el mayor contenido de polifenoles (2,21 ± 0,01 mg / 1 g de esporas de *Ganoderma*) y carbohidratos con alto contenido en glucosa (77,73%). En el paso 5 se llevó a cabo una mayor purificación de los extractos BR y MBR1. Se eligió el extracto BR porque presentaba el mayor número de bandas de proteínas (analizado con sodio dodecil sulfato de poliacrilamida en gel -SDS PAGE). También, el extracto MBR1 fue elegido porque tenía el mayor contenido de carbohidratos y la mayor cantidad de extracto producido. El residuo de las esporas fue expuesto a diferentes temperaturas (60, 90 y 100 °C) y se realizó diálisis (membrana de 12 - 14 kDa) obteniendo las fracciones eluidas BR2 y MBR2 y las fracciones retenidas BR1 y MBR1. Los extractos BR1, MBR1 y MBR2 presentaron carbohidratos tipo glucano. La fracción MBR2 fue la única que presentó glucanos de bajo peso molecular y la fracción BR2 fue la única con el mayor contenido de bandas de proteínas (análisis realizado por SDS PAGE). El paso 6 del algoritmo fue la evaluación de la actividad biológica. El extracto acuoso de esporas de *G. lucidum* que se adiciono al cultivo *in vitro* de folículos preantrales estimulóel crecimiento folicular, ya que aumentó el diámetro folicular (38,36 ± 05,03 µm) y el diámetro del ovocito (24,60 ± 03,48 µm). Por otro lado, la adición de la fracción MBR2 en la prueba antioxidante en espermatozoides permitió mantener la viabilidad celular (19,20 ± 0,13 %). La actividad más importante de los extractos de *G. lucidum* fue la acción antioxidante. Los extractos de las esporas fueron útiles para mantener el equilibrio osmótico celular en los medios de cultivo; porque sus carbohidratos ayudan a controlar los radicales libres y porque sus micro y macronutrientes (Na, K, Ca, Mn, Mn, Mg, P)

pueden proporcionar un mayor apoyo a la viabilidad celular. finalmente, el paso 7, donde se caracteriza y explica la fracción MBR2, ya que su glucano 1,4-α tiene efectos contra la peroxidación lipídica de la membrana celular. Sin embargo, se necesitan más estudios para purificar y describir este tipo de carbohidratos. Como conclusión general, los extractos de *G. lucidum* pueden añadirse como adyuvantes a los medios de cultivo celular porque su poder antioxidante es tan efectivo como el ácido ascórbico.

Palabras clave: *Ganoderma lucidum,* espermatozoide, folículo preantral, citotoxicidad, antioxidante

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GENERAL INTRODUCTION

Human fertility rates in Brazil have declined between 2000 and 2015, from 2.4 to 1.8, according to data collected on the website of the Brazilian Institute of Geography and Statistics (IBGE) in 2013. This phenomenon is also reflected in global statistics, where global fertility rates are declining, most prominently in Western European countries where population can decline dramatically over the next 50 years (CIA, 2018). Among the causes of fertility decline are the increase in the age to have children and the greater availability of contraceptives (CIA, 2018). In addition, the decrease in fertility comes along with infertility, as it has been increasing in recent years, reaching about 30% of couples.

Each woman born with 2 million primordial follicles (reproductive cells), about 99.9% of these cells will be eliminated by mechanisms of apoptosis activated by physiological processes (FIGUEIREDO et al., 2009). However, different autoimmune and inflammatory diseases accelerate the loss of follicular reserve in the ovary with the consequent loss of fertility (FRANKS et al., 2015; ORSI et al., 2017). In men, the same diseases that affect women, produce in the semen a poor quality of seminal parameters (motility, viability and fertilizing capacity), affecting the fertilizing capacity and producing embryos without the ability to complete their development (GUERRIERO et al., 2014; HOMA et al., 2015).

Several laboratories around the world investigate the development of new in vitro methodologies that can rescue a greater number of preanthral follicles (FIGUEIREDO et al., 2009) and that can preserve the quality of semen (VICTOR et al., 2000). Similarly, the use of antioxidants to treat diseases and maintaining of antioxidant system in cell cultures in vitro are investigated. For example, ascorbic acid is the antioxidant most used to protect reproductive cells against endotoxic shock (VICTOR et al., 2000), dyslipidemias and drugs that affect seminal quality (LEITE et al., 2017). In bulls, these drugs, improves sperm concentration and motility (LUCK et al., 1995). Likewise, the ascorbic acid optimal seminal parameters after freeze/thaw cycles (WAGNER et al., 2017). In women, ascorbic acid is used in in vitro culture of preanthral follicles with the purpose of rescuing a greater number of cells that can be fertilized in vitro (GIOMETTI, 2003; FIGUEIREDO et al., 2009).

One of the key points to improve reproductive performance is decrease the production of free radicals (AGARWAL et al., 2005; GUERRIERO et al., 2014). In nature, mushrooms have

homopolysaccharides, heteropolysaccharides, proteins, amino acids, glycoproteins, lipids and antioxidant phenols (BOWMAN; FREE, 2006; SYNYTSYA; NOVAK, 2014).

Ganoderma lucidum is a mushroom of the family *Ganodermataceae*, widel used in the treatment of different diseases (CHENG et al., 2010). In Eastern culture extracts of *G. lucidum* are consumed in infusions to increase longevity, to prevent hepatitis, chronic bronchitis, gastritis and immunity disorders (RIOS-CAÑAVATE, 2008). Studies have demonstrated the action of *G. lucidum* metabolites on the modulation of the immune response and its potential to inhibit neoplastic cells (PATERSON, 2006). The antioxidant action of *G. lucidum* may protect cells by reducing damage caused by oxidative stress (AGARWAL; ALLAMANENI, 2004). The Ceramide is an intracellular lipid mediator that responds to various stimuli, including stress. The oxidant H_2O_2 induces a rapid increase in ceramide levels due to the hydrolysis of sphingomyelin (SM). KAO et al., 2012, showed that the activity of β-1,3-glucan can interfere with Smashes. Thus, the authors identified β-1,3-glucan of *G. lucidum* as a potential source of antioxidants.

The triterpenes and polysaccharides of *G. lucidum* too have beneficial health activities because they induce apoptosis in different cell lines of leukemia, lymphoma, myeloma and HL-60 cells of acute myeloblastic leukemia (EL-MEKKAWY et al., 1998; ZHU et al., 2012). The antioxidant activity of *Ganoderma* was extensively documented for its high content of polysaccharide and ganodermic acids, even more present in the fruiting bodies of the mushroom (HUIE; DI, 2004; HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012). This research aims to evaluate extracts of *G. lucidum* in reproductive cells (preanthral follicle and sperm) to evaluate their antioxidant capacity in vitro culture systems, aiming at the development of an antioxidant product with properties like ascorbic acid.

DEFINING THE PROBLEM

Physiologically all woman loses 99.9% of primordial follicles by physiological mechanisms during her reproductive life (FIGUEIREDO et al., 2009). However, this cell death program is accelerated by the emergence of different diseases, exposure to toxic substances and poor socio-environmental conditions (FRANKS et al., 2015; ORSI et al., 2017). In men, the same variables that affect women produce alterations in semen motility, viability and in the

fertilizing capacity of semen. These factors in men and women decrease the birth rate because the embryos generated are not compatible with life (GUERRIERO et al., 2014; HOMA et al., 2015).

HYPOTHESIS

- If the *Ganoderma lucidum* spores have antioxidant action, is it possible to increase the survival of preanthral follicles and spermatozoa in vitro?
- Is it possible that *Ganoderma lucidum* spores have a higher antioxidant effect compared to ascorbic acid used in in vitro cell culture systems?

OBJETIVES

GENERAL

Evaluate different extracts of *Ganoderma lucidum* spores in vitro culture systems for reproductive cells (espermatozoid and oocyte).

SPECIFIC

- Create a database of the functional metabolites from each extract to identify fractions that may be useful in medicine.
- To evaluate different methodologies of extraction of phenols, triterpenes, fatty acids and carbohydrates to evaluate the antioxidant effects by DPPH.
- Characterization of soluble extracts in different solvents to assess functional compounds and determine the methodology for the best extract
- Compare extracts of *G. lucidum* prepared with broken spores and without breaking.
- Evaluate the survival, activation, development and growth of preanthral follicles, after the addition of *Ganoderma* extracts.
- Evaluate DNA motility, viability and fragmentation.

STRUCTURE OF RESEARCH PROGRESS

To test the antioxidant activity of *G. lucidum* spores, we have developed an algorithm (see flowchart below) for screening the metabolites of natural substances (Figure. 1). This algorithm contains eight progress points:

- The step 1 the literature was examined, in particular the taxonomy and bioactive molecules of *G. lucidum*. The purpose of this review was to examine the major antioxidant compounds.
- The step 2, an aqueous extract of *G. lucidum* was produced to assess the cytotoxicity of the extract in reproductive cells using MOEPF biotech.
- The step 3 different methodologies were evaluated to extract the antioxidant power of *Ganoderma* spores. The methodologies evaluated were: ultrasonic cellular disruptor, microwave, Soxhlet and maceration.
- The step 4, the best extraction methods were combined and at the same time the spores were broken. The extracts obtained were evaluated by infrared and chemically characterized.
- The step 5, the extraction of the best extracts from point 4 was amplified and the first chemical structures were identified.
- The step 6, biological activities were carried out. The fractions obtained were evaluated in an antioxidant test for sperm cells. It is also explained how crude extracts of *G. lucidum* have an antioxidant effect on the growth of pre-antral follicles.
- The step 7 analyses the structure of the best fraction of *G. lucidum* extract and specifies the potential use of spores on cell viability in vitro cultures.
- Finally, the bibliographic supports will be presented at the end of the algorithm steps.

ALGORITHM FOR SCREENING OF PRIMARY AND SECONDARY METABOLITES

The algorithm to study biological substances was created during the development of this work. At the beginning, the importance of collecting information on the organism to be studied is shown, followed by the preparation of a crude extract that was evaluated bythe in vitro culture system of preanthral follicles. Then, the purification of different molecules in several extracts that were evaluated for their antioxidant capacity in in vitro culture of sperm under an environment of cellular stress is shown. Figure. 1.

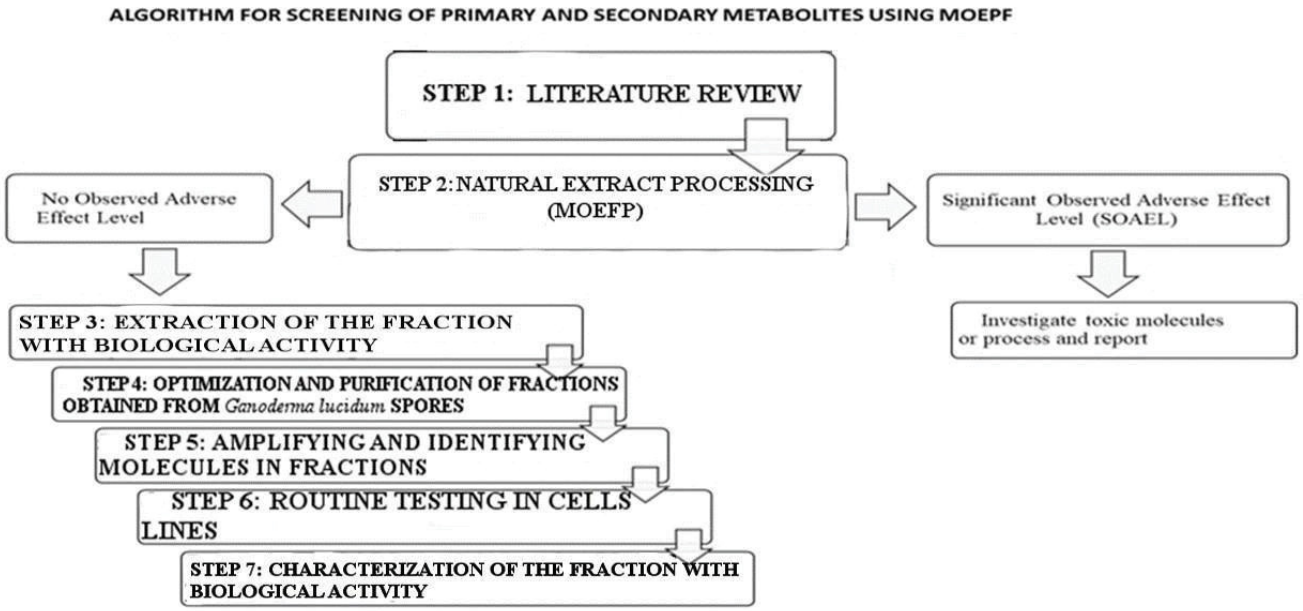

FIGURE. 1 - ALGORITHM FOR MOLECULE SCREENING

STEP 1: LITERATURE REVIEW

1.1 IDENTIFY THE USES OF *Ganoderma lucidum* SPORE EXTRACTS

In this step a general review of the uses found in the literature for *G. lucidum* extracts was developed. Also, a general review of all bioactive molecules that have been identified to date for *Ganoderma* was performed.

1.1.1. GENERAL COMMENTS

Ganoderma lucidum is a *Basiodiomcetes* mushroom that belongs to the *Ganodermataceae* family. It has been used for years to treat different diseases (CHENG et al., 2010). About 100 species of this fungus are described in China, but only *G. lucidum* and *G. sinense* are used in medicine. *Ganoderma* is popularly known as *Lingzhi* in China and *Reishi* in Japan (WU et al., 2013).

Ganoderma spp. has polysaccharides and triterpenes, derived from steroids, lecithins, and adenosine analogues (RÍOS-CAÑAVATE LUIS, 2008). The polysaccharides of *G. lucidum* have antioxidant properties, fact that has been evidenced by the enzymatic activity against radical hydroxyl and radical superoxide. Besides *G. lucidum* has lowering effects and chelating effects on ferrous ion. *G. lucidum* stimulates the increase of antioxidant enzymes such as superoxide dismutase (SOD) which catalyzes the dismutation of superoxide anion to hydrogen peroxideand the catalase enzyme (CAT), which degrades hydrogen peroxide and glutathione peroxidase (GPx). Moreover, *Ganoderma* enhances the expression mRNA, SOD and glutathione peroxidase (HELENO; BARROS; MARTINS; JOÃO; et al., 2012).

In the literature it is reported that the best antioxidant activity is presented in the extracts prepared from the spores of the *Ganoderma* species (HELENO; BARROS; MARTINS; JOÃO; et al., 2012). In *G. atrum* an isolated polysaccharide presented cytotoxicity against tumor cells in in vivo studies because it increased cAMP and PKA and stimulated lymphocyte proliferation and phagocytic macrophage activity (ZHANG et al., 2014).

Ultrasound has been used to extract polysaccharides from *Ganoderma lucidum* whose antioxidant activity against DPPH radicals, hydrogen oxide and ABTS radicals were observed. It was found that there was stimulation in macrophage proliferation and phagocytosis after treatments

with doxorubicin, a drug used in chemotherapies (SHI et al., 2014). In addition to polysaccharides, the extract may have flavonones, naringin and hesperetin, which inhibit the cytochrome p450 enzyme involved in chemical reactions of oxidase enzymes (HELENO; BARROS; MARTINS; JOÃO; et al., 2012).

1.1.2. HISTORY

Ganoderma is known as immortality Mushroom (LI, et al., 2013). This fungus has a history of over 2000 years and has been used in traditional medicine of China, Japan and Taiwan (SHIAO, 2003). Taoist monks were the first in using it to improve meditation and to lead a long and healthy life. In Japan *Ganoderma* has been considered as *sarunokoshikake*, the most important polypore in medicine. In China it is known as *Reishi* to prolong life, prevent old age and boost qi. In Korea it is called *Young ji* and in Vietnam is *Ling chi* (UPTON et al., 2006).

1.1.3. TAXONOMY

The genus *Ganoderma* was first identified by Karsten in 1881 with *G. lucidum* as sole representative specie. After the taxonomic review of Cao & Yuan in 2013, *G.* was recognized as cosmopolitan (HAPUARACHCHI et al., 2015). This genera belongs to the family *Ganodermataceae* which is a group of basidiomycetous that form fruiting bodies with pores in their lower part (WACHTEL-GALOR S, YUEN J, BUSWELL JA, 2011). *Ganoderma* is a white-rot polypore fungi and its basidiocarps have many colors, among orange-red to black and also has brownish gradations (MONCALVO et al., 1995; HENNICKE et al., 2016). The structure of *G. lucidum* spores is distinguished by pores in its surface that can fuse with their neighbors and have a circular to ovoid contour. The spores known as basidiospores have a hyaline layer called *myxosporium* and a brown layer that has spines that protrude called as *exosporium*. *G. lucidum* spores can vary between 9 - 11 μm on length and between 5.5 - 7 μm in width (HENNICKE et al., 2016). Figure. 2.

1.1.4. CULTURE

It is necessary several months to cultivate the fungus and prolonged culture times produce variations in the content of medicinal compounds. Hence different fermentations process has been developed to accelerate the culture time. The liquid fermentation was used to produce

ganoderic acids (FANG; ZHONG, 2002) and triterpene (FENG et al., 2017). The combination of liquid fermentation and static culture produces a hyperproduction of polysaccharides and ganoderic acids. This fermentation process needs high levels of oxygen to stimulate the sporulation of the mycelia (ZHANG; ZHONG, 2010). To produce triterpenes in a submerged liquid fermentation medium, a decreasing temperature and fermentation times of process vary from 32C (0 – 16 h), 31-30 °C (62 – 127 h) and 29 °C (128 h) (FENG et al., 2016). Using sucrose in fermentation an increase of intra and extracellular polysaccharides occurs because it regulates the activities of phosphoglucomutase and phosphoglucose isomerase enzymes. Under same fermentation conditions, production of biomass of intracellular and extracellular polysaccharides was increased by 25.5, 2.9 and 4.8 $g.L^{-1}$ respectively (WEI et al., 2016). For liquid fermentation, the most common media culture used was potato dextrose agar medium: 200 $g.L^{-1}$ potato tissue, 20 $g.L^{-1}$ glucose and 20 $g.L^{-1}$ agar. For solid fermentation, 75 % of sawdust, 20 % of bran, 3 % de corn flour, 1 % de gypsum and 1 % of light calcium carbonate is used (CHEN et al., 2017).

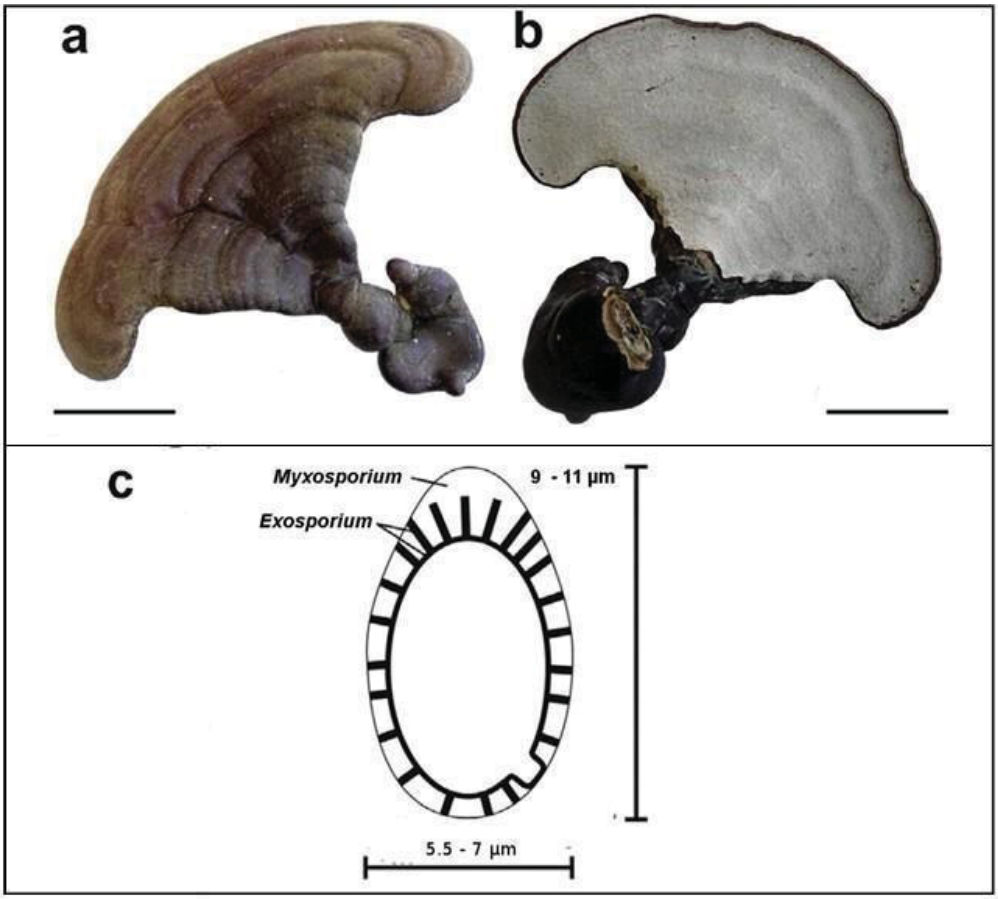

FIGURE. 2 - MORPHOLOGY OF *G. lucidum*
(A – B) BASIDIOCARP (SCALE BARS 3 CM) AND (C) SPORE. A- PILEAL SURFACE AND STIPE, B- PORE SURFACE AND STIPE, C- SCHEMATIC ILLUSTRATION OF A BASIDIOSPORE, SEE FROM OUTSIDE TO INSIDE MYXOSPORIUM AND EXOSPORIUM. THE EXOSPORIUM HAVE SPINES THAT PROTRUDE INTO MYXOSPORIUM. THE SPORE HAS MEASURED 9 – 11 X 5.5 - 7 µM. GRAPHIC MODIFIED FROM (HENNICKE ET AL., 2016).

1.1.5. MICRO AND MACROELEMENTS

Ganoderma spores contains magnesium, aluminum, silicon, phosphate, sulfur, chlorine, potassium, calcium, iron, nickel (SOCCOL et al., 2016). Likewise, sodium, phosphorus, carbon, zinc, chromium, arsenic, copper, manganese, cobalt, molybdenum, lead, fluorine, selenium and germanium is found (AHMAD, 2018). Mineral content is resumed in TABLE.

1, where the amounts of calcium, copper, zinc and magnesium seem to be the most reported elements. It is known that several minerals are important in human diet. In goats iron and protein levels change significantly before and after birth (TANRITANIR et al., 2009). Calcium is necessary for ossification, blood coagulation, heart rate control and secretion of hormones and enzymes. Magnesium participates in cellular metabolism pathways. Copper participates in the enzymatic aerobic metabolism of cytochrome C oxidase and superoxide dismutase. Sodium and potassium ions maintain blood pH (RADWINSKA; ZARCZYNSKA, 2014). Zinc plays an important role against diabetes mellitus type 2. Zinc is a cofactor of superoxide dismutase enzyme because it modulates the metabolism glutathione and the expression metallothionein and is part of RNA and DNA polymerases (CRUZ et al., 2015).

TABLE. 1 - CONCENTRATION OF MINERALS FOUND IN *Ganoderma* (mg.100 g^{-1})

MINERALS	mg. 100 g^{-1}	AUTHORS
Al (aluminium)	08.00	(SHARIF et al., 2016)
	2.38-2.46	(MHANDA et al., 2015)
B (Boron)	06.00	(SHARIF et al., 2016)
	0.014-0.74	(OBODAI et al., 2017)
Ca (Calcium)	109.2	(SHARIF et al., 2016)
	832	(CÖR et al., 2018a)
	56.8-248	(OBODAI et al., 2017)
	23200	(MHANDA et al., 2015)
Cu (copper)	01.20	(SHARIF et al., 2016)
	0.29-3.2	(OBODAI et al., 2017)
	4300	(MHANDA et al., 2015)
Fe (iron)	12.10	(SHARIF et al., 2016)
	82.60	(CÖR et al., 2018a)
	4.99-299	(OBODAI et al., 2017)
Li (lithium)	00.20	(SHARIF et al., 2016)
Mg (magnesium)	89.10	(SHARIF et al., 2016)
	1030	(CÖR et al., 2018a)
	43-204.8	(OBODAI et al., 2017)
Mn (manganese)	0.56-4.22	(OBODAI et al., 2017)
	01.10	(SHARIF et al., 2016)
Na (sodium)	20.50	(SHARIF et al., 2016)
	375	(CÖR et al., 2018a)
	0.838-4.53	(OBODAI et al., 2017)
Zn (zinc)	02.20	(SHARIF et al., 2016)
	0.56-3.06	(OBODAI et al., 2017)
	2890	(MHANDA et al., 2015)
P (phosphorus)	502.5	(SHARIF et al., 2016)
	4150	(CÖR et al., 2018a)
	37.7-427	(OBODAI et al., 2017)
K (potassium)	742.1	(SHARIF et al., 2016)
	3590	(CÖR et al., 2018a)
	100-637.9	(OBODAI et al., 2017)
Mo (molybdenum)	0.029-0.072	(OBODAI et al., 2017)
S (sulphur)	111.2-187	(OBODAI et al., 2017)

Vitamin B1	3.49	(SHARIF et al., 2016)
Vitamin B2	17.10	
Vitamin B6	0.71	
Choline	1150	
Niacin	61.9	
Inositol	307	(SHARIF et al., 2016)

Among the consulted authors the presented data are very different, because the taxonomy of Karsten in 1881 shows that *Ganoderma lucidum* is the only representative of the genus. This means that there is a great variety of fungi within the same group. Such a difference can be seen between the data presented by *Ganoderma* cultivated in Namibia (MHANDA et al., 2015), and the group of mushrooms *Ganoderma lucidum* of the variety red reishi (CÖR et al., 2018a).

1.1.6. TRITERPENOIDS

Usually extracted in solvents such as methanol and chloroformium, triterpenes are lanostate-type whose structure is based on lanosterol, an intermediary of steroid andtriterpene biosynthesis (SOCCOL et al., 2016). In the ethanol fractions has been found ganopsoreric acid A that decreases the levels of transaminase GTP in liver injuries (MA et al., 2011). Triterpenes regulate interleukin 8 (IL-8) and matrix metalloproteinase (MMP)-9 by suppressing metastasis in cancer cells (AHMAD, 2018). The lucidenic acids A, B, C and N inhibit growth and stimulate apoptosis in HL-60 cells (CÖR et al., 2018a). Ganoderic acids are related to decreasing of urokinase-type plasminogen (uTP) activator that converts inactive plasminogen to active plasmins that alters the extracellular matrix and controls adhesion and cell migration (JIANG et al., 2008). The action on uTP regulates the genes expression in the cell cycle progress, survival cell, angiogenesis and vascular endothelial growth factor - VEGF (JIANG et al., 2008; DIAZ; YEPES, 2018).In laboratory conditions, after supplementing wealing pigs with 50 mg.kg-1 of food, growing performance increased and immunocompetence by the

regulation of cytokines (Interleukin-2, Interferon-γ, tumor necrosis factor-α) produced by cells (CHEN et al., 2008). Triterpenes stimulate the activity of the immune system and act on several cancer lines. The activities for each of the compounds found in the *G. lucidum* spores is summarized in Table. 2.

TABLE. 2 - BIOACTIVITY OF TRITERPENES IN *G. lucidum* SPORES.

TRITERPENOIDS	ACTIVITY	AUTHORS
Ganopsoreric acid A	Decreases GPT transaminase levels in mice with iartrogenically injured liver.	(MA et al., 2011)
Ganoderic acid B	Inhibition of farnesyl protein transferase	(SLIVA, 2004a)
Ganoderic acid C_1	Decreases TNF-α in macrophages and in human blood mononuclear cells. in Cron'h disease decreases TNF-α, IFN-γ, IL-17.	(LIU et al., 2016)
Ganoderic acid E	Liver cancer cells (Hep G2),	(CÖR et al., 2018a).
Ganodermanontriol	Hepatoprotective effect in Murine liver cells.	(HA et al., 2013)
Ganosporelactone B	N.	N.
Lucidumol A	Citotoxic in LLC and Meth-A cancer cells	(SLIVA, 2004a)
Ganoderic acid β	Inhibition of HIV-1 protease	(SLIVA, 2004a)
Lucidumol B	Inhibition of HIV-1 protease, Citotoxic in LLC and Meth-A cancer cells. Inhibe 5α-reductase activity of rat microsomes	(SLIVA, 2004a) (JIANG et al., 2008)
Ganodermanondiol	Against breast and colon cancer cells (MDA-MB-231; HCT-116, HT-29)	(CÖR et al., 2018a).
Ganoderiol F	Lung cancer lines (LLC) and sarcoma-180, carcinoma T-47D.	(CÖR et al., 2018a).
Ganoderic acid A	Cell of Breast (MDA-MB-231)	(CÖR et al., 2018a).
Ganoderic acid γ, δ, ε,η, Θ, ξ, $C_{6,}$ G	Decrease urokinase-type plasminogen activator	JIANG et al., 2008
Ganolucidic acid D	N.	N.
Ganoderic acid C_2	Inhibition on the induction of the early antigen of the Epstein-Barr virus. Group	h y

droxyl or acetoxy groups in 3, 7, 15 positions with activity breast cancer cells by modulating AP-1 and NF-kB signaling. (JIANG et al., 2008)

Lucidenic acid SP1	N.	N.
Ganoderic acid D	Cervical cancer cells (HeLa).	(CÖR et al., 2018a).
Ganoderic acid H	Breast cancer cells (MDA-MB-231)	(CÖR et al., 2018a).

Methyl ganoderate A Mehtyl ganoderate B	Decreases lipid deposition, study in adipocyte, differentiation in 3T3-L1 cells.	(DURU; ÇAYAN, 2015)

N. data not found in the literature.

1.1.7. STEROLS AND FATTY ACIDS

The main lipid markers are ergosterol and ergosteryl (AHMAD, 2018). Ergosterol is a precursor of vitamin D and spores possess 41.9 % of these in its structure (SOCCOL et al., 2016). Ergosterol inhibited growth of Madin Darby canine kidney cells infected with influenza virus (BASNET et al., 2017). Likewise, is capable to suppress cancer cells regulating the Foxo3 tumor suppressor (LI et al., 2015). As for 19-carbon fatty acids have physiological effects (GAO et al., 2012). The most predominant fatty acids are oleic acid (57.5 %), linoleic acid (13.4 %), palmitic acid (19.6 %), hexadecenoic acid (2.2 %) and linolenic acid (0.5 %)(SOCCOL et al., 2016). Table. 3 lists the main constituents of sterols and fatty acids in *G. lucidum* spores.

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TABLE. 3 - MAIN FATTY ACIDS AND STEROLS IN *G. lucidum* SPORES.

FATTY ACIDS	STEROLS
C14:0	Ergostan-7,9,22-trien-3-ol
C15:0	Ergosterol
C16:0	Ergosta-7,22-dien-3β-ol
C17:0	Ergost-7-en-3-ol
C18:0	
C18:1	
C18:2	
C19:0	
C19:1	
C20:0	

(GAO et al., 2012; AHMAD, 2018).

1.1.8. PROTEINS

One of the proteins this mushroom is Fungal Immunomodulatory Protein (FIP) are small molecules that regulate the immune system activity. FIP have 13 kDa and a structure of 110 to 114 amino acids. Theyare rich in aspartate and valine but poor in histidine and cysteine. They generally form complexes with glucose units and regulate the response of the immune system because they are immunoglobulin-like in structure (LI et al., 2011). Most fungal walls are constituted by about 30 to 50 % of proteins. Many of the cytosolic proteins before considered as contaminants in wall preparations, now are considered part of the structure because they are

cross-linked into the cell wall. The structure of the proteins is linked by N-linked or O-linked to oligosaccharides forming glycoproteins. This structures confer resistance as well as biological activities to the wall (BOWMAN; FREE, 2006). However, more studies of these proteins are mandatory, especially in the type of chemical structure and biological action.

1.1.9. CHEMICAL STRUCTURE ON THE WALL OF *G. lucidum* SPORES

The compounds of the walls of mushrooms are mostly chitin, glucans, mannans and glycoproteins. The structure of the wall contains mainly many glucan-chitin complexes and has been described in the literature several glycoproteins and polysaccharides (BOWMAN; FREE, 2006). The chitin that composes the wall has β-1,4-linked N-acetylglucosamine residues and despite its small amount is important for the integrity of the wall because it forms inter-chain hydrogen bonding which form microfibers. The glucan contained in the walls of mushroom is the main constituent polysaccharide because it makes up 50 to 60 % of the total. The proteins can constitute 30 to 50 % of the mushroom wall; in the case of filamentous mushrooms they represent 20 to 30 % (BOWMAN; FREE, 2006).

Many of the polysaccharides are composed of units of glucose α and β in combinations of linear structures of glucans →4)-α-D-Glu*p*-(1→ y →3)-α/β-D-Glu*p*-(1→ with branches →6)-α/β-D-Glu*p*-(1→ (ZHANG et al., 2007; SYNYTSYA; NOVAK, 2014). In general, the superficial structures and weakly bounded to the extracellular matrix of the wall are glucans of type 1,3-linked with 1,6-branched (ZHANG et al., 2007). This explains why many of the soluble spore extracts of *G. lucidum* have biological activity (i.e. the structure $\{[3)$-α-D-Glu*p*-$(1\rightarrow]_m 4)$-α-D-Glu*p*-$(1\rightarrow\}_n$ is soluble in cold water and is 2000 kDa) (SYNYTSYA; NOVÁK, 2013). The 1→3-β-glucan and 1→4-β-glucan are helical and flexibleand participate in cellular recognition, whereas 1→3-β-glucan with 1→6-β-glucan branches are moderately chain compact with anticancer properties (ZHANG et al., 2007).

The glucan-chitin complexes in *Ganoderma* are diverse depending on how they were extracted. In extracts with alkali, insoluble forms of basidiomycetes (1→3) -β-D/ (1 → 6)-β-D glucans bound to chitin are obtained (poly-β-(1→4)-N-acetyl-D-glucosamine). The amino acids present in mushrooms vary between serine, threonine, glutamate, proline, glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine, tyrosine, lysine, arginine and histidine. This type

of complex can be applied on skin to accelerate wound closure and delay formation of scabs (LEE et al., 2001). There are also heterogeneous structures of glucose and mannose with 1,6-glucan linkage that stimulate cytokines and TNF-α. Similarly, heterogeneous oligosaccharides [-α-1,4-Glu-(β-1,4-$GluA_3$-]$_n$ stimulate NK cells and T-lymphocytes by induction of interleukin 2 (TSAI et al., 2012).

In the wall of *G. lucidum* there are also proteins with anti-allergy and anti-tumor function with weights of 13 kDa and formed by 110 to 114 amino acids rich in histidine, cysteine and methionine. The LZ-8 structure with amino acids and bound glucose units has the same function as immunoglobulins since they function in antigen-antibody junctions in the immune response, stimulating lymphocyte proliferation and aiding in cytokines regulation (LI et al., 2011).

Values of studied signals of fungal glycans using nuclear magnetic resonance (NMR) are visible between 4.9 to 5.2 ppm. Values found in ^{13}C depend on the type of glucan than can be α or β. The α-glucans show signals between 98 to 101 ppm while β-glucans are 103 to 104ppm. The ^{1}H signals that show participation in glycosidic bonds can range from 4.5 to 8 ppm (SYNYTSYA; NOVAK, 2014). Table. 4 and 5.

TABLE. 4 - ASSIGNMENTS OF ^{1}H NMR CHEMICAL SHIFTS IN GLUCANS.

Units	Chemical dislocations of proton (ppm)							Authors
	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B	
→2)-α-D-Glc*p*-(1→	5.4	4-3.2	3.8	3.4	3.9	3.8	3.7	(LEEUWEN et al., 2008)
→3)-α-D-Glc*p*-(1→	5.2	4-3.2	3.7-3.9	3.6	4.0	3.8	3.7	
→4)-α-D-Glc*p*-(1→	5.2	4-3.2	3.9	3.6	3.9	3.7-3.9	3.9-3.8	
→4)-β-D-Glc*p*-(1→	4.6	4-3.2	n/d	3.6	3.6	n/d	n/d	
→6)-α-D-Glc*p*-(1→	5.2	4-3.2	n/d	3.5-3.4	3.7	3.8-3.7	4.0-3.9	
α-D-Glc*p*-(1→	5.2	3.5	3.7	3.4	3.8	3.8	3.7	
α-D-Glc*p*-(1→	5.1	3.4	3.5	3.2	3.6	n/d	n/d	(HOBLEY et al., 1996)
→4)-α-D-Glc*p*-(1→	5.4	4.2	4.4	4.5	5.2	4.2	n/d	(TSAI et al., 2012)
→4)-β-D-Glc*p*-(1→	4.9	3.7	4.1	4.1	4.3	n/d	n/d	
→4)-α-D-Glc*p*-(1→	5.1	3.8	3.9	3.6	4.1	3.9	3.8	(VETVICKA; NOVÁK, 2011)
→4)-β-D-Glc*p*-(1→	4.9	3.8	4.0	3.5	3.7	4.1	3.7	
→6)-α-D-Glc*p*-(1→	4.9	3.8	3.5	3.6	3.9	4.1	3.8	
GlcN	4.8	3.1-3.2	3.6-3.5	3.5-4.0				(KASAAI, 2010)
GlcNAc	4.6	3.1-3.2	2.0-2.1					
GlcN	4.9	3.2	Overlap					(KUMIRSKA et al., 2010)
GlcNAc	4.6	2.1						
GlcN	4.8-4.9	3.2-3.2	3.5-3.8	CH_3: 1.8-2.1				(KIM, 2011)
GlcNAc	4.6-4.8	3.3-3.7	3.5-3.7	Metil group: 2.0-2.1				
GlcN	5.2α / 4.9β			N-acetyl: 2.1				(LAVERTU et al., 2003)
GlcNAc	5.2α / 4.7β							
GlcNAc	5.2α / 4.7β							(LIU et al., 2011)

n.d, undetermined.(H =proton,C=carbon). Thedifferentdisplacements weretakenfrom differentmushrooms that reportdifferenttypes ofglucans.

TABLE. 5 - SIGNALS OF 13C NMR CHEMICAL SHIFTS IN GLUCANS.

Units	Chemic al dislocations f carbon (ppm)						Authors
	C-1	C-2	C-3	C-4	C-5	C-6	
α -D-*Glcp*-(1→	93.3-92.9	72.7	74.0	70.8	72.6	n/d	(BUBB, 2003)
α- D-*Glcp*-(1→	93.5	71.9	73.0	70.6	72.4	n/d	(HOBLEY et al., 1996)
→3)-α-D-*Glcp*-(1→	97-1	70.3-71.4	80.1-83.8	69.8-70.8	73.1-73.5	61.2-61.8	(SYNYTSYA; NOVÁK, 2013)
→3)-α-D-*Glcp*-(1→	99.0	70.5	82.7	69.3	71.8	60.0	(VETVICKA; NOVÁK, 2011)
→4)-α-D-*Glcp*-(1→	98.8-100.7	71.5-72.1	71.4-73.9/75.3	77.7-79.8	71.2-73.6	60.1-61.8	(SYNYTSYA; NOVÁK, 2013)
→4)-α-D-*Glcp*-(1→	98.0	71.4	71.7	75.7	69.6	60.6	(VETVICKA; NOVÁK, 2011)
→4)-β-D-*Glcp*-(1→	102.6	102.4	n/d	80.1	n/d	60.4	
→6)-α-D-*Glcp* -(1→	97.6-101.1	71.3-73.3	72.7-74.9	69.5-73.9	70.1-75.9	65.5-68.1	(SYNYTSYA; NOVÁK, 2013)
→6)-β-D-*Glcp*-(1→	103.0-104.7	73.1-74.7	76-76.6	69.5-71.1	74.9-77.3	67.2-70.5	
→6)-α-D-*Glcp*-(1→	100.1-100.2	72.7	75.9	72.0	73.9	66.2-68.1	(VETVICKA; NOVÁK, 2011)
→4,6)-α-D-*Glcp*-(1→	102.4	72.4	75.3	78.6/76.4	69.9	71.3	
→3,6)-β-D-*Glcp*-(1→	103.0-104.7	72.8-75.2	84.4-87.2	68.7-71.8	76.3-75	68.6-71.4	(SYNYTSYA; NOVÁK, 2013)
→4,6)-α-D-*Glcp*-(1→	98.5	72.4	73.1	77.4	69.9	68.7	(VETVICKA; NOVÁK, 2011)
α-D-*Glcp*-(1→	98.7-99.7	71.8-72.1	72.8-74.0	69.7-70.0	71.3-72.7	60.8	(SYNYTSYA; NOVÁK, 2013)
β-D-*Glcp*-(1→	102.7-107.0	73.0-75.2	76.2-78.2	68.8-71.8	74.7-78.1	60.7-63.4	
GlcN / GlcNAc	95.0-110.0	55.2-57.6	73.1-75.7	80.9-85.7	73.1-75.7	59.6-60.8	C metil: 15-25 (KASAAI, 2010)
GlcNAc	107.7-105.7	55.2-57.6	73.1-75.7	80.9-85.7	73.1-75.7	59.6-60.8	C metil: 22.8-23.3 (KIM, 2011)
GlcN	89.3α / 93.9β						(TZOU, 2005)
GlcNAc	93.0α / 96.8β						

n.d, undetermined.(H= proton,C=carbon). Thedifferentdisplacements weretakenfrom differentmushroomsthatreportdifferent types ofglucans.

1.2. PHYSIOLOGY OF REPRODUCTIVE CELLS

A general description of the physiology of reproductive cells (oocytes and spermatozoid) was necessary to understand the mechanisms involved during their development. Since the antioxidant effects of *G. lucidum* spore extracts were studied, it was necessary to include an explanation of the mechanisms of action of free radicals and their effects on reproductive cells.

1.2.1. DEVELOPMENT OF OOCYTES IN MAMMALS

The development of reproductive cells has two phases. The first begins with fetal development in females. The reproductive cells in this phase are germ cells that specialize in primordial follicles. These follicles have a central structure called oocyte that is surrounded by some granulosa cells that are in the form of flattened. It has been documented that this follicular development begins between 75 to 80 days of gestation in sheep and it is known that in mice the formation ends between 4 and 5 days after birth. The initial follicular development is important because it formed the cellular reserve of all the reproductive life of the females. This phenomenon of follicular growth is called initial folliculogenesis because after the primordial follicles are formed, the cellular reserve will enter quiescence or numbness until puberty (YU; ROY, 1999; ARAÚJO et al., 2014). Folliculogenesis has been studied in several classes of mammals to understand the reproductive cycle in women. The arrival of puberty in mammals is the second phase of folliculogenesis that ends with the formation of an ovulatory follicle or *Graafian*. Although the exact mechanisms that activate the growth of primordial follicles are not yet known, it is known that there are leptins derived in adipocytes that initiate signaling. This process stimulates the secretion of gonadotropic hormones in the pituitary gland that trigger signals at the level of the ovary to initiate follicular development (ORSI et al., 2017).

The growth of the primordial follicles begins with the transformation of granulosa cells (cells located around the oocyte). These cells will multiply be forming a layer and changing from a flattened form to a cuboidal shape. An oocyte with a full layer of granulosa cells is called a primary follicle. The follicle granulosa cells will initiate the production of hormones, cytokines and growth factors that increase the follicular diameter. This growth is accompanied by the formation of a second layer of granulosa cells and the appearance of a new cell layer called theca cells, thus, this leads to the appearance of a secondary follicle. In this follicle there is antrum

formation due to the accumulation of follicular fluid and proliferation of the theca cells and granulosa, together with the appearance of pelucide zone. All the structures will continue to grow and shape the tertiary follicle. Afterwards, the maturation of the oocyte and its ovulation will continue (ARAÚJO et al., 2014). For detail see Figure. **3** and Figure. **4**.

The follicular reserve in women is 2 million primordial follicles at birth. Follicular number decreases to 100.000 or 200.000 as a result of follicular atresia (FRANKS et al., 2015). Atresia is a physiological process during follicular growth. Many primordial follicles are activated in each menstrual cycle and only 1 follicle will become ovular because there is dominance between the growing follicles. In each stage of growth, the dominant follicles secrete substances that activate the apoptosis of the follicles that grow next to it. This factor is called transforming growth factor beta (TGFβ). The cellular reserve in the ovary declines between 40 and 55 years (FRANKS et al., 2015). However, there are processes that affect the cellular reserve and accelerate apoptosis processes, also, ovulation can be interrupted by inflammatory, infectious, autoimmune and iatrogenic processes; in the case of treatments in diseases such as cancer. This accelerated decrease and anovulation cause fertility problems in women and animals of productive importance (ORSI et al., 2017).

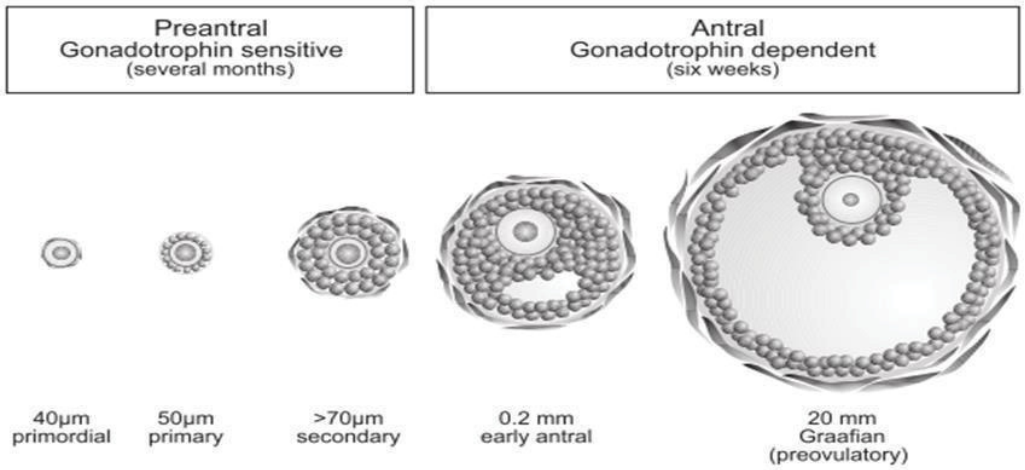


FIGURE. 3 - DEVELOPMENT STAGES.
(FRANKS ET AL., 2015).

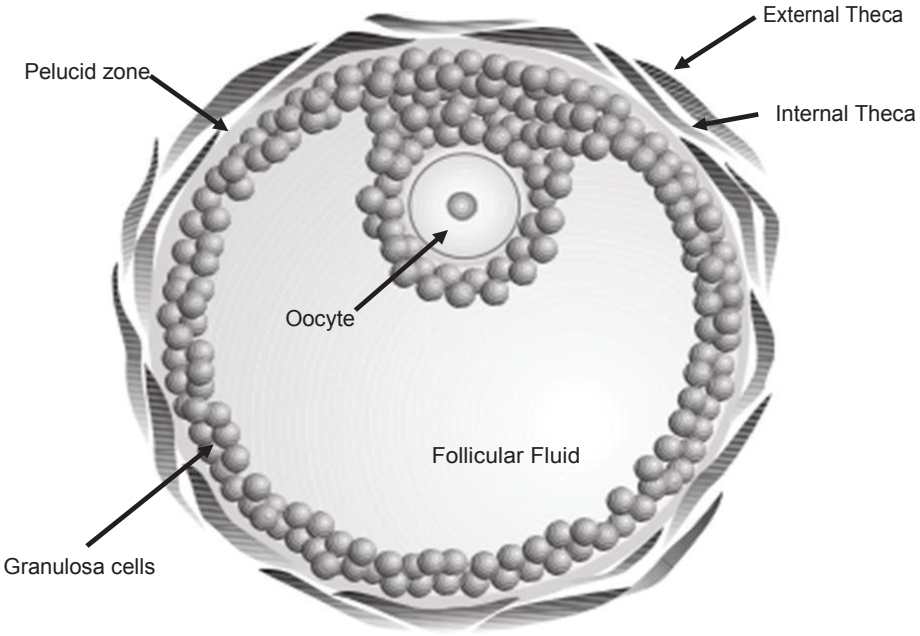


FIGURE. 4 - ANATOMIC STRUCTURE OF THE FOLLICLE.
(FRANKS ET AL., 2015).

1.2.2. DEVELOPMENT OF SPERM

In male, the development of sperm is continuous. The formation starts in the testicles in a structure called seminiferous tubules. In these tubules are cells called Sertoli that assist the reproductive cells (spermatogonia) to nourish themselves and stimulate their maturation from the surface of the tubules to their light. This development takes 74 days.

The formation of spermatogonia begins with the stem cells in the testes. Each stem cell undergoes a process of mitosis where it divides to maintain a reserve and for future sperm. Spermatogonia are primary sperm cells with 46 chromosomes that increase the size of their cytoplasm and their organelles. Then, the spermatogonia is divided to form secondary sperm cells. The secondary cells have half of the chromosomes (23). After the division each cell must mature. The nuclear material turns oval and is covered by a cap (acrosome) to pierce the ovule during fertilization. Derived from the cytoplasm, a tail develops from the secondary cells that will allow the sperm to move. From the seminiferous tubules, the cells go to the epididymis and mature to form the sperm (NISHIMURA; HERNAULT, 2017; UTIGER, 2018) Figure. **5**.

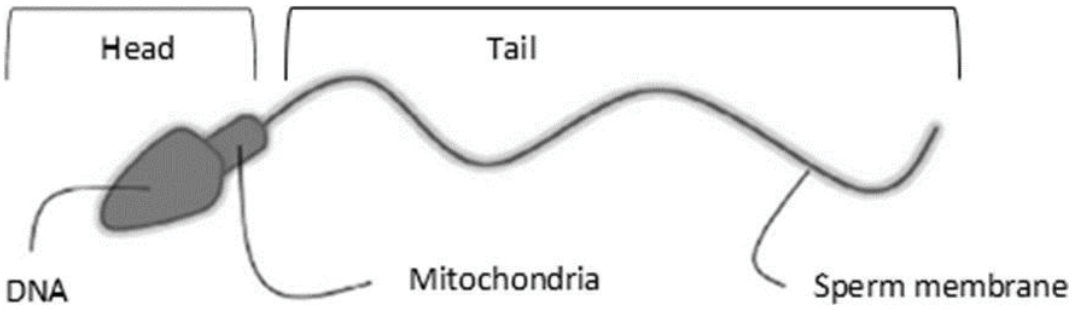


FIGURE. 5 - STRUCTURE SPERMATOZOID.
(WAGNER ET AL., 2017).

1.2.3. OXIDATIVE STRESS IN REPRODUCTION

Reactive species are divided into two classes: the reactive oxygen species (ROS) and the reactive nitrogen species (RNS). The chemical structures of these two types of radicals are unstable. The ROS lose an electron and look for its chemical balance in lipids, proteins and nucleic acids, whereas the RNS are only unstable (AGARWAL et al., 2005). The sub classes of ROS are: superoxide anions (O_2^-), hydroxyl radicals (OH^-), hydrogen peroxide (H_2O_2), (BASINI et al., 2008) and the classes of RNS are: nitric oxide (NO) and peroxynitrite anion ($ONOO^-$), (WAGNER et al., 2017).

The reactive nitrogen species are synthesized during the enzymatic conversion of L-arginine to L-citrulline by the nitric oxide synthase (AGARWAL et al., 2005). The reactive oxygen species (ROS) are synthesized in four pathways. In the first pathway there is mitochondrial oxidative phosphorylation, where nicotinamide adenine dinucleotide (NADH) is used as an electron donor and oxygen is used as an electron acceptor in the electron transport chain. Consequently, a reduction and oxidation reaction occurs with synthesis of ATP (adenosine triphosphate) where the oxygen consumed ends in the form of ROS. In the second one, ROS are generated by leukocytes through anaerobic synthesis. The third is generated in inflammations to attract leukocytes, for example, in varicoceles of males and in patients with diabetes. Finally, the fourth route occurs only in sperm, because these cells produce ROS in their cytoplasm by means of glucose-6-phosphate dehydrogenase (G-6-PDH), (WAGNER et al., 2017). In addition to

intrinsic pathways, there is an extrinsic production of ROS by exposure to metals such as cadmium, lead, iron, copper, pesticides, phthalate and pollution (WAGNER et al., 2017).

Reactive species have physiological functions in the case of ovarian involved in ovulation and fertile men in small concentrations were recorded in sperm (FRANKS et al., 2015; HOMA et al., 2015). Meanwhile, excessive production of ROS has been related to the loss of motility and fragmentation of DNA (GUTHRIE; WELCH, 2012) in men, whereas in women it is reported apoptosis of preanthral follicles and interruption of angiogenesis, necessary for the development of all follicular structures (BASINI et al., 2008).

The antioxidant system responsible for eliminating free radicals has two control mechanisms. The first are the enzymatic catalysts known as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (BASINI et al., 2008). The second, is based on vitamin C and E, selenium, zinc, taurine, hypotaurine, beta carotene and carotene that prevent peroxidative propagation (AGARWAL et al., 2005).

1.2.4. FREE RADICALS IN THE FOLICULOGENESIS

The regulation of gonadotropins is altered by primary and secondary factors. Deregulation interferes with the normal development of the ovarian follicles causing loss of fertility. Primary factors include irradiation and chemotherapy. Among the secondary ones are weight loss, exercise and other idiopathic causes. Infertility is also generated by pathogens such as bacteria and fungi, autoimmune diseases (lupus erythematosus and vitiligo) and endocrine disorders of the thyroid and adrenal glands (FRANKS et al., 2015; ORSI et al., 2017).

Due to the inflammatory process of several diseases on the reproductive system, the production of free radicals and lipid peroxidation increase. This process affects the entire follicular structure, because its cell membranes are rich in lipids and because the production of endogenous hormones that are important for follicular development is interrupted. Studies report that the concentrations of O_2^-, H_2O_2 and ROOH (hydroperoxides) decrease with follicular development as well as the concentrations of antioxidant enzymes (SOD and GPx). This phenomenon occurs because follicular angiogenesis requires hypoxia process to stimulate the development of theca and granulosa cells (BASINI et al., 2008). The ovary also has an antioxidant system in the ovarian follicle. This system is based on copper-zinc SOD (Cu-Zn SOD) and manganese superoxide

dismutase (MNSOD) located in the granulosa and theca cells (AGARWAL et al., 2005). Consequently, any alteration of the antioxidant system is related to a lesser development of hormonal receptors and less synthesis of estrogen of the cells that make up the follicle, ending in the failure of ovulation.

It is necessary to highlight that Infertility can be defined as the inability to conceive after 12 months or more of unprotected sex. In addition, any disease that increases ROS production decreases angiogenesis, increases apoptosis in granulosa cells, causing DNA fragmentation with consequent follicular rupture and loss of follicular reserve (AGARWAL et al., 2005).

1.2.5. FREE RADICALS IN THE SEMEN

50% of infertility problems are attributed to men. Deficiencies of selenium (antioxidant) are associated with decreased testosterone synthesis, alterations in sperm morphology and loss of motility in sperm. This occurs because under normal conditions selenium is incorporated into selenocysteine proteins and is associated with GPx to protect liposomes and cell membranes from lipid oxidation (GUERRIERO et al., 2014; HOMA et al., 2015).

Motility is reduced by overproduction of ROS in the semen that ends up depleting the energy reserve by glycolysis (GUTHRIE; WELCH, 2012) and because they overactive the flagellum by the induction of cyclic adenosine monophosphate (cAMP) that inhibits tyrosine phophatase by initiating phosphorylation by tyrosine. This tyrosine is also responsible for the binding of the plasma membrane to the ZP-3 protein with pelucid zone in the oocyte that promotes the fusion of sperm with the ovule (LAMIRANDE; GAGNON, 1993; AITKEN, 1995).

Toxic elements such as iron, lead, cadmium, copper and pesticides damage sperm DNA because there are not known mechanisms of self-repair and because they affect the hypothalamic-pituitary-gonadal axis system causing testicular atrophy and decreasing the production of gametes in the testes (WAGNER et al., 2017).

Lipid peroxidation is another mechanism initiated by ROS that affects sperm because its cell membranes are rich in lipids (WAGNER et al., 2017). In contrast, a feature a characteristic of the sperm is that its genome has nucleoprotamines that protect it against free radicals. It is known that in some infertile men this protamination is deficient, further harming the sperm DNA (MARCON; BOISSONNEAULT, 2004).

STEP 2: NATURAL EXTRACT PROCESSING

The growing demand for functional foods and new natural medicines motivates the search and evaluation of the antioxidant power of *G. lucidum*, a fungus has been used for many years for several medical treatments (CHENG et al., 2010). In eastern cultures *G. lucidum* is consumed as an infusion to increase longevity and immunity (RIOS-CAÑAVATE, 2008). In addition, this mushroom can be used as a coadjuvant in cancer treatments because it has cytotoxicity against cervical cancer cell lines (EL-MEKKAWY et al., 1998).

Techniques such as the manipulation of oocytes enclosed in pre-antral follicles (MOEPF) are widely used to evaluate the safety of different natural substances, hormones and toxic agents (AMBE et al., 2005; FIGUEIREDO et al., 2007, 2009). This methodology could be an alternative as 3Rs (replacement, reduction and refinement) policy regards laboratory animals use in drugs preliminary studies.

The spores of *G. lucidum* have evident antioxidant activity due to their high content of polysaccharides and ganoderic acids (XIN et al., 2005; HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012). Therefore, a study on the toxicity of preantral follicles is necessary, since its innocuous in reproduction is unknown.

There is the possibility of using MOEPF to evaluate the effects of *G. lucidum* on reproduction and citotoxicity. Under this rationale, the action of different dilutions of an aqueous extract of *G. lucidum* on the survival, were evaluated and described. All experiments were performed in vitro culture of preantral pig follicles in 10 prepubertal swine females for one and seven days.

2.1. METHODOLOGY

2.1.1. NATURAL EXTRACT PROCESSING

Ten (10) grams of *G. lucidum* spores were diluted in 100 mL of sterile water and disrupted on the sonicator for 15 min (HELENO; BARROS; MARTINS; JOÃO; et al., 2012). After, the suspension was centrifuged twice at 12.000 x*g* (10 min, 4 °C) and its sediments were discarded. The supernatant was filtered on a 0.22 μm filter and the final solution stocked at -80 °C until use. One day before being used in culture in vitro of follicles preanthral, the extract was thawed, and

aliquots were prepared at concentrations of 10, 25, 50 and 100 % and kept in the refrigerator until use.

2.1.2. CITOTOXITY STUDY: IN VITRO TEST BY PREANTHRAL FOLLICLES CULTIVATION MOEPF

Ovaries from pigs were collected in a slaughterhouse in Fortaleza-Ceará, Brazil. Ten complete ovaries from ten prepubescent Yorkshire gilts (*Sus scrofa domestica*) were removed. The ovaries were washed once in 70 % ethanol and twice in Minimum Essential Medium (MEM) containing 100 µg/mL penicillin, 100 µg/mL streptomycin, and transported to the laboratory in MEM at 37 °C (MAO et al., 2004). From each ovaries pair were obtained 11 pieces to compose the three treatments (control group and *G. lucidum* extract treated group) during cultivation for 7 days.

The ovarian cortex tissue in situ cultivation was according to MAO et al., 2004 methodology. The cultivation medium used was alpha MEM (pH 7.2 - 7.4), 100 mg/mL ascorbic acid, 1000 mg/1 mL BSA, 29,200 µg/mL hypoxanthine, 10 µg/mL transferrin, 1 mM pyruvate, 100 µg/mL penicillin and 100 µg/mL streptomycin. The organ culture system used herein has been previously described (CELESTINO et al., 2009; MAGALHÃES-PADILHA et al., 2012). Ovarian cortex tissue from each ovarian pair from the same animal was cut into 11 slices (approximate size: 5 mm × 5 mm x 1 mm) using a scalpel under sterile conditions. One slice was immediately fixed for histological and ultrastructural analysis (fresh control) and the other 10 were placed in culture for 7 days in individual wells. The cortex tissue samples were transferred to 24-well culture dishes containing 1 mL of culture medium and 10 µL of aqueous extracts of *G. lucidum*. Four extract concentrations were tested (10, 25, 50 and 100 %). In the control group was medium extracts free. Subsequently, was performed histological analyses.

Samples were fixed in 4 % paraformaldehyde solution in PBS at pH 7.2 for 12 h at 4 °C. They were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Histological samples were cut into 7 µm sections and stained with periodic acid – Schiff (PAS) – hematoxylin. Each section was evaluated using an optical microscope (Nikon, Tokyo, Japan) at 400x magnification. In reading were considered the living follicles, based on oocyte and granulosa cells

integrity, presence or absence of pyknotic bodies, ooplasmic retraction and organization of the granulosa cells (FIGURE. 27).

The developmental stages of follicles have been defined previously (TELFER, 1996) as primordial (one layer of flattened) or growing follicles (primary – one layer of cuboidal granulosa cells, and secondary– two or more layers of cuboidal granulosa cells around the oocyte). Follicles were classified histologically as: normal, when an intact oocyte was observed; surrounded by granulosa cells, that were well arranged in one or more layers, and lacked a pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. The percentage of morphologically normal follicles, before (day 0) and after culture, indicated the percentage of follicle survival.

2.2. RESULTS

The *G. lucidum* spore extract in concentrations of 10, 25, 50 and 100 % had no effects cytotoxic on the survival and development of preanthral follicles during days of in vitro culture. Here we present an example of follicle (FIGURE. 6) after submission to *G. lucidum* spore extract at the four concentrations.

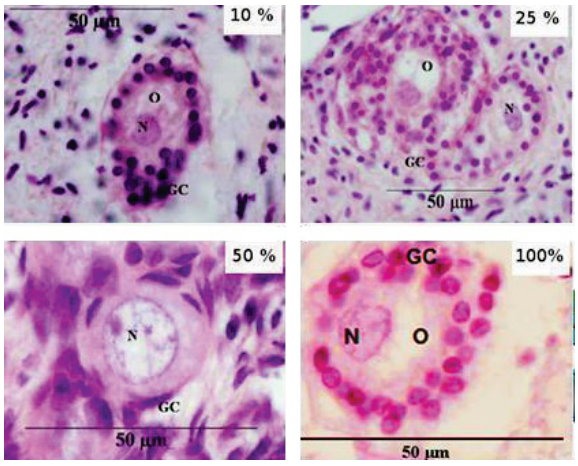


FIGURE. 6 – MORPHOLOGY OF FOLLICLE WITH *G. lucidum*
MORPHOLOGY OF FOLLICLE AT 7 DAYS OF CULTURE, (GC) GRANULOSA CELLS, (O) OOCYTE, (N) NUCLEUS. SCALE: 50 µm.

Our results encourage to continue characterization and purification of the biomolecules of the *G. lucidum* spores extract. Step 3 will focuse on extraction of the antioxidant power from spores.

2.3. CONCLUSIONS

G. lucidum spores extract have no observed adverse effect on survival when comparing results from groups exposed to different suspensions of extract and the control group. This result encourages to go to continuing purification from aqueous extract of *G. lucidum* spores. For this reason, it will be necessary to study different methodologies of extraction of the antioxidant power from spores.

STEP 3: EXTRACTION OF THE ANTIOXIDANT POWER FROM *G. lucidum* SPORES

The spores have different bioactive compounds and different extraction technique can produce different contents of lipids, carbohydrates and proteins (ZHAO et al., 2014). The ultrosinc cell disruptor extraction technique can extract higher quantity of lipids and carbohydrates (ZHAO et al., 2014). Furthermore, microwave technique can extract different polysaccharides (ZHU et al., 2012; ZHAO et al., 2014) and soxlhet technique can extract fatty acids and triterpenes (USMAN et al., 2012). On the other hand, it is known that the macrofungal *G. lucidum* has several molecules with antioxidant power that can help in several pathologies and that these substances are also found in higher concentration in the spores (HELENO; BARROS; MARTINS; JOÃO; et al., 2012).

In addition to the antioxidant power described for various pathologies, antioxidants have been used to preserving the viability of sperm and ovules has focused on maintaining viability, DNA integrity and fertilizing capacity. (SUN et al., 1997; LOPES et al., 1998; TWIGG et al., 1998; MARTÍNEZ et al., 2007; PERDICHIZZI et al., 2007). Ascorbic acid (AA) is included in supplements administered during processes that affect the reproductive system. The AA exerts influence on the tubular structure and functionality of sperm. Also, it is related to semen quality because it helps maintain motility, viability and sperm concentration. Studies on deficiencies of AA demonstrated degeneration of the epithelium germinal testicular, alters the effects of gonadotropins and affects the morphology and function of sperm. In addition, the AA is important as part of follicular development and growth because it participates in tissue remodeling and collagen synthesis. AA has been found in theca cells and follicular basement membrane. One of the main mechanisms of AA is the decrease of oxidation and the positive stimulus on neutrophils (LUCK et al., 1995). It was proven that AA decreases the damage in the systemic shock caused by the endotoxin of several bacteria because it regulates the phagocytosis process (VICTOR et al., 2000). Similarly reduces the cytotoxic effect of drugs on dyslipidemias when obesity problems delays puberty and decrease androgens (LEITE et al., 2017). The AA is important for various cell functions, particularly to support against the production of free radicals. AA is a known adjuvant to preserve reproductive cells. Compare the antioxidant activity of AA against extracts of *G. lucidum* spores would be a challenge.

In this part of the work it was necessary to carry out two steps to isolate the probable molecules with activity of *G. lucidum* spores. The first step required identifying a methodology to prepare extracts with high antioxidant value and the second step was devoted to improving the extraction technique and quantifying the amount of carbohydrates and proteins present.

Consequently, the aim of this work was to assess different extracting methodologies of the spores of *G. lucidum* to elucidate their antioxidant compounds and compare them the antioxidant capacity using an ascorbic acid curve as a parameter.

3.1. MATERIALS AND METHODS

Different extracting methodologies were used to obtain antioxidants from *G. lucidum* spores (GLS). The antioxidant power of the different extracts was evaluated based on the content of phenols and the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the ascorbic acid curve in µM. Figure. 7. It is important to highlight that the methodologies used and evaluated were taken from several protocols of the literature, where extracts of *Ganoderma* with high antioxidant value were obtained.

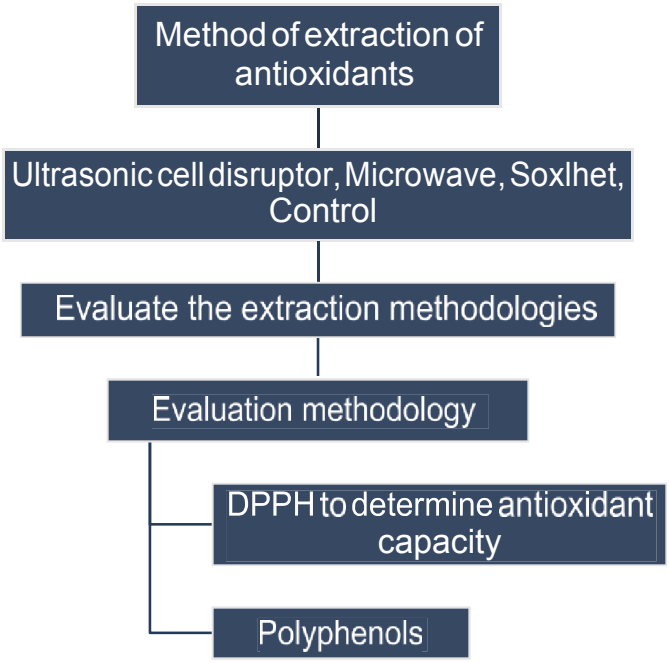


FIGURE. 7 - EXTRACTION PLAN FOR ANTIOXIDANT *G. lucidum* SPORES.

3.1.1. PRODUCTION OF ANTIOXIDANT EXTRACTS

0.7 mg.mL^{-1} concentrations of *G. lucidum* spores were prepared and different extracting methodologies was assessed to extract antioxidants; they were:

1. Ultrasonic cell disruptor
2. Microwave
3. Soxhlet
4. Maceration

To evaluate the breakdown of spore wall of each extracting methodology, samples were observed using a Nikon E200 light microscope.

3.1.1.1. ULTRASONIC CELL DISRUPTOR

Based on the methodology of (ZHAO et al., 2014), a solution of 0.7 mg.mL^{-1} of spores of *Ganoderma lucidum* (GLS) was made. The spores were diluted in distilled water and placed in an ice bath. The mixture was then placed in the ultrasonic cell disruptor (BJED-20150), where the spores were exposed to cycles of 4 min, for a total time of 90 min. The temperature was controlled in an ice bath to not exceed 40 °C. After the spores were submitted to ultrasonic, the extracts were prepared with methodology A and B, as explained below.

3.1.1.1.1. METHOD A:

After exposing the mixture to the cell disruptor, it was centrifuged at 4000 g for 10 min and the fractions were separated: supernatant and residue. First, the supernatant **(A1)** was precipitated in ethanol for 48 h (3:1, v / v) and centrifuged for 10 minutes in 4000 *g*. In this fraction the ethanolic **(A1.E)** and its precipitated **(A1.P)** fraction were analyzed to then suspended in distilled water (80:20, v / w). Second, the residue **(A2)** was washed in ethanol 3:1 (v / w) for 24 h at 4 °C to obtain an ethanolic fraction **(A2.E)**. Finally, the A2 was diluted in water (3:1, v / w) for 12 h with shaking at 4 °C and centrifuged to obtain the soluble fraction **(A2.S)**. Figure. **8**.

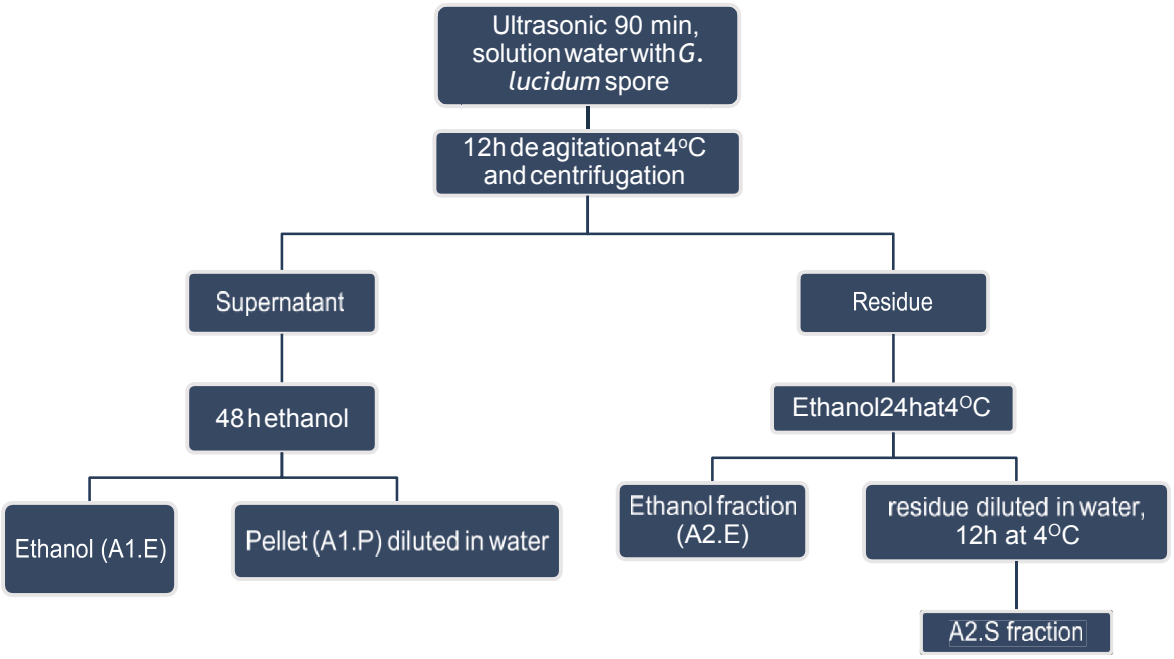


FIGURE. 8 - EXTRACTS OBTAINED WITH PROCESS A AFTER ULTRASONIC CELL DISRUPTOR FOR 90 MIN.

3.1.1.1.2. METHOD B:

The mixture was exposed to the cell disruptor and centrifuged at 4000 g for 10 min obtaining a supernatant and residue. First: the supernatant **(A31)** was precipitated in ethanol (3: 1, v / v) for 24 h at 4 °C. The ethanolic pellet was diluted in water (80:20, v / v) to form the **A31.P** fraction and the ethanolic supernatant was named **A31.E**.

Second, the residue **(A32)** was suspended in water and heated for 2 h ranging from 90 to 100 °C (HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012). This process was repeated twice more. After centrifugation, the supernatant was mixed with absolute ethanol (3:1, v/v) for 24 h to form the precipitated fraction **A32.P**. On the other hand, the residue was resuspended in water for 12 h with stirring at 4°C and centrifuged to obtain the **A32.S** fraction. Figure. **9**.

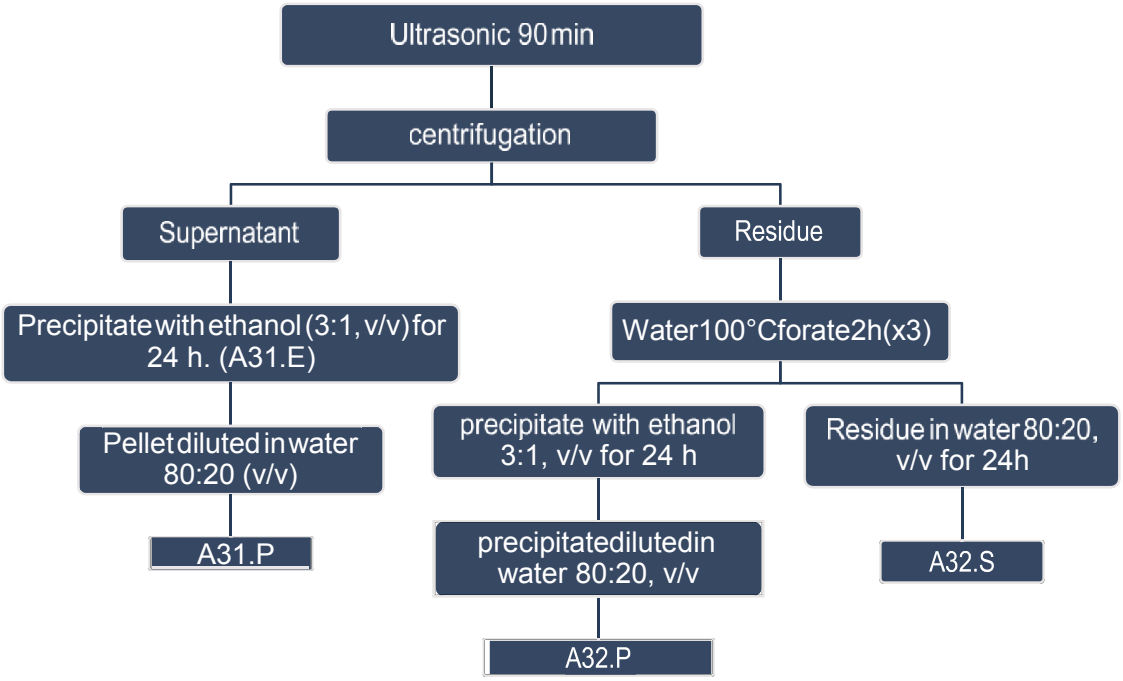


FIGURE. 9 - EXTRACTS OBTAINED WITH PROCESS B AFTER ULTRASONIC CELL DISRUPTOR FOR 90 MIN.

3.1.1.2. MICROWAVES METHODOLOGY

1 g of *G. lucidum* spores per 100 mL of distilled water was used to heat in a microwave oven (Carrefour Home, model HMO22E-13-127V) for a time of 5 and 10 min (1200 watts), (ZHANG et al., 2008; HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012; ZHU et al., 2012). After heating, the solution was centrifuged for 10 min in 4000 *g* to separate supernatant and residue. The supernatant was precipitated with ethanol for 48 h (3:1 (v/v) to form the soluble fraction **5.A** and the precipitate **5.D** (diluted on water, 80:20, v/p).

The centrifuged spore residue was warmed and then washed in ethanol to be diluted once more in ethanol for 24 h. After centrifugation the ethanolic supernatant was named **5.B** and the ethanolic precipitate named **5.C.** Figure. **10**.

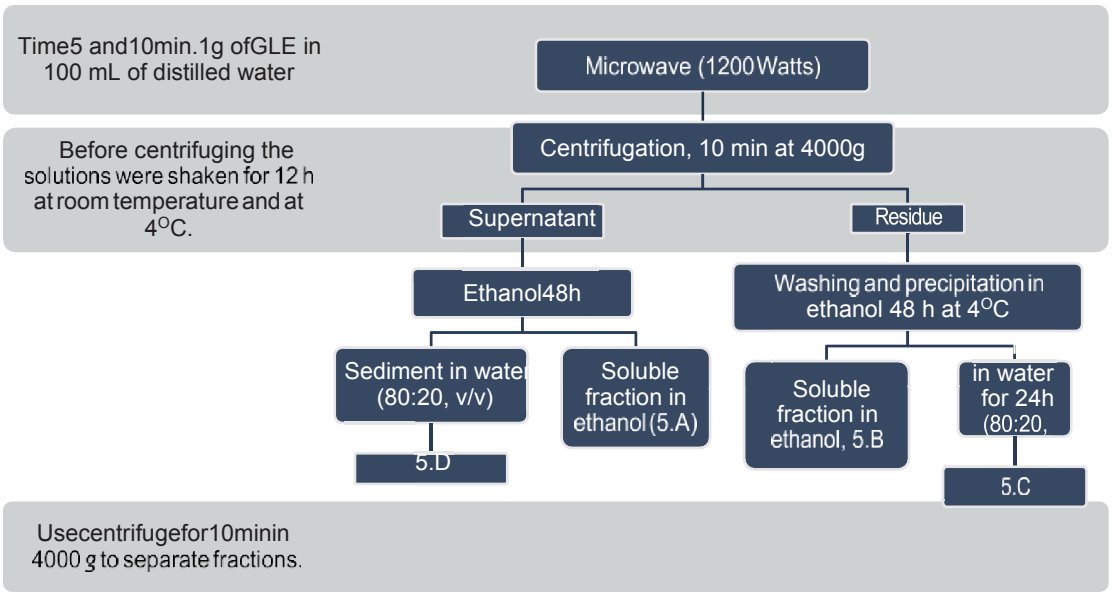


FIGURE. 10 - EXTRACTS OBTAINED WITH MICROWAVE HEATING FOR 5 AND 10 MIN.

3.1.1.3. SOXHLET METHODOLOGY

2g of *G. lucidum* spores were placed in Soxhlet with 100 ml of absolute ethanol as solvent. The experiment was run at 40 °C for 24 h and then the GLS residue was washed with ethyl acetate and n-butanol. The ethanol fraction (**S.E.L**), the ethyl acetate fraction (**EE**) and the fraction in n-butanol (**NB**) were evaluated (ZHANG et al., 2008; USMAN et al., 2012). Figure. 11. The fractions in NB and EE were used due to their polarity by flavonoids, cardiac glycosides, anthraquinone, saponin glycosides and terpenoids (USMAN et al., 2012).

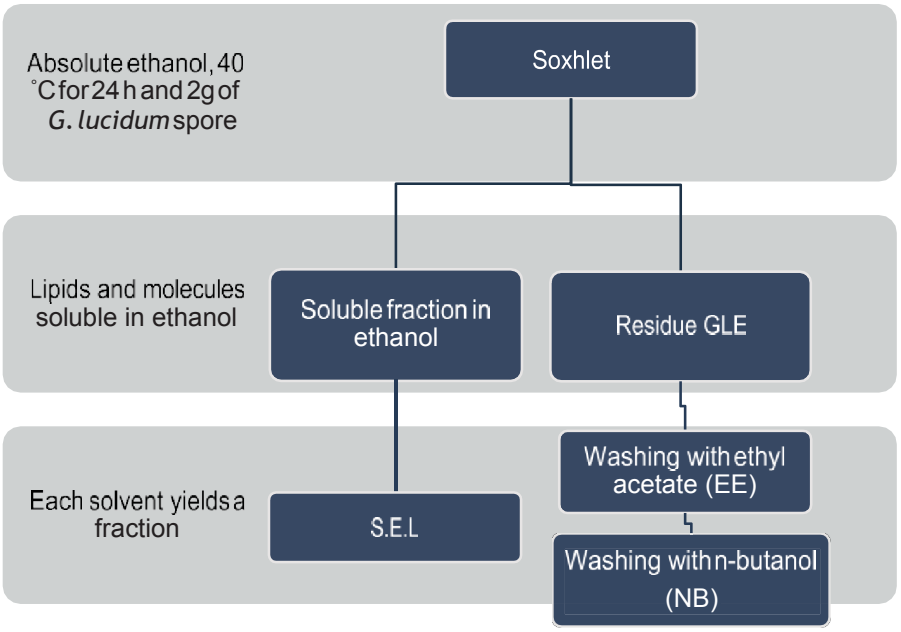


FIGURE. 11 - EXTRACTS OBTAINED BY THE SOXHLET METHODOLOGY.

3.1.1.4. MACERATION METHODOLOGY

Based on the work of NOVAK, (2013), because the extract has an important antioxidant action. In this study a crude extract with *G. lucidum* spores was prepared applying manual maceration of 1g of spores for 20 minutes. The macerate was then diluted with water pH 7.0 (1:40, w/v) and the solution held in shaker at 120 rpm for 24 h at 30 °C. The mixture was then centrifuged for 15 min at 10.000 rpm and the supernatant (RM) filtered with Whatman # 1 filter paper to remove the spores of *G. lucidum* (x3). Figure. **12**.

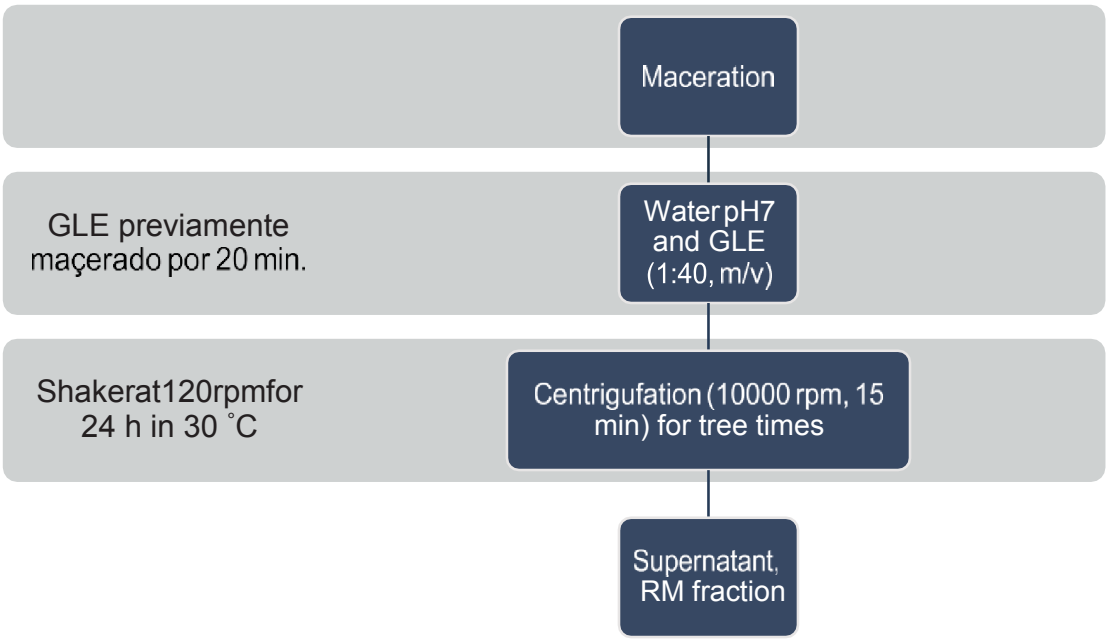


FIGURE. 12 - MACERATION METHODOLOGY.

3.1.2. COMPARING METHODOLOGIES

The best fractions of each methodology were compared between each other. The antioxidant power, the content of phenolic compounds and the mg extracted from 1 g of *Ganoderma lucidum* spores were evaluated among the methodologies. A control extract with 1 g of *G. lucidum* spores in 16 mL of water pH 7.0 was considered. The control extract was left 24 h in water at 30 °C and then centrifuged to remove the supernatant (**Control extract**).

3.1.3. TOTAL POLYPHENOLS

The total content of phenolic compounds in each extract was determined spectrophotometrically according to the method of Folin-Ciocalteau (SINGLETON; ROSSI, 1965). For reading on the spectrophotometer, a 250 µL aliquot of the sample was diluted and mixed with 125 µL of Folin-Ciocalteau reagent (0.1 N) and 625 µL sodium carbonate (7.5 g.100 mL^{-1}) after 20 min of reaction in the dark, 0.2 mL was transferred to a microplate and the absorbance was

read at 750 nm. The results were expressed in milligrams of gallic acid equivalents per liter of sample (mg. eq of gallic acid. mL^{-1}).

3.1.4. DETERMINATION OF ANTIOXIDANT ACTIVITY BY 2.2-diphenyl-1-picrylhydrazyl (DPPH).

The DPPH method is based on the determination of the ability to scavenging an antioxidant compound by the stable DPPH free radical in ethanolic medium in the UV-VIS region of λ = 515 nm (FÉRNANDEZ-PACHÓN et al., 2006).

The solutions DPPH (0.004 mg.100 mL^{-1}) were prepared and used only on the day of analysis, being the standard curve prepared with ascorbic acid solution in ethanol, with concentrations ranging from 0 to 200 μM. For each point of the standard curve, triplicate samples of absorbance were determined every 5 min, until obtain a regression coefficient (R^2= 0.991).

The radical scavenger activities of each sample were calculated according to the inhibition percentage of the DPPH • (% Inb) radical, as shown in Equation 2:

$$(\%III) = \frac{[II - II]}{II} * 100 \quad (IIIIIIII\ 2)$$

Where:

AC: Values of absorbance of DPPH solution in ethanol

AR: Values of absorbance of ascorbic acid + DPPH (reaction), respectively, at the end of the reaction.

The value of the antioxidant activity expressed in μM (Equivalent ascorbic acid-AA) from the regression coefficient of the calibration curve (equation 3):

$$IIII\ (\mu I°II) = (\%III - \frac{IIIIIIII}{IIIIIII(I2)}.) * II\backslash II\backslash III \quad (IIIIIIII\ 3)$$

3.1.5. ESTATISTIC ANALYSIS

All variances were compared using ANOVA and Tukey's test to find the best methodology for extraction of antioxidants and phenolic compounds. The data were processed in the program Statistica 7.0 in triplicates.

3.2. RESULTS

Gallic and ascorbic acid graphs were made to assess the polyphenol content and the antioxidant power between the methodologies of *G. lucidum* spore extracts. O R^2 was 0.9947 for gallic acid and 0.9951 for ascorbic acid in the calibration curves. Figure. **13** and Figure. **14**.

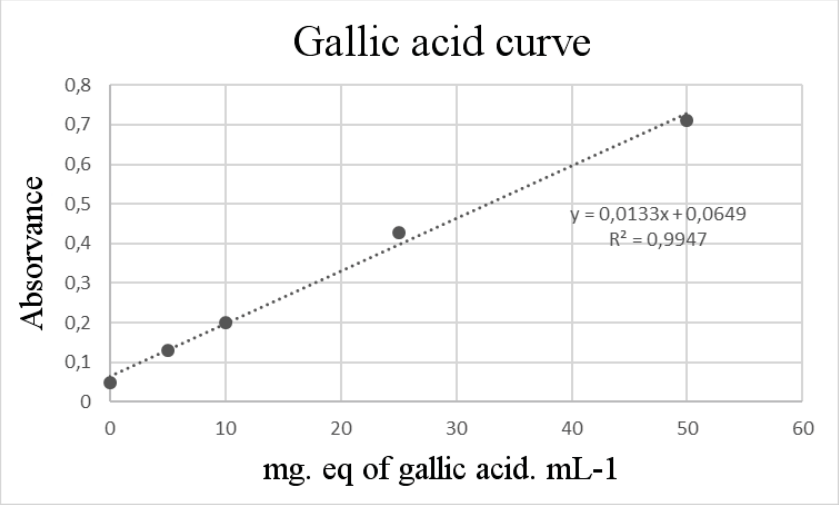


FIGURE. 13 - CALIBRATION CURVE OF ASCORBIC ACID TO ASESS POLYPHENOLS CONTENT.

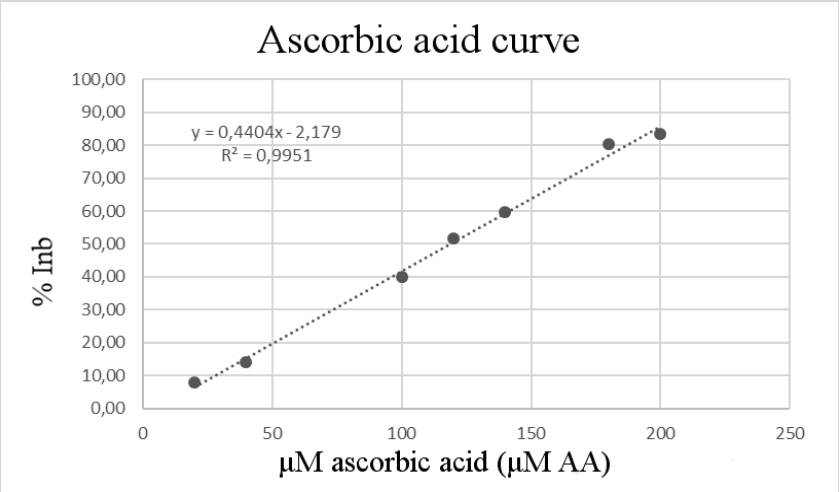


FIGURE. 14 - ASCORBIC ACID CALIBRATION CURVE TO ASSESS THE INHIBITION OF DPPH (%).

3.2.1. ULTRASONIC CELL DISRUPTOR METHOD

The group with the highest antioxidant activity was A1.E (07.50 ± 0.04 µM ascorbic acid, Figure. 8), and for total polyphenols, none of the groups presented values within the ascorbic acid curve. Table. 6. The nomenclatures can be seen in the Figure. **8** and Figure. **9**.

TABLE. 6 - RESULTS FOR ULTRASONIC CELL DISRUPTOR.

METHODOLOGY	NAME	% INB	µM_AA	mg_eq gallic acid/mL
Ultrasonic cell disruptor	A1.P	10.41±00.07^{AB}	01.40	n.d.
	A1.E	**14.53±00.04^{B}**	**07.50**	n.d.
	A2.S	3.73±00.02^{A}	0.0	03.10 ±00.09
	A2.E	n.d.	n.d.	n.d.
	A31.P	3.81±00.08^{A}	0.0	08.32 ±00.08
	A31.E	6.35±00.11 ^{AB}	0.0	03.55 ±00.05
	A32.P	13.63± 00.08 ^{AB}	06.17	n.d.
	A32.S	n.d.	n.d.	n.d.

Note: The table describes the methodology, extract name, percent inhibition of DPPH (% INB), micromolar of the sample in the ascorbic acid curve (µM_AA) and milligrams of gallic acid equivalents per milliliter (mg. eq. gallic acid.mL^{-1}), nd- not determined. The nomenclatures can be seen in the Figure. **8** and Figure. **9**.

3.2.2. MICROWAVE

The 10.C extract had the higher reducing power of DPPH (31.37 ± 0.07 µM ascorbic acid), followed by **5.B** (12.01 ± 0.07 µM ascorbic acid). The extracts with high presence of total polyphenols were **5.B** and **10.A** with 11.34 ± 0.7 and 24.83 ± 0.28 mg.eq of gallic acid.mL^{-1}, respectively. Table. 7. The nomenclatures can be seen in the Figure. **10**.

TABLE. 7 - RESULTS FOR MICROWAVE.

METHODOLOGY	NAME	% INB	µM_AA	mg_eq gallic acid/mL
MICROWAVE	**10.A**	**11.43±00.07^{A}**	**02.91**	**24.83 ± 00.28^{C}**
	10.B	17.13 ±00.03^{AB}	11.35	n.d.
	10.C	**30.65±00.07^{B}**	**31.37**	**n.d.**
	10.D	n.d.	n.d.	n.d.
	5.A	12.13±00.09^{A}	03.94	n.d.
	5.B	**17.58±00.07^{AB}**	**12.01**	**11.34 ± 00.07^{BC}**
	5.C	16.47±00.07^{A}	10.37	n.d.
	5.D	16.13±00.07^{A}	09.86	n.d.

Note: Methodology description, name of the extract with time 5 and 10 min, percentage of inhibition of DPPH (% INB), micromolar of the sample in the curve of ascorbic acid (µM_AA) and milligrams of gallic acid equivalents per milliliter (mg eq. gallic.mL^{-1}), nd- not determined. The letters highlighted in black are significant results. The nomenclatures can be seen in the Figure. **10**.

3.2.3. SOXLHET

Only S.E.L fraction presented antioxidant values for the S.E.L fraction of 10.67 ± 0.12 µM ascorbic acid and a polyphenol content of 5.29 ± 0.1 mg.eq of gallic acid.mL^{-1}, Table. 8. The nomenclatures can be seen in the Figure. **11**.

TABLE. 8 - RESULTS FOR SOXHLET.

METHODOLOGY	NAME	% INB	µM_AA	mg_eq gallic acid/mL
SOXHLET	EE	n.d.	n.d.	n.d.
	NB	n.d.	n.d.	n.d.
	S.E.L	10.67 ±00.12^{A}	01.78	05.29 ±00.01

Note: Methodology, extract name, percent inhibition of DPPH (% INB), micromolar of the sample in the ascorbic acid curve (µM_AA) and milligrams of gallic acid equivalents per milliliter (mg eq. gallic acid.mL^{-1}), nd- not determined. The nomenclatures can be seen in the Figure. **11**.

3.2.4. MACERATION EXTRACT

The macerated extract had 0.17 ± 0.07 mg_eq of gallic acid.mL^{-1} of total polyphenols and 18.4 ± 0.06 μM of antioxidant power ascorbic acid. Table. 9. The nomenclature can be seen in the Figure. **12**.

TABLE. 9 - RESULTS FOR THE CONTROL METHODOLOGY.

METHODOLOGY	NAME	% INB	μM_AA	mg_eq gallic acid/mL
Maceration	RM	21.90±00.06	18.40	00.17 ±00.03

The nomenclature can be seen in the Figure. **12**.

3.2.5. COMPARING METHODOLOGIES

Control, maceration (**RM**) and microwave extracts (**10.A** and **10.C**) were compared. Among the extracts, the best antioxidant power was found for **10.C** (100.60 ± 0.01 μM of ascorbic acid), followed by **Control** (94.48 ± 0.07 μM of ascorbic acid) and **RM** (67.57 ± 0.06 μM of ascorbic acid). The content of polyphenols was only important in the control extract (0.14 ± 0.01 mg of gallic acid in 1 g of GLE), Table. 10. The yields of the extracts were measured after drying the extracts and obtaining the dry weight, the **RM** extract was the most concentrated followed by **10.C** and **10.A**, Table. 11.

TABLE. 10 – COMPARISON OF METHODOLOGIES.
COMPARISON OF EXTRACT METHODOLOGIES: MACERATION, MICROWAVE AND CONTROL.

Extract	DPPH (μM AA)	mg gallic acid/1 g GLE dw
CONTROL	94.48 ±0.07 ^{A}	0.14 ± 0.01 ^{c}
10.C	100.60 ±0.01 ^{A}	n.d.
10.A	21.82 ±0.20 ^{B}	0.51 ± 0.01 ^{a}
RM	67.57 ±0.06 ^{AB}	0.05±0.01^{b}

Note: the letters show the statistical significance ($p < 0.05$).

TABLE. 11 - YIELD IN MG OF THE COMPARED EXTRACTS.

Extract	Yield(mg)	%
CONTROL	36.8	3.7
10.A	220.8	22.1
10.C	394.7	39.5
RM	641.2	64.1

3.3. DISCUSSION

The fraction with the highest antioxidant power was A1.E, because it contained 7.5 µM_AA (µM of antioxidant power in the ascorbic acid curve). This value was obtained when the *G. lucidum* spores were exposed to ultrasonic cell disruptor treatment. This result is confirm by ZHAO et al., 2014. He reports that the use of sonication could the extraction of polysaccharides, proteins and lipids. In addition, the A1.E fraction represents the fraction separated by ethanol after the precipitation of the supernatant. A1.E reflects a better result because ethanol separated several compounds responsible for the antioxidant effect. According SHI, 2016 ethanol is a common solvent that removes lipids and phenolic compounds. In addition, it is known that ethanol is used in the extraction of antioxidant compounds in various plants and foods (SULTANA et al., 2009).

The microwave fraction 10.C had an antioxidant power of 31.37 µM AA. The 10.C Fraction is the result of 10 min of exposure of the spores to the microwave. The spores were washed in ethanol and suspended in water for 24 h. It is possible that there is a separation of polysaccharides in the soluble fraction analyzed by the high antioxidant value and because the ethanol removed the lipids. Also, the microwave process is presented by several authors, who explain that the high temperatures produced by the microwave facilitate the release of compounds (ZHANG et al., 2008; HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012; ZHU et al., 2012).

Ethanol fraction obtained by the Soxhlet methodology was the only one with antioxidant power, 1.78 µM_AA. This happens since ethanol has an affinity for compounds such as fatty and phenolic acids (SHI, 2016). After several cycles in the Soxhlet system, the extraction of phenolic compounds was evident (5.29 ± 0.01 mg equivalents of gallic acid) and the antioxidant power may be related to them.

During maceration the antioxidant action was 21.9 ± 0.06 µM_AA. The alteration of the spore wall may increase the release of antioxidant compounds supported by others previously works

(HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012; SOCCOL et al., 2016). Maceration for 20 min was able to release more antioxidant molecules. For this reason, this methodology has an important antioxidant value compared to the other methodologies.

Also, the best antioxidant activity among the groups was for maceration and microwaves, because they presented the greatest reducing activities of DPPH.

Among the control samples, microwave (10.C) and maceration (RM) the best value within the ascorbic acid curve was the extract prepared for 10 min in the microwave (100,6 ± 0.01 μM_AA). Reports describe that chemical changes in the walls of the spores increase the antioxidant activity (LIN et al., 1995; MIN et al., 1998; YEN; WU, 1999). The microwave produces the expansion of the particles, contract and dehydrate (SINGH et al., 2012). Nevertheless, strong structures such as the wall of the spores of *G. lucidum* were altered and released compounds, which correspond mostly to α and β glucan polysaccharides with antioxidant activity (SYNYTSYA; NOVAK, 2014). The structure of the cell wall of several fungi is rich in heteroglycan, glycopeptide, mannoglucan and several peptides (BOWMAN; FREE, 2006; ZHANG et al., 2007). Many of these chemical structures are responsible for the anticancer effects and the stimulation of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase that eliminate several free radicals of cellular metabolism (HELENO; BARROS; MARTINS; JOÃO; et al., 2012). It is possible that beta glucans are released from the wall of the spores increasing the antioxidant power of the extract 10.C. However, more complex molecules such as glycopeptides that can be inside the extract can participate in the antioxidant effect.

The control extract presented an important content of polyphenols (0.14 ± 0.01 mg_eq gallic acid) and antioxidant activity (94.48 ± 0.07 μM_AA) by probably having liberated highly soluble carbohydrates. Also, because it released phenols with stable antioxidant structure that may have flavonones that stimulate vascular endothelial growth factor (VEGF) during angiogenesis and protect nervous system cells in rats during oxidative processes (HELENO; BARROS; MARTINS; JOÃO; et al., 2012).

In summary extraction in mg from 1 g of spores was best when macerated (RM: 641 mg) and microwave used (10.C: 394.7). Among the results, the importance of exposing the spores to a physical extraction process to increase the extracted molecules is observed. Also, the temperature and water during the extraction of molecules increase the antioxidant value.

3.4. CONCLUSIONS

For the extraction of molecules with medical potential from the spores of *G. lucidum*, it is important that they go through a rupture process followed by exposure to high temperatures, because the RM, control and 10.C extracts had an important antioxidant power.

Water at pH 7.0 was an effective solvent when compared with absolute ethanol, ethyl acetate and n-butanol because among the evaluated methodologies, the water-soluble extracts had a higher value of antioxidant power and polyphenols. The best concentration of *G. lucidum* spores in the extracts was 1 g in 16 mL because it increased the antioxidant values when they were compared in the ascorbic acid curve.

STEP 4: OPTIMIZATION AND PURIFICATION OF FRACTIONS OBTAINED FROM *Ganoderma lucidum* SPORES

The extraction and study of several lipids and polysaccharides of *G. lucidum* are important, because this mushroom has biomolecules active against several diseases such as arthritis, bronchitis, gastric ulcer, hyperglycemia, insomnia, nephritis, diseases with inflammatory courses and cancer (SLIVA, 2003, 2004b). They have antioxidant activity (HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012) decrease the migration of PC-3 cells in prostate cancer (SILVA et al., 2003), have triterpenes and ganoderic acids A, B, H, T, C2 and Me with hepatoprotective, antihypertensive, hypochondrial, anti-HIV-1 and antihistamine activities related in the literature (BOH et al., 2007; KEYPOUR et al., 2010). *Ganoderma lucidum* is known in traditional Chinese medicine and has been used in the form of extracts and spores for various medical treatments. This *Basidiomycota* fungus, of the *Ganodermataceae* family (BOH et al., 2007) has a ductile structure in its spores, this property makes them resistant and is considered one of the hardest structures to break in nature (ADASKAVEG; GILBERTSON, 1988; CHAU; WU, 2007; HENNICKE et al., 2016). Due to this difficulty several methodologies have been applied to breaking spores, e. g. micronizer, air currents, knife mills, press, enzymes, sonicator, mechanical chemical extraction by mill and use of reagents such as sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3) (HELENO; BARROS; MARTINS; JOÃO; et al., 2012; ZHU et al., 2012). However, despite of several studies regarding the breaking and description of several molecules of *G. lucidum* spores, the mechanical, chemical and enzymatic breaking produce variations in the release of compounds. Such variations are not well known in the literature. Therefore, it was important to carry out a study to analyze the alterations that can produce the fragmentation of the spore wall and which types of molecules can be extracted. Are there differences when applied several techniques as manual or chemical rupture in the spore wall? Does the extraction of biomolecules depend on how the spore wall has been fragmented? Because of these questions, the aim of this research was to compare three breaking techniques for *G. lucidum* spores. A mechanical manual breaking assisted mechanical breakage using different types of spheres and the use of microwaves. The best isolated and combined spore-breaking techniques

were then evaluated comparing the extracted content of proteins, carbohydrates, lipids and antioxidant power using FTIR, light microscopy, scanning electron microscopy and chemical characterization techniques.

4.1. MATERIAL AND METHODS

4.1.1. BREAKING OF *G. lucidum* SPORES

Different spore-breaking approaches of *G. lucidum* were compared using 1g of spores in each essay. In the first technique the spores were macerated manually for 20 min. In the second, were macerated with 20 g of spheres for 20 min. Spheres made with different materials as, such as steel/chrome, airsoft sphere, glass, silica gel and ceramics were used. In the third technique, the spores were exposed to the microwave for 10 min in 1200 watts, 127 V60 Hertz and 2450 MHz frequency (Carrefour Home, model HMO22E-13-127V).

The light microscope and Neubauer camera (American Optical Co.) of 0.1 mm depth were used to analyze the breakage. To each breakage analysis 0.1050 g of spores were used and then suspended in 30 mL of distilled water. A pipette Pasteur droplet was then placed in the Neubauer chamber and a count was performed under the microscope with a 40X lens. Counting was done in five (5) small squares and the samples were evaluated in quadruplicate, adding the data and applying the following equation to know the percentage of breakage:

$$\frac{IIIIII\ II\ IIIII\ I\ II\ IIIIIII\ I\ IIIIII}{IIIIII\ II\ IIIII\ III\ IIIIIII} x\ 100\ (IIIIIII\ 1)$$

After this analysis, one portion of the sample was used for scanning electron microscopy (SEM) to show what types of changes occurred in the spore walls. For this process, the samples of *G. lucidum* were dried in air stoves at 80 °C by 24 h. Then the samples were divided into two parts. The first was coated superficially with gold (Au) for microscopic analysis TESCAN VEGA3 LMU. The second part of the spores was stored for further analysis.

4.1.2. *G. lucidum* EXTRACTS PRODUCTION

Besides evaluating different spore-breaking techniques of *G. lucidum*, it was necessary to compare and characterize which methodology was best for the extraction of biomolecules. In the Table. 12 all the procedures are explained by major detail.

TABLE. 12 - METHODOLOGIES USED TO ELABORATE THE EXTRACTS CONTROL, RM, BR, MBR1 OF *G. lucidum*.

Extract	Breaking methodology	Extraction
CONTROL	Unbroken spores	The spores were diluted in 16 mL of pH 7 water and incubated for 24 h at 30 °C at 120 rpm.
RM	Manual maceration during 20 min of 1 g of spores.	
BR	1 g of spores macerated with the aid ofspheres.	
MBR1	1 g of spores exposed to microwave for 10 min in 1200 watts, 127 V60 Hertz and 2450 MHz frequency.	

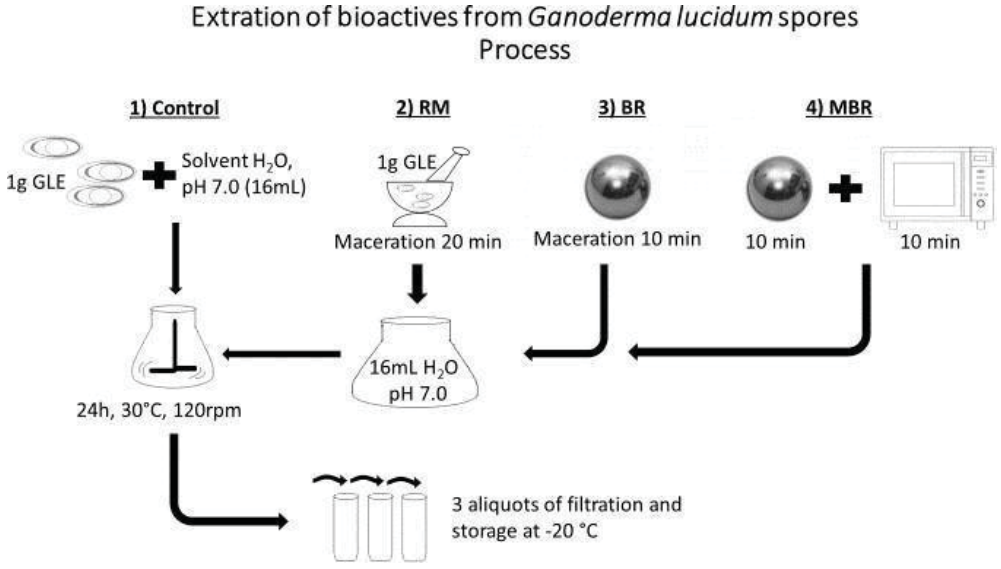


FIGURE. 15 - METHODOLOGIES OF EXTRACTION.

4.1.3. FUNCTIONAL GROUPS FOR MIDDLE INFRARED FOR FOURIER TRANSFORM (FTIR)

The functional groups present in the spore samples and aqueous extracts of *G. lucidum* were determined by means of the Medium Infrared Spectroscopy (MIS), on a VERTEX 70 (Bruker) equipment, with the DRIFT accessory with 64 scans, 4 cm^{-1} resolution, without losing atmospheric compensation in the region between 4000 and 400 cm^{-1}. The samples were crushed, pulverized and oven dried. At determination, about 20 mg were mixed and homogenized with 100 mg of spectroscopic potassium bromide (KBr) to carry out the measures. The analysis for regions of lipids, carbohydrates and proteins was performed with methodology of (CHEN et al., 2012; WANG et al., 2012).

4.1.4. FTIR ANALYSIS FOR SPORE BREAKAGE

Band stretches and functional groups in the FTIR spectrum need to be defined to recognize the contribution of proteins, carbohydrates and fatty acids (STUART, 2006). In the case of *G. lucidum*, literature describes that the breaking of the spore wall can be distinguished by regions. The first region 3000 to 2800 cm-1 receives the contribution of carbohydrates, lipids and proteins where the stretching band 2955 cm-1 is recognized, this band is due to asymmetric of CH3. The second and third region 1660 to 1600 cm-1 and 1400 to 1200 cm-1 the protein structure is identified with the bands 1642 cm-1 (Amide I) and 1240 cm-1 (unordered structures the Amide III) respectively. The fourth region 1100 to 1000 cm-1 where the carbohydrate band is in 1080 cm-1, this band is due to C – O stretching vibration. The fifth region 1750 to 1700 cm-1 for lipid contribution in the band 1746 cm-1, associated to C = O stretching vibration (CHEN et al., 2012). Due to the detectability of stretching bands, samples of spores that had breakage by light microscopy were taken and analyzed to describe wall changes.

4.1.5. FTIR ANALYSIS TO CHARACTERIZE EXTRACTS OF *G. lucidum*

The CONTROL (intact spores), manual maceration (RM), steel/chromium sphere with maceration (BR) and microwave with maceration plus steel/chromium sphere maceration (MBR1) extracts were analyzed by FTIR using stretching bands to describe changes in lipid, carbohydrate and protein extraction (STUART, 2006). Extracts were evaluated usingfive regions:

the first region 3000 to 2800 cm-1 (asymmetric stretching of CH3 and symmetric stretching of CH2), the second region 1660 to 1600 cm-1 (Amide I), the third region 1400 to 1200 cm-1 (α-Helix in the Amide III and unordered structures the Amide III), fourth region 1100 to 1000 cm-1 (C – O stretching if sugars) and the fifth region 1750 to 1700 cm-1 (C = O stretching and α/β unsaturated carboxylic acids). In each of the regions, a bandwidth peak was analyzed. The first region receives contributions of carbohydrates, lipids and proteins showing bands in 2925 (asymmetric stretching of CH2) and 2855 cm-1 (symmetric stretching of CH2), for the second and third region the protein structure in 1641 cm-1 (Amide I) and 1240 cm-1 (unordered structures the Amide III). The fourth sample shows structure of sugars, related to glucopyranose group in 1078 cm-1 (polisacharide band C - O) (BAO et al., 2001; GUO et al., 2009) and the fifth region receiving fatty acids and lipids contribution in 1746 cm-1 (C = O stretching) (CHEN et al., 2012; WANG et al., 2012).

4.1.6. CHEMICAL CHARACTERIZATION

4.1.6.1. TOTAL SOLUBLE PROTEIN (TSP) - BRADFORD METHOD

Bradford reagent (350 mg of R-Brilliant Blue/100 mL of 95% ethanol/200 mL of 88 % H_3PO_4) was prepared as stock solution, filtered on Whatman number 1. In test tube, 900 μL of Bradford's reagent and 100 μL of each sample were added and followed by vortex homogenates. The TSP concentration was determined by UV-VIS spectroscopy in the region of λ = 595 nm (SP2000 Visible SP 1105UV Spectrophotometer, China) (BRADFORD, 1976), with Bovine Serum Albumine (BSA) as standard.

4.1.6.2. TOTAL PHENOLIC COMPOUND CONTENT (TPC)

The TPC content in each extract was determined by using the method of Folin-Ciocalteau (SINGLETON; ROSSI, 1965). An aliquot of 250 μL of the sample diluted in 125 μL of Folin-Ciocalteau reagent (0.1 N) and 625 μL Na_2CO_3 (7.5 g/100 mL). After 20 min of reaction in the dark, 0.2 mL of the solution was transferred to a microplate, and a spectrophotometric determination was performed on UV-VIS equipment at λ = 750 nm (BioTek`s PowerWave HT Microplate Spectrophotometer). The results were expressed in milligrams (mg) gallic acid equivalent/ L sample (R^2 0.9947).

4.1.6.3. TOTAL CARBOHYDRATE CONTENT (TCT) - ANTHRONE'S METHOD

The anthrone suspended in sulfuric acid is a method that is used to determine carbohydrates and starches in plants. These sugar compounds can be identified by the color difference in the reaction.

The antrhone solution was prepared at the time of use (0.2 g anthrone/100 mL H_2SO_4). 900 µL of solution was added to a test tube in 100 µL of the sample. The solutions were homogenized in vortex, and placed in a water bath at 100 °C for 3 min, then the TCT concentration was determined by UV-VIS spectroscopy in BIOTEK equipment with λ = 620 nm (SP2000 Visible SP 1105UV Spectrophotometer, China) (LOEWUS, 1952).

4.1.6.4. COMPOSITION ANALYSIS

The extracts was freeze-dried and (2 mg) hydrolyzed in 2 M of Trifluoroacetic acid (TFA) at 110 °C for 2 h (DONG et al., 2012). The determination of monosaccharide composition was carried out by reductive hydrolysis (STEVENSON; FURNEAUX, 1991) using extra reducing agent [borane.4-methylmorpholine complex (4-MMB)] before and after pre-hydrolysis and hydrolysis steps (FALSHAW; FURNEAUX, 1994). The hydrolytic process was performed as described by (FERREIRA et al., 2012). After acetylation (STEVENSON; FURNEAUX, 1991) the alditol acetate derivatives were analyzed by Gas chromatography–mass spectrometry (GC–MS) in the same conditions described by [(FERREIRA et al., 2012) and were identified by their typical electron-impact fragmentation profiles and GC retention times (JANSSON et al., 1976). A double hydrolysis-reductive amination method was used to determine the absolute configuration of the monosaccharide constituents (NAVARRO; STORTZ, 2003). Chiral 1-amino-2-propanol was used to determine the ratio of d- and l-galactose and its 6-*O*-methyl derivative, whereas the configuration of 2-*O*-methylgalactose, 3,6-anhydrogalactose and their 2-*O*-methyl derivatives were determined using chiral α-methylbenzylamine. The resulting alditol acetates derivatives were analyzed by GC–MS using the conditions described by (FERREIRA et al., 2012).

4.1.6.5. DETERMINATION OF ANTIOXIDANT ACTIVITY BY 2.2-diphenyl-1-picrylhydrazyl (DPPH).

The DPPH method is based on the determination of the ability to scavenging an antioxidant compound by the stable DPPH free radical in ethanolic medium in the UV-VIS region of λ = 515 nm (FÉRNANDEZ-PACHÓN et al., 2006).

The solutions DPPH (0.004 mg.100 mL^{-1}) were prepared and used only on the day of analysis, being the standard curve prepared with ascorbic acid solution in ethanol, with concentrations ranging from 0 to 200 μM. For each point of the standard curve, triplicate samples of absorbance were determined every 5 min, until obtain a regression coefficient (R^2= 0.991).

The radical scavenger activities of each sample were calculated according to the inhibition percentage of the DPPH • (% Inb) radical, as shown in Equation 2:

$$(\%III) = \frac{[II - II]}{II} * 100 \quad (IIIIIIII\ 2)$$

Where:

AC: Values of absorbance of DPPH solution in ethanol

AR: Values of absorbance of ascorbic acid + DPPH (reaction), respectively, at the end of the reaction.

The value of the antioxidant activity expressed in μM (Equivalent ascorbic acid-AA) from the regression coefficient of the calibration curve (equation 3):

$$IIII\ (\mu I°II) = (\%III - \frac{IIIIIIII}{IIIIIII(I2)}.) * IIIIIIII\ (IIIIIIII\ 3)$$

4.1.6.6. CONTENT OF MICRO AND MACRO NUTRIENTS BY METALLIC ATOMIC OPTICAL EMISSION IN INDUCTIVELY COUPLED PLASMA-ICP-OES

The used glassware was previously placed in a solution of nitric acid (HNO_3) 20% (m/v) for 24 h, next, it was washed with deionized water. All reference solutions were prepared with

deionized water with resistivity of 18.2 MΩ cm^{-1}, using a Milli-Q system of water purification, (Milipore, Berford, MA, USA), coupled to a distiller of water glass model 534.

One gram of material was transferred to a volumetric flask of 250 mL followed by adding 5 mL of concentrated HNO_3 and 4 mL of hydrogen peroxide, H_2O_2 (30%). After reaching boiling-point, the system was left in heating for 40 min. Then, the solution was allowed to cool down to room temperature, and it was filtered on a filter paper quantitatively. Finally, it was completed to 100 mL in a volumetric flask. The same procedure was performed for all samples. The three samples from the control group (whites) were prepared in the same way.

Quantifying the levels of metals (micro and macro nutrients) by ICP-OES was performed on equipment VARIAN, model 720 ES; in conjunction with the axial arrangement and detector of solid state.

4.1.6.7. LIPID CONTENT (LT) IN GLS

1.0 g sample of broken and not broken spores were assessed in triplicate for determination of LT. In a Falcon tube, 5 mL chloroform ($CHCl_3$), 10 mL of CH_3OH and 4 mL of water were added. Then, it is placed in a shaker for 30 min at 120 rpm. Next, 5 mL of $CHCl_3$ and 5 mL of Na_2SO_4 (1.5%) and the samples were agitated in a vortex for 2 min, and centrifuged at 1500 *g* for 2 min. After extraction, the solvent was evaporated in an oven at 50 ºC for 8 h (BLIGHT; DYER, 1959).

4.2. STATISTICS

Results are presented as means ± standard deviation (S.E.) Significance at ($p < 0,05$) was assessed by one- and two-way analysis of variance (ANOVA) using the statistical package Statistica.7.0 (2002) for Windows.

4.3. RESULTS

The results will be presented in two parts, the first to define the best technique for breaking *G. lucidum* spores and the second on extraction and characterization of *G. lucidum* extracts obtained through different extraction methodologies.

4.3.1. BREAKING OF *G. lucidum* SPORES

Manual maceration of *G. lucidum* spores did not produce any visible breakage under the evaluation of light microscopy (MO). The maceration with the aid of 1 mm diameter steel/chrome spheres produced a breakage rate of 97.58 ± 1.41%. Other types of spheres, such as airsoft sphere, glass, ceramics and silica gel, did not produce any breakage of the spores Figure. 16

After obtaining the mechanical break with the steel/chrome spheres, different maceration times of 5, 10 and 15 min were tested. For each time a break up percentage was obtained 89.10 ± 6.66%, 97.48 ± 3.61% and 98.58 ± 2.89% respectively. Ten min of maceration was sufficient to obtain almost 100% breakages of the spores.

The following methodology, microwave at 1200W for 10 min, did not produce visible light microscopic rupture, but did observe slight fissures in the spore wall by electron microscopy (SEM) Figure. 17. In the same way, when the images of the maceration technique were analyzed with and without the aid of steel/chrome spheres, the best breakage obtained was using the spheres because it fragmented the entire wall.

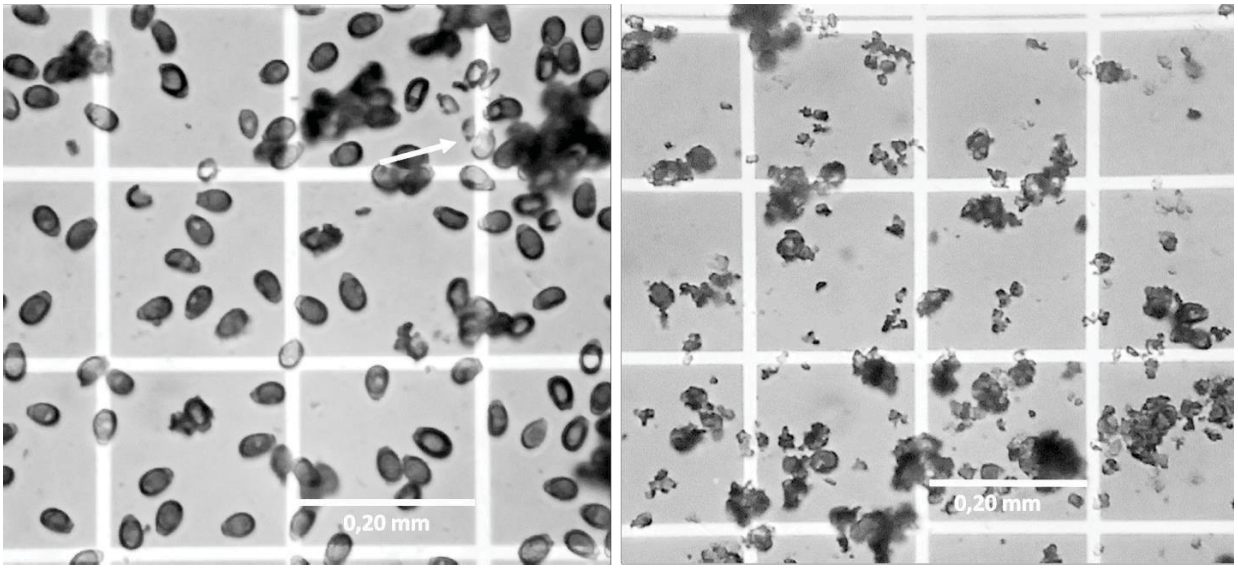


Figure. 16 - *G. lucidum* spores seen through light microscopy.

Left- spores macerated for 20 min (6% break); arrow shows the breaking of some spores. Right- spores with total wall breakage. The breaking was produced by maceration with help steel/chrome spheres for 10 min.

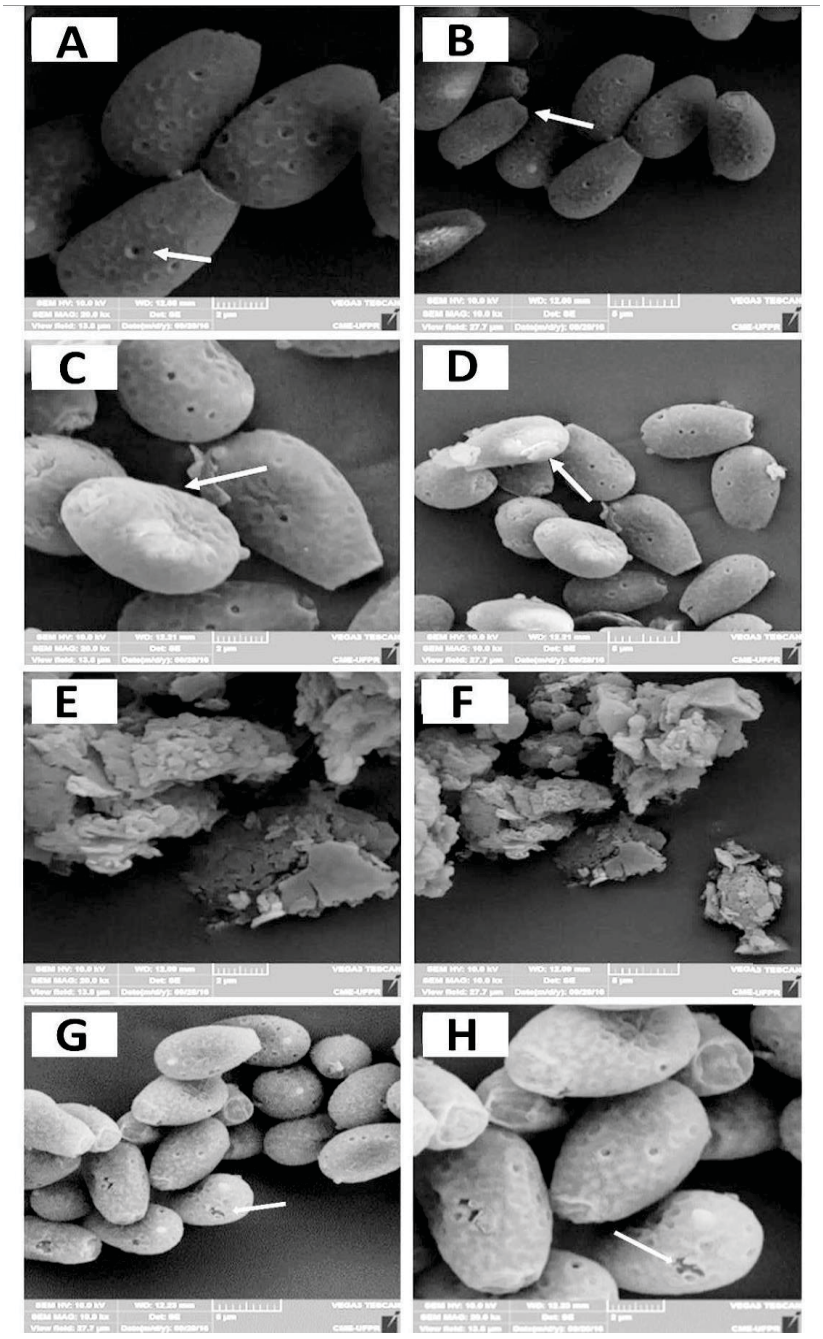


Figure. 17 - spores g. lucidum.

Breakage of the spore walls analyzed by SEM. Spores with scale bars 5 µm, HV 10 kV, MAG 10 kx, view field 27.7 µm and Det SE. a-b) control spores without any breakage (WD 12.08 mm). c-d) mild fracture and spore wall sinking by the maceration technique for 20 min (WD 12.21 mm). e-f) complete fragmentation of the spore wall when macerated with steel/chrome spheres (WD

12.09 mm). g-h) fissures and sinking wall of all spores exposed to 10 min of microwave (WD 12.24 mm).

4.3.2. ANALYSIS OF SPORE BREAKING BY FTIR

The wavelengths found for the samples obtained by maceration with the aid of steel/chromium spheres showed changes in the structure of fatty acids, lipids and proteins. This due to difference in asymmetric stretching of CH3 from band 2955 cm-1 with found band 2967 cm-1, indicating difference in the general extracts structure. The spectral vibration from unordered structures the Amide III in 1240 cm-1 had change to 1231 cm-1. Contributions of C = O spectral vibration from fatty acids and lipids in 1746 cm-1 were found in 1738cm-1. For microwave exposed spores, the changes more important were observed in spectral vibration for proteins. This due to stretching vibration from unordered structures the Amide III and Amide I, the spectral vibrations were found in 1630cm-1 and 1629cm-1 relatively. The change in the different wavelengths shows that there was a rupture in the chemical structure of the walls of the G. lucidum spores (Figure. 18, Figure. 19, Table. 13)

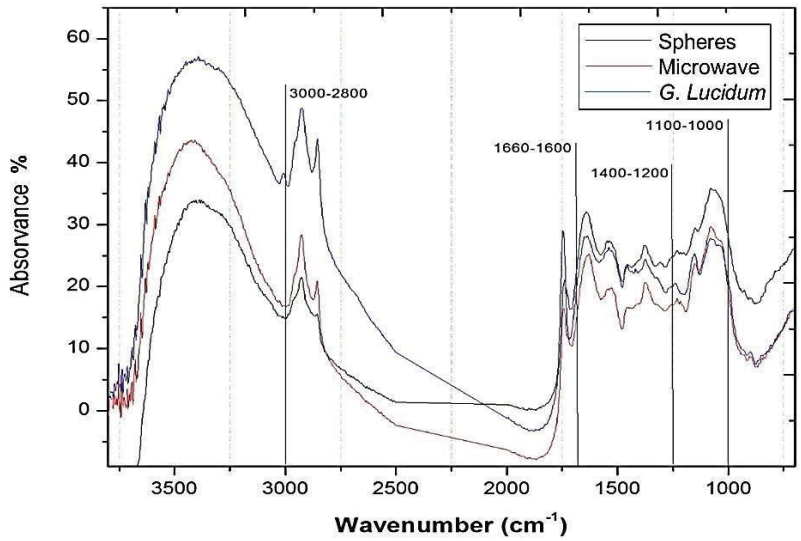


FIGURE. 18 – FTIR SPECTRUM.

FTIR SPECTRUM OF THE *G. lucidum* SPORES TREATED BY MACERATION BY: SPHERES STEEL/CHORME AND MICROWAVE. *G. lucidum* – INTACT SPORES. OBSERVE THE FOUR REGIONS OF THE SPORE WALL ANALYSIS: 3000-2800 CM^{-1}, 1660-1600CM^{-1}, 1400-1200CM^{-1} E 1100-1000CM^{-1}.

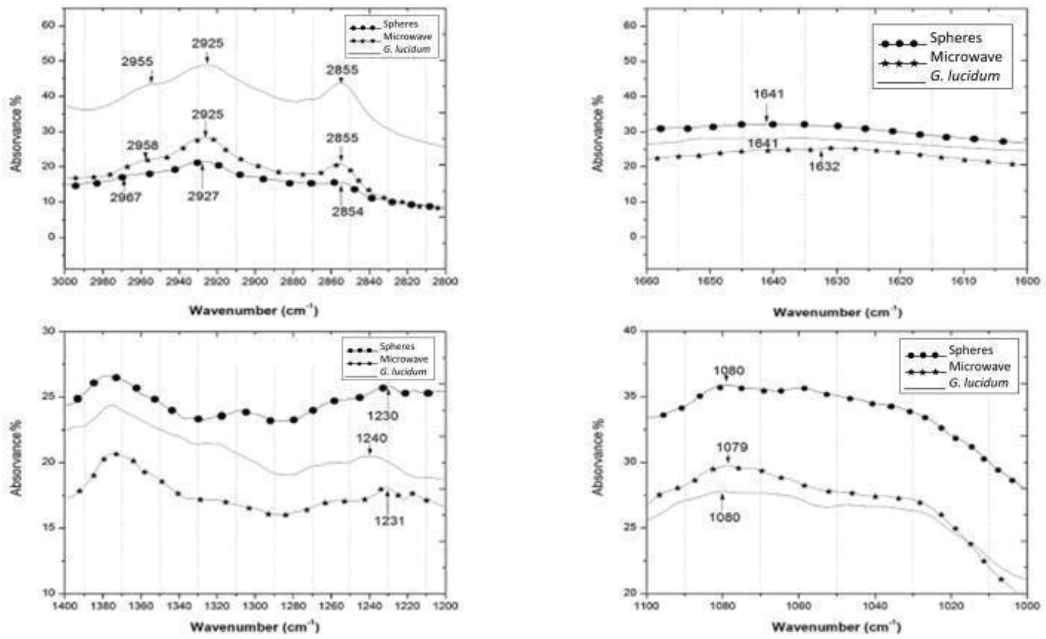


FIGURE. 19 - FTIR SPECTRA SHOW CHANGES IN THE WALL OF *G. lucidum* SPORES WITH BREAKAGE TREATMENTS (STEEL/CHROMIUM SPHERES AND MICROWAVES).
UPPER LEFT FIGURE - STRETCH 2955 CM^{-1} FOR FATTY ACIDS, LIPIDS AND PROTEINS FROM THE SPORE WALL. TOP RIGHT FIGURE - THE 1641 CM^{-1} STRETCH SHOWS CHANGES IN THE PROTEINS OF THE SPORE WALL. BELOW LEFT - SHOW CHANGES IN PROTEINS IN STRETCH 1240 CM^{-1}. BOTTOM RIGHT- SHOWS THE CHANGES IN THE CARBOHYDRATE BAND IN 1080 CM^{-1}.

4.3.3. FTIR EVALUATION FOR *G. lucidum* EXTRACTS

After evaluating the breakingtechniques, there was a need to assess the extraction capacity of biomolecules, which in this case the water could extract for each type of spore extraction methodology. For the analysis of the CONTROL extract lipids, fatty acids and proteins were observed in 2932 cm^{-1}, proteins in 1641 and 1228 cm^{-1} and sugars in 1079 cm^{-1}. For RM proteins were extracted in 1635 cm^{-1} and carbohydrates in 1079 cm^{-1}, for BR lipids, fatty acids and proteins in 2929 and 2852 cm^{-1}, proteins in 1641 and 1235 cm^{-1} and sugars in 1078cm^{-1}. Finally, for MBR1

the contribution of fatty acids, proteins and lipids was 2933 cm^{-1}, for proteins in 1647 and 1227 cm^{-1} and for carbohydrates in 1078 cm^{-1}.

In addition to identifying stretching bands for different compounds, by FTIR it is possible to compare the proportions between carbohydrates, lipids and proteins in the attempt to understand even more what type of compounds were extracted in each methodology. For this purpose, the band heights (H) of the stretches were analyzed in 1748cm^{-1} for α and β lipid esters (C=O, baseline: 1771-1723 cm^{-1}), in 1642 cm^{-1} for amide I protein (baseline: 1700-1581cm^{-1}) and 1079cm^{-1} for carbohydrates (baseline: 1130-951cm^{-1}) according to the methodology of literature (YANO et al., 2000; WANG et al., 2012), where the proportions are evaluated by comparing proteins with lipids (H1642/H1748), carbohydrates in relation to proteins (H1079/H1642) and carbohydrates between lipids (H1079/H1748). We found significantly a higher proportion of proteins over lipids for the CONTROL extract, a higher proportion of carbohydrates over proteins for the MBR1 extract and carbohydrates over lipids for CONTROL. Table. 14 and Table. 15, Figure. 20.

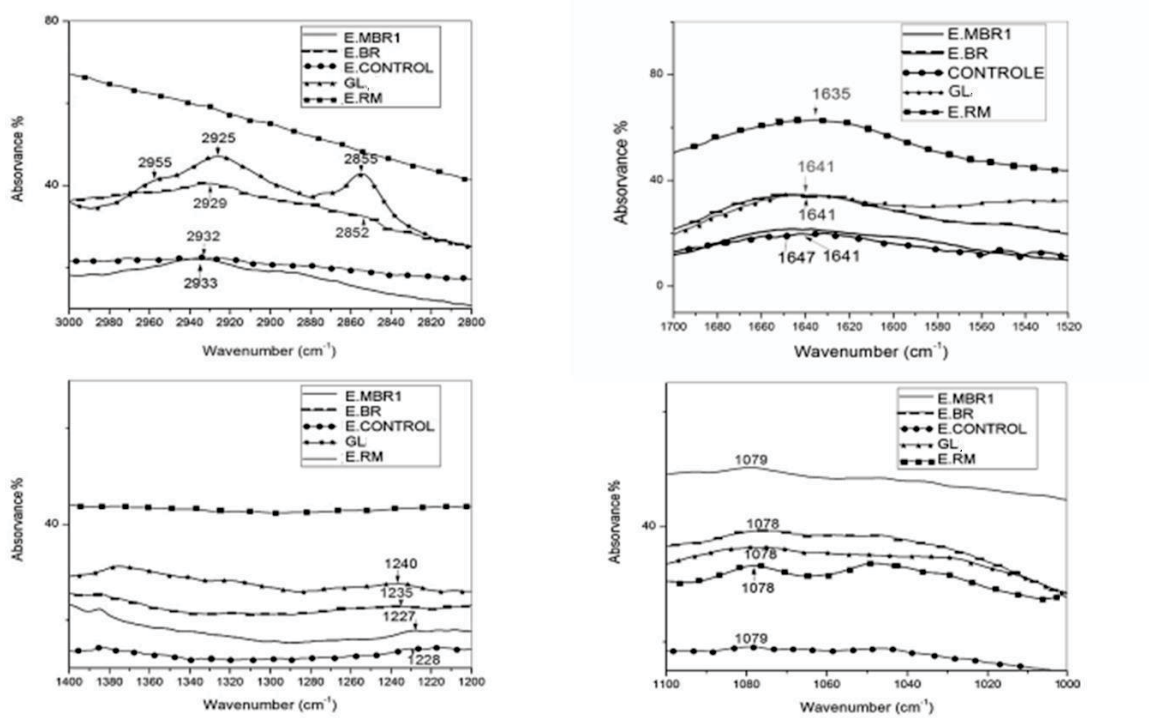


FIGURE. 20 - FTIR SPECTRA SHOW CHANGES IN THE EXTRACTS OF *G. lucidum* SPORES WITH TREATMENTS (MBR1, RM, BR, CONTROL AND *G. lucidum* SPORES).
UPPER LEFT - STRETCH 2932 CM^{-1} FOR FATTY ACIDS, LIPIDS AND PROTEINS BETWEEN EXTRACTS.
TOP RIGHT FIGURE - THE 1641 CM^{-1} STRETCH SHOWS THE CHANGES IN THE PROTEINS BETWEEN

EXTRACTS. BELOW LEFT - SHOWS CHANGES IN PROTEINS IN STRETCH 1228 CM^{-1}. BOTTOM RIGHT- SHOWS THE CHANGES IN THE CARBOHYDRATE BAND IN 1079 CM^{-1}.

TABLE. 13 - DIFFERENCES BETWEEN WAVELENGTHS IN THE WALLS OF UNBROKEN SPORES AND BROKEN SPORES WITH STEEL/CHROME SPHERES EXPOSED TO MICROWAVES.

GLS Stretching region	**GLS control**	**GLS Broken Wall**	**Wavenumber**	**Functional groups**
3000-2800 cm^{-1}	2955 cm^{-1}	Steel/Chrome spheres	2967 cm^{-1}	- Fatty acids, lipids and proteins.
		Microwave	2967 cm^{-1}	-Asymmetric stretching of CH_3
1660-1600 cm^{-1}	1642 cm^{-1}	Steel/Chrome spheres	1641 cm^{-1}	Proteins -Assignment: Amide I
		Microwave	1629 cm^{-1}	
1400-1200 cm^{-1}	1240 cm^{-1}	Steel/Chrome spheres	1231 cm^{-1}	-Proteins -Assignment: Unordered structures the Amide III
		Microwave	1230 cm^{-1}	
1100-1000 cm^{-1}	1080 cm^{-1}	Steel/Chrome spheres	1080 cm^{-1}	Sugars - Assignment: Polissacharide band C - O
		Microwave	1079 cm^{-1}	
1750-1700 cm^{-1}	1746 cm^{-1}	Steel/Chrome spheres	1738 cm^{-1}	-Fatty acids and lipids. - Assignment: C = O stretching
		Microwave	1743 cm^{-1}	

Data compared with papers (CHEN et al., 2012; WANG et al., 2012).

TABLE. 14 - PRESENCE OF DIFFERENT BIOMOLECULE EXTRACTION BANDS IN CONTROL, RM, BR AND MBR1 EXTRACTS.

GLS Stretching region	**GLS control**	**GLS Broken Wall**	**Wavenumber**	**Functional groups**
3000-2800 cm^{-1}	2925 cm^{-1}	CONTROL	2932 cm^{-1}	-Fatty acids, lipids and proteins.
		RM	-----	-Asymmetric stretching of CH_2 for 2925 cm^{-1}
		MBR1	2933 cm^{-1}	
		BR	2929 cm^{-1}	
	2855 cm^{-1}	CONTROL	------	-Symmetric stretching of CH_2 for 2855 cm^{-1}
		RM	------	
		MBR1	------	
		BR	2852 cm^{-1}	
1660-1600 cm^{-1}	1641 cm^{-1}	CONTROL	1641 cm^{-1}	-Proteins
		RM	1635 cm^{-1}	-Assignment: Amida I.
		MBR1	1647 cm^{-1}	
		BR	1641 cm^{-1}	
1400-1200 cm^{-1}	1240 cm^{-1}	CONTROL	1228 cm^{-1}	-Proteins
		RM	-----	-assignment: Unordered structures the Amide III
		MBR1	1227 cm^{-1}	
		BR	1235 cm^{-1}	
1100-1000 cm^{-1}	1078 cm^{-1}	CONTROL	1079 cm^{-1}	-Sugars
		RM	1079 cm^{-1}	-Assignment: Polisacharide band C – O.
		MBR1	1078 cm^{-1}	
		BR	1078 cm^{-1}	
1750-1700 cm^{-1}	1746 cm^{-1}	CONTROL	------	-Fatty acids and lipids.
		RM	------	-Assignment: C = O stretching.
		MBR1	------	
		BR	------	

Data compared with papers (CHEN et al., 2012; WANG et al., 2012).

TABLE. 15 – RATIO CHAINS.

MEAN VALUES OF THE RATIO CHAINS H1642/H1748, H1079/H1642, H1079/H1748 OF *G. lucidum* SPORE IN EXTRACTS.

RATIOS	**CONTROL**	**BR**	**MBR1**	**RM**
H1642/H1748	01.18	n.d.	n.d.	n.d.

H1079/H1642	01.23[D]	00.83[C]	40.51[E]	00.26[B]
H1079/H1748	01.46	n.d.	n.d.	n.d.

Different letters in rows indicate statistically significant differences at $p<0.05$. **n.d:** not determined

4.3.4. ANALYSIS OF PROTEIN, ANTIOXIDANTS, CARBOHYDRATES AND POLYPHENOLS CONTENT

Without importing the methodology of extraction, the extracted proteins were among 12.24 and 15.55 mg of 1g of spores, without statistical difference. Table 6.5. For polyphenols the extract MBR1 was statistically the most significant with 2.21 ± 0.01 mg extracted of 1 g of broken spores, followed by BR with 1.79 ± 0.01 mg of 1g of spores, while CONTROL and RM did not have detectable levels. In the analysis for the methodology of anthrone the extract with major extraction of carbohydrates was MBR1 with 19.80 ± 0.03 mg of 1g of spores of *G. lucidum*. The antioxidant power, the extract with major power of inhibition was BR with 57.22 ± 0.09 % (Table. 16).

During the monosaccharide analysis, extraction depended directly on the methodology. Fucose and arabinose were absent in the control. Rhamnose and fucose were not detected in MBR1 and RM. Arabinose was not present in BR. In BR, galactose was the second more abundant. MBR1 has quite same proportion of arabinose and mannose. There were not equal proportions of monosaccharides in RM although xylose was the second abundant. Glucose was abundant in all the extracts, however, in the extracts of BR and MBR1 were found other abundant monosaccharides as galactose and mannose respectively. These two extraction methods had more effectiveness to isolate mayor heterogeneity of monosaccharides (Table. 16). Monosaccharides found correspond to those reported by (SOCCOL et al., 2016; YUE et al., 2008).

TABLE. 16 - CONTENT OF PROTEINS, POLYPHENOLS, CARBOHYDRATES, ANTIOXIDANT POWER AND MONOSACCHARIDES IN THE EXTRACTS CONTROL, RM, BR AND MBR1.

	PROTEINS mg/1 g GLS	**POLYPHENOLS mg / 1 g GLS dw**	**CARBOHIDRATES mg / 1 g GLS dw**	**DPPH %Inb**
CONTROL	12.24 ± 0.09 ^{A}	n.d.	01.24 ± 0.03^{B}	46.83 ± 0.08 ^{AB}
RM	15.55 ± 0.05 ^{A}	n.d.	11.92 ±0.01 ^{A}	47.85 ± 0.07 ^{AB}
BR	13.08 ± 0.00 ^{A}	1.79 ± 0.01^{A}	11.26 ±0.06 ^{A}	57.22 ± 0.09 ^{B}
MBR1	14.03 ± 0.06 ^{A}	2.21 ±0.01^{B}	19.80 ±0.03 ^{C}	45.13 ± 0.03 ^{A}

*a, b show statistical differences between extracts. A, B show statistical differences between samples. **n.d:** not determined

MONOSACCHARIDES	BR %	MBR1%	RM%	CONTROL%
Rhamnose	1.761	n.d.	n.d.	4,911
Fucose	4.194	n.d.	n.d.	n.d.
Arabinose	n.d.	11.726	1.332	n.d.
Xylose	3.904	1.415	9.880	10.925
Mannose	10.157	12.076	4.390	5.831
Glucose	62.190	73.545	77.738	67.746
Galactose	17.215	1.238	6.336	9.362

n.d: not determined

4.3.5. MACRONUTRIENTS AND MICRONUTRIENTS

Due to the use of steel/chromium spheres, it was necessary to analyze the heavy metal content of MBR1 and BR extracts. The metal content of unbroken *G. lucidum* spores and broken spores with spheres were evaluated. MBR1 and BR extracts presented less than 0.01 mg/kg for metals V, Se, Pb, Ni, Mo, Cd, Cu e Co. The MBR1 extract had the lowest B content (9.95 mg/kg) between the two extracts.

High values for metals were found in broken spores: Al (1071.53 ± 00.14 mg/kg), B (57.75 ± 00.01 mg/kg), Ba (57.75 ± 00.00 mg/kg), Ca (30.31 ± 00.19 mg/kg), Fe (2321.24 ± 00.09 mg/kg), K (2349.59 ± 00.39 mg/kg), Mn (41.28 ± 00.00 mg/kg), P (1886.87 ± 00.08 mg/kg) and Zn (99.96 ± 00.00 mg/kg). Broken spores with steel/chromium spheres had slightly higher Cu (17.34 ± 00.01 mg/kg) content compared to the unbroken spore content (Cu: 16.08 ± 00.01 mg/kg). Table. 17.

The Na values (1053.15 ± 00.06 mg/kg) were higher for intact spores and lower for MBR1 (189.91 ± 00.04 mg/kg). The integral and broken walls of the spores had less than 0.01 mg/kg of Co, Cd, Mo, Ni, Se e V. All spore and extract samples had low contents of Co e Pb (<0.01 mg/kg). Table. 17.

TABLE. 17 - MACRONUTRIENTS AND MICRONUTRIENTS CONTENT IN EXTRACTS AND SPORES OF *G. lucidum*.

Metal	MBR1 * (mg/kg)	*Spore of G. lucidum* (mg/kg)	BR * (mg/kg)	*Spores of G. lucidum* broken (mg/kg)
Al	30.18 ± 00.02^{A}	69.57 ± 00.02^{A}	23.82 ± 00.01^{A}	1071.53 ± 00.14^{B}
B	09.45 ± 00.01^{B}	40.45 ± 00.01^{A}	24.92 ± 00.01^{A}	57.75 ± 00.01^{C}
Ba	00.90 ± 00.00^{A}	05.35 ± 00.00^{A}	24.92 ± 00.00^{A}	57.75 ± 00.00^{B}
Ca	162.99 ± 00.01^{A}	119204 ± 00.01^{B}	270.05 ± 00.03^{A}	3009.31 ± 00.19^{C}

Co	< 0.01	< 0.01	< 0.01	< 0.01

Cu	< 0.01	16.08 ± 00.01[A]	< 0.01	17.34 ± 00.01[A]

Cd	< 0.01	< 0.01	< 0.01	< 0.01
Fe	06.85 ± 00.00^{A}	51.43 ± 00.00^{A}	13.30 ± 00.00^{A}	2321.24 ± 00.09^{B}
K	114.44 ± 00.03^{A}	1976.34 ± 00.21^{B}	152.70 ± 00.04^{A}	2349.59 ± 00.39^{C}
Mg	21.32 ±^{A}	277.36 ± ^{B}	42.40 ± ^{AB}	899.30 ± ^{C}
Mn	02.16± 00.00^{A}	17.91 ± 00.00^{B}	02.79 ± 00.00^{A}	41.28 ± 00.00^{C}
Mo	< 0.01	< 0.01	< 0.01	< 0.01
Na	189.91 ± 00.04^{A}	1053.15 ± 00.06^{C}	233.52 ± 00.04^{A}	780.01 ± 00.05^{B}
Ni	< 0.01	< 0.01	< 0.01	< 0.01
P	153.78 ± 00.03^{A}	1814.20 ± 00.15^{B}	199.24 ± 00.03^{A}	1886.87 ± 00.08^{C}
Pb	< 0.01	< 0.01	< 0.01	< 0.01
Se	< 0.01	< 0,01	< 0.01	< 0.01
V	< 0.01	< 0.01	< 0.01	< 0.01
Zn	05.68 ± 00.00^{A}	61.01 ± 00.01^{AB}	09.87 ± 00.00^{A}	99.96 ± 00.00^{B}

*a, b- show statistical differences between extracts. A, B show statistical differences between samples.

4.3.6. LIPID CONTENT IN SPORES

Determination of lipids was performed only for broken and unbroken *G. lucidum* spores, which were 25.09 ± 3.65 % and 7.0 ± 1.41 % respectively, showing greater release of lipids after spore rupture with assisted maceration with spheres.

4.4. DISCUSSION

The spores of *G. lucidum* have two internal caps: *exospoirum* and *myxosporium* (ADASKAVEG; GILBERTSON, 1988; HENNICKE et al., 2016). These structures have a high ductility, property that provides with the spore resistance (CHAU; WU, 2007). Due to this the spores are one of the structures most difficult to break, reason for which on the market different methodologies of breach have developed like: ultrasonography, ultra-fine grinding, high-speed centrifugal shearing pulverizer and enzymatical. With these methodologies a break-up is obtained among 80 and 100 % of the spores (MIN et al., 2000; BAO et al., 2002; LIU et al., 2002; MA et al., 2007; YUE et al., 2008). Nevertheless, it is necessary complex equipment whereas the maceration with aid of spheres of steel/chrome shows an easy and more economical way of break-up for the spores of *G. lucidum*.

Though the break of the spores not always is visible for microscopy of light, such it is the case of the exhibition of these to the microwave. Several methodologies of break can be analyzed and compared using the stretching bands in 2955, 1642, 1240, 1080 y 1746 cm^{-1}. Because, so much the break with the spheres of steel/chrome and the break of the wall with the microwave they

showed alterations in the proteins of the wall in the bands1642 y 1240 cm^{-1}. The change in structures of lipid occurred in the bands 2955 y 1746 cm^{-1}. This result shows that different methodologies of break of spores of *G. lucidum* must be studied because they are different biomolecules that are extracted from the spores.

FTIR's use for analysis of the extracts CONTROL, RM, MBR1 and BR can compare the structure of the walls of the spores and the biomolecules that were extracted for every extract. The stretching bands 2925, 2855, 1641, 1240, 1078 y 1746 cm^{-1} are useful to evaluate the principal compounds found in the spores because they concern to contributions of lipids, proteins and carbohydrates. The most soluble compounds under the studied conditions were the carbohydrates and some proteins. Also, they can be compared proportionally the content of the principal stretching bands in 1748, 1642 and 1079 cm^{-1} related with lipids, stretching of amide I and carbohydrates respectively. The extract MBR1 showed a major proportion for carbohydrates according to the studied regions demonstrating that the tool FTIR can be used to evaluate the breach of the spores and the extraction of its biomolecules.

The results of the chemical analyses show that the extracts did not present important differences in the extraction of proteins. Several proteins *G. lucidum* are important because they have function immunomodulatory. In medicine, several proteins have a similar structure with the immunoglobulin superfamily. This type of structures has a role in treatments for cancer and other biological activities. The proteins can be formed by several amino acids like: serine, alanine, glycine, threonine, aspartic acid, glutamic acid, proline y valine (WAGNER et al., 2011; LI, D. et al., 2016).

For the chemical quantification of polyphenols, the methodology used in extracts MBR1 and BR favored the amount extracted of phenols. The spores of *G. lucidum* report in the literature an amount of phenols of 0.61 ± 0.05mg/100g of dry weight (MIN et al., 2000; HELENO; BARROS; MARTINS; JOÃO; et al., 2012). This amount is less than that found in extracts MBR1 and BR. Polyphenols are important because they have lanostane-type triterpenes with function against several types of cancer cells (MIN et al., 2000; HELENO; BARROS; MARTINS; JOÃO; et al., 2012).

The extraction of carbohydrates for the MBR1 extract was higher than the reported of8.65 ± 0.46g of 100 g of dried spores (HELENO; BARROS; MARTINS; JOÃO; et al., 2012). *G. lucidum* carbohydrates are important in antitumor therapies due to their polysaccharides, which are

β-D-glucan chains (DONG et al., 2012). MBR1 y BR these two extraction methods had more effectiveness to isolate a mayor heterogeneity of monosaccharides. The most common monosaccharides correspond to those reported in the literature (YUE et al., 2008; SOCCOL et al., 2016) and the largest amount was identified as glucose, mannose, and galactose.

The BR extract had the best result for the analysis of antioxidant activity. The spores possess the highest concentrations of antioxidants compared to other parts of the fungus (HELENO; BARROS; MARTINS; JOÃO; et al., 2012). Different molecules such as carbohydrates, phenols, and proteins also have antioxidant activity. Therefore, the breaking of the spores increases the release of antioxidant compounds.

As soon as the analysis of heavy metals neither the extracts nor the spores presented risks to public health. Al, Pb, and Cd are of interest because they can be absorbed by the plants and by the soil from contaminated water flows. These metals are toxic because they increase oxidative stress in cells and affect the metabolism of Ca, P and Fe. Due to these metabolic changes, cellular communication decreases, affecting their secretory function (JAISHANKAR et al., 2014). The toxicity of these elements is described by the European Commission 1881/2006 of December 19, 2006, where the contents of Pb for edible fungi are set at a minimum of 0.10 mg/kg (FW) and for Cd at 1.0 mg/kg. In Brazil, Portaria number 685 of August 27, 1998, does not include levels of Pb and Cd in mushrooms, but in fruits, vegetables and cocoa establish a minimum of 10 mg/kg and 2 mg/kg for Pb and Cd. In Brazil, Portaria number 685 of August 27, 1998, does not include levels of Pb and Cd in mushroom, but in fruits, vegetables and cocoa establish a minimum of 10 and 2 mg/kg for Pb and Cd.

Although the extracts did not show band stretches for the lipid content, analysis of broken spores for lipids showed that broken spores could release higher lipid concentration. 23.74 % of spore lipids from *G. lucidum* can be extracted by Sonication, 30 % by Soxhlet, and 29.50 % by supercritical CO_2 under 35 MPa (LI, L. et al., 2016). Since in this work, the Blight & Dyer method was used to determine lipids, this proved to be fast and practical, once there were a greater number of lipids. The percentage of 25% matches with that previously reported for *G. lucidum*, in which a percentage in the range between 26.9 and 23.3 % of lipids was depicted (LIU et al., 2007).

TABLE. 18 - COMPARISON BETWEEN DIFFERENT SPORE BREAKING TECHNIQUES OF *G. lucidum.*

TECHNIQUE	BREAKING (%)
Manual maceration	<10
Airsoft, glass, ceramic and silica-gel spheres	< 10
Steel/Chrome spheres	97.48
Microwave	Fissures in all spore walls (detectable by SEM).

4.5. CONCLUSIONS

The best method used to break the *G. lucidum* spore walls was the mechanical assisted maceration with steel/chromium spheres.

The analysis by FTIR regarding the chain lengths was an easy tool for the preliminary study of qualification and monitoring of extract methods of the different chemical compounds present in the spores and *G. lucidum* extracts, showing some changes in the protein structure in MBR1 extract, while for BR the proteins preserved structure without modification. In addition, although there was a loss of lipids and fatty acids, all extracts conserved the carbohydrate regions

Concerning lipid, protein and carbohydrate contents, MBR1 produced the highest proportion of carbohydrates and polyphenols, maintaining antioxidant potential; but for BR, the protein structure was conserved.

Similar results were obtained between FTIR and chemical characterization, however FTIR elucidate structural changes in both carbohydrates and proteins depending on the extracting methods. The carbohydrate analysis by anthrone method was useful to quantify them. FTIR was the best tool to notice that structural changes in the bio compounds occurred in each extracting method confirmed through monosaccharide analysis.

It was confirmed that there was no significant contamination of the steel/chromium spheres in all extracts, since the analyzed stages and elements were among the requested parameters by the European and Brazilian commission on food control.

Finally, the rupture and the chemical extraction by microwave, regarding GLS samples, especially MBR1 and BR extracts, maintained the antioxidant activity, and extracted greater amount of compounds from *G. lucidum* spores.

STEP 5: AMPLIFYING AND IDENTIFYING MOLECULES IN FRACTIONS

The extracts RM, BR and MBR1 and Control, were selected to amplify the potential for biological activity. It is known that the best extraction methods require spore rupture and heat exposure. In this case, the protein bands and molecular weight structure were reviewed to purify extracts with higher antioxidant potential. The analysis was carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and performance size exclusion chromatography coupled with multi-angle laser light scattering and refractive index detector (HPSEC-MALLS-RID), in addition to the comparison between the averages of substances extracted between extracts. The best extracts were fractionated by different temperature and dialysis exposures. The aim was to amplify the extraction of antioxidant molecules.

5.1. MATERIALS AND METHODS

5.1.1. EXTRACTION QUANTITY BETWEEN CONTROL, RM, BR AND MBR1 EXTRACTS

Several flasks of glass were left to dry for 24 h in a 100 °C stove. After drying, the flasks were kept in the desiccator for 60 min and weighed before the *G. lucidum* spore extracts were placed. The extracts were then overnight frozen and placed in a freeze-dryer to dehydrate the biological material (24 h). Then, each flask was weighed, and the original weight of the glass flask was removed to know the amount of organic material extracted from the spores. The evaluation of all weights was carried out by keeping the glass flasks in a freeze-dryer throughout the process. Each evaluation was done in triplicate for each extract.

5.1.2. PROTEIN ANALYSIS

The protein analysis was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The samples protein concentration was evaluated following the

Lowry-Folin and Ciocalteu method (F9552 Sigma-Aldrich) by triplicate for each concentration. A suspension was prepared adding to 500 µg from *G. lucidum* extract 1 mL of distilled water. The absorbance for each suspension was measured at 750 nm and the concentration was obtained using a standard curve for Bovine Serum Albumin (BSA) (PETERSON, 1979). After reading, the values have been converted into percentage. Values of standards were plotted and compared to the absorbance to achieve the protein content from extracts.

The protein values of *G. lucidum* spores extract was determined by Lowry et al., (1951). For the SDS PAGE a suspension of 15 µL were used in polyacrylamide gel electrophoresis (concentration 12%, voltage 30 mA) to determine the mass of proteins in each extract and comparing with a PMM pattern (Broad Range Protein, V849A and size 500 µL - 100 lanes). Gels were stained with Coomassie Blue using routine protocol.

5.1.3. COMPOSITION ANALYSIS

The extracts was freeze-dried and (2 mg) hydrolyzed in 2 M of Trifluoroacetic acid (TFA) at 110 °C for 2 h (DONG et al., 2012). The determination of monosaccharide composition was carried out by reductive hydrolysis (STEVENSON; FURNEAUX, 1991) using extra reducing agent [borane.4-methylmorpholine complex (4-MMB)] before and after pre-hydrolysis and hydrolysis steps (FALSHAW; FURNEAUX, 1994). The hydrolytic process was performed as described by (FERREIRA et al., 2012). After acetylation (STEVENSON; FURNEAUX, 1991) the alditol acetate derivatives were analyzed by Gas chromatography–mass spectrometry (GC–MS) in the same conditions described by FERREIRA et al., 2012 and were identified by their typical electron-impact fragmentation profiles and GC retention times (JANSSON et al., 1976). A double hydrolysis-reductive amination method was used to determine the absolute configuration of the monosaccharide constituents (NAVARRO; STORTZ, 2003). Chiral 1-amino-2-propanol was used to determine the ratio of d- and l-galactose and its 6-*O*-methyl derivative, whereas the configuration of 2-*O*-methylgalactose, 3,6-anhydrogalactose and their 2-*O*-methyl derivatives were determined using chiral α-methylbenzylamine. The resulting alditol acetates derivatives were analyzed by GC–MS using the conditions described by (FERREIRA et al., 2012).

5.1.4. HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY (HPSEC)

The heterogeneity of the material was determined by high performance size exclusion chromatography (HPSEC). A Waters type chromatograph equipped with differential refractive index detectors (IR, Waters model 2410), static light scattering (SLS, Wyatt Technology model DAWN DSP), and ultraviolet (UV, Pharmacia LKB model Uvicord VW 2251) was used. Four gel permeability columns were used (Waters model Ultrahydrogel 2000, 500, 250 and 100) connected in series, with exclusion limits of 7x106, 4x105, 8x104 and 5x103, respectively. A solution of sodium nitrite ($NaNO_3$) 0.1 M with sodium azide (200 ppm) was used as a mobile phase at 20 °C.

1.0 $mg.mL^{-1}$ of the analyzed extract was solubilized in the mobile phase of the HPSEC system and then placed in a cellulose filter of 0.22 μm pore diameter. The volume 100 μL was injected into the system with flow of 0.6 $mL.min^{-1}$ and after the results were analyzed with the ASTRA version 4.7 program.

5.1.5. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

The extracts were solubilized in deuterated water (D_2O). 15 mg of each extract for analysis ^{1}H and 30 mg of each extract for analysis ^{13}C. Each "samples" were diluted on 0.5 mL with D_2O and then centrifuged to eliminate non-soluble contaminants. The samples were analyzed on the Bruker model DRX 400, Avance® series spectrometer. The extracts were placed in 5 mm diameter tubes at 30 °C. The chemical shifts (δ) were expressed in ppm using acetone for the ^{13}C experiments (δ = 30.20 ppm) and the HOD signal for ^{1}H experiments (δ = 2.20 ppm).

For magnetic resonance of carbon 13, the samples were concentrated from 15 to 30 $mg.mL^{-1}$. The coupled and decoupled NMR ^{13}C spectra were obtained at the base frequency of 100.61 MHz, with a signal acquisition interval of 0.6 s to a measure of 6.600 acquisitions in 0.1 s intervals between pulses.

For the hydrogen resonance, NMR ^{1}H, the samples were concentrated between 10 to 20 $mg.mL^{-1}$. The spectra were obtained at the base frequency of 400.12 MHz. In two-dimensional methods, the heteronuclear single quantum coherence (HSQC) was used to determine which protons were bound to the carbons from signals already described in the literature.

5.1.6. PURIFICATION OF MBR1 AND BR

G. lucidum spores were broken using two methodologies. In the first methodology the spores were macerated with the aid of spheres; in the second, maceration with spheres was combined with microwave exposure. After breaking the spore walls, they were suspended in water (pH 7.0) at 120 rpm with 30 °C for 24 h. Each of the solutions separatelywere centrifuged and the residue was exposed for 2 h to three hot water baths with temperature 100 °C. Once baths finished, the supernatants resulting were combined, frozen and lyophilized separately by type of break. The lyophilized material was diluted in Miliq water and placed inside a filtration membrane (porosity between 12 to 14 kDa) with constant agitation and closed water system for 24 h. The retentate and the eluted material were concentrated in a rotary evaporator (RE-100A, 10L) and then lyophilized. The extracts were labeled by breaking methodology and by the type of fraction filtered as follows (FIGURE. 21):

- **BR1:** break with maceration assisted by spheres; filtrated retained fraction.
- **BR2:** break with maceration assisted by spheres, filtrated eluted fraction.
- **MBR1:** breaking by exposure to microwaves and maceration with the aid of spheres, filtrated retained fraction.
- **MBR2:** breaking by exposure to microwaves and maceration with the aid of spheres, filtrated eluted fraction.

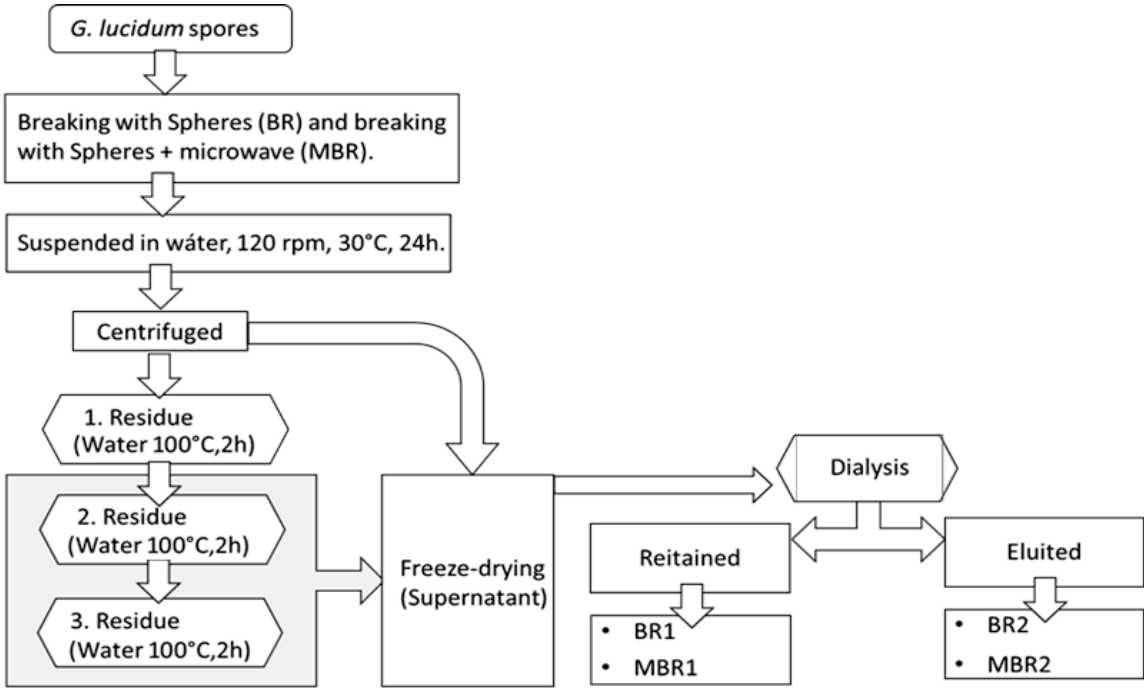


FIGURE. 21 - METHODOLOGY OF BREAKING AND EXTRACTION OF BIOMOLECULES OF THE EXTRACTS BR1, BR2, MBR1, MBR2.

5.2. RESULTS

5.2.1. TOTAL mg EXTRACTED FROM *G. lucidum* SPORES FOR EXTRACTS CONTROL, RM, BR AND MBR1

Bearing in mind that all extracts were prepared with 1 g of *G. lucidum* spores, the results for each prepared extract were: for the control extract the volume extracted was 7.7 mg. For RM extract was 14.4 mg, BR 11.2 mg and MBR1 20 mg.

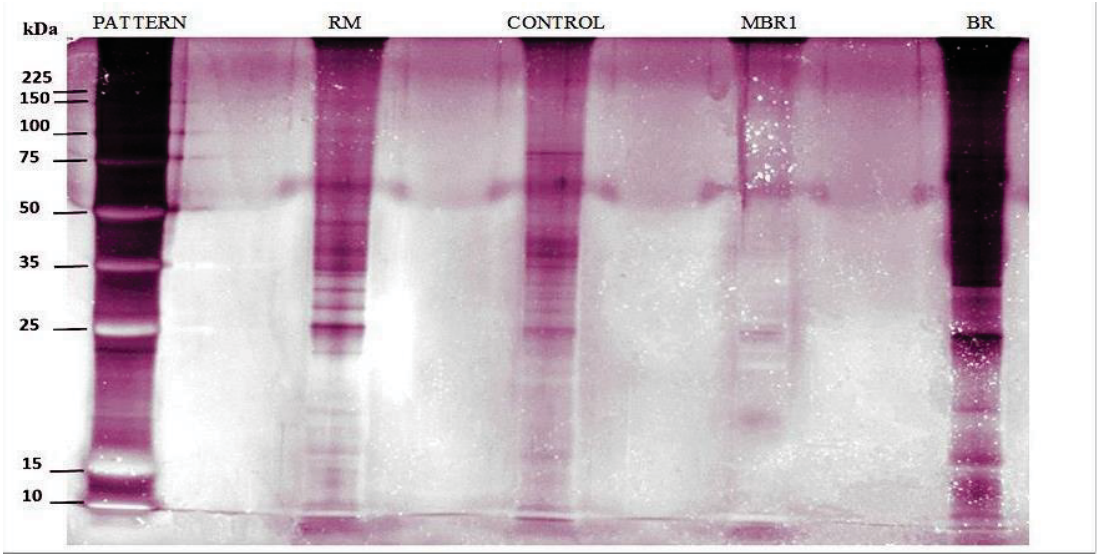


FIGURE. 22 - SDS PAGE RESULTS FROM RM, CONTROL, MBR1 AND BR EXTRACTS.
THE FIRST COLUMN ON THE LEFT SHOWS BROAD RANGE PROTEIN MOLECULAR WEIGHT MARKERS. THE PATTERN COLUMN SHOWS THE PROTEIN BANDS: 225, 150, 100, 75, 50, 35, 25, 15 AND 10 kDa. GEL 12 % POLYACRYLAMIDE. SILVER COLORATION.

5.2.2. PROTEIN ANALYSIS FOR THE EXTRACTS CONTROL, RM, MBR1 AND BR

Considering the band protein pattern, bands present between the different extracts prepared with *G. lucidum* spores were described. In the RM extract, 3 bands between 50 and 35 were observed kDa. Four bands between 35 and 25 kDa appeared with the 25 kDa band markedly more defined. Four poorly defined bands between 25 and 15 kDa and a last weak color band about 10 kDa. The control extract showed: 1 band in 75 kDa well defined, 3 bands between 50 and 35 kDa, 4 poorly defined bands between 35 and 25 kDa and a band off about 15 kDa. The MBR1 extract shows only 1 band in 25 kDa. For BR extract: 1 band in 225 kDa, 1 in 150 kDa, 1 in 75 kDa, 3 bands between 50 and 35 kDa, being the 35 kDa band the most defined. There is also 1 band defined at 25 kDa, 2 bands near 15 kDa and a weak band at 10 kDa, (FIGURE. 22).

5.2.3. HPSEC-MALLS-RID OF RM, CONTROL, MBR1 AND BR EXTRACTS

Each of the four extracts were analyzed for their elution profile in HPSEC-MALLS-RID to evaluate their degree of homogeneity. This analysis was performed using high performance size exclusion chromatography (HPSEC) and multi-angle laser light scattering (MALLS) to determine the molar masses contained in the extracts. The refractive index detector (RID) was also used to determine the concentration of the different molar masses.

In the RM extract (FIGURE. 23), a polydisperse light scattering (MALLS / laser 90 °, blue line) was found. This indicates that the extract is heterogeneous. The sample is constituted by high molecular weight molecules at 35 min and low molecular weight at 45 min. In RID (black line) the concentration of the molecules at 35 min were low and at 45 min were moderate. At the 60 and 65 min, two peaks of high concentrations appeared. UV (red line) peaks related to amino acids, nucleotides, pigments and beta-carotene appeared. In the ultraviolet only a slight peak is shown that coincides at minute 60 with RID and MALLS.

In the control extract (FIGURE. 23), the spectrum has a heterogeneous profile. The light scattering detector (MALLS / laser 90 °) showed that the extract is polydisperse with low molecular weight molecules at 40 and 45 min. In the RID refractive index, there is little concentration of molecules in 45 min, in 60 minutes there is a peak of high concentration and in 70 min a peak of medium concentration. For the ultraviolet index (UV) there is only a slight stretch in 60 min.

MBR1 extract was also polydisperse (FIGURE. 23). The peak with the largest molecular size (MALLS / laser 90 °) were at 40 min and decreases towards 50, 60 and 65 min. The highest concentration (RID) was found at 65 min, followed by 60 min and progressively decreased to 40 min. In the ultraviolet (UV) there were no defined peaks to describe.

BR extract was polydisperse and heterogeneous because it has several sizes of molecules (MALLS / laser 90 °, FIGURE. 23). The largest molecules in the spectrum were at 40 min, followed by small molecules at 45 min and even smaller molecules at 60 and 65 min. The highest molecular concentration occurred at 60 min, followed by 65 min and low molecular concentrations were between 40 and 60 min. The least perceptible concentration was at 40 min. For ultraviolet (UV) there was only a minor peak at 60 min.

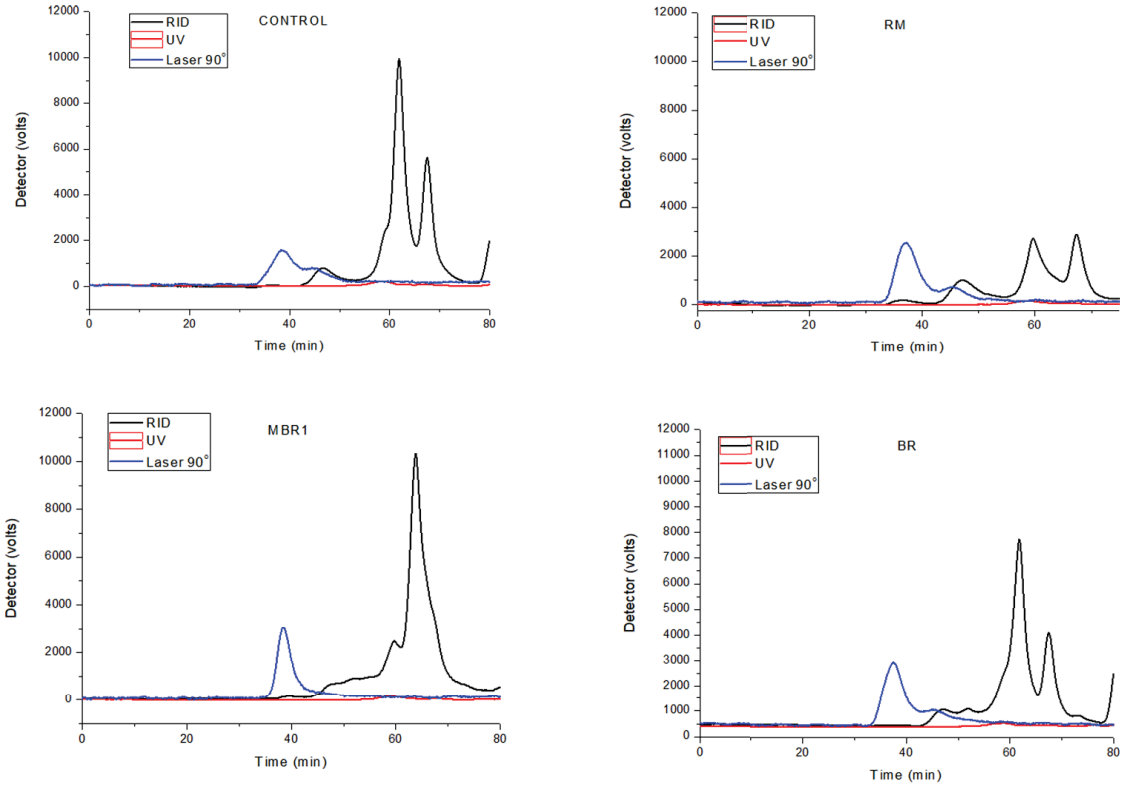


FIGURE. 23 - HPSEC-MALLS-RID OF THE EXTRACTS CONTROL, RM, MBR1 AND BR.
RID- BLACK LINE SHOWS THE CONCENTRATION IN THE EXTRACTS OF EACH MOLECULAR SIZE. UV- LINE IN RED SHOWS PROTEINS PRESENCE. LASER 90 °- MALLS, BLUE LINE SHOWS THE MOLECULAR SIZES INSIDE THE EXTRACTS. MEASURING RANGE FROM 0 TO 80 MIN.

5.2.4. NMR FOR ^{13}C AND ^{1}H OF MBR1 AND BR FRACTIONS

Among the extracts MBR1 and BR were found for the ^{13}C spectrum dislocation at 102.66, 97.17, 95.93 and 92.12 ppm. In the spectrum of ^{1}H the presence of glucose dislocation α and β was analyzed. For the carbon spectra the MBR1 extract only showed dislocation at 102.66 and 93.15 ppm. In the BR extract, dislocation was present at 102.66, 97.17, 95.93 and 92.12 ppm. For the ^{1}H spectrum the glucose terminal dislocation α and β were found in the BR extracts (α at 5.4 ppm and for β at 4.6 ppm). In the MBR1 extract the dislocation produced by the glucose terminal β was not present, (Figure. 24).

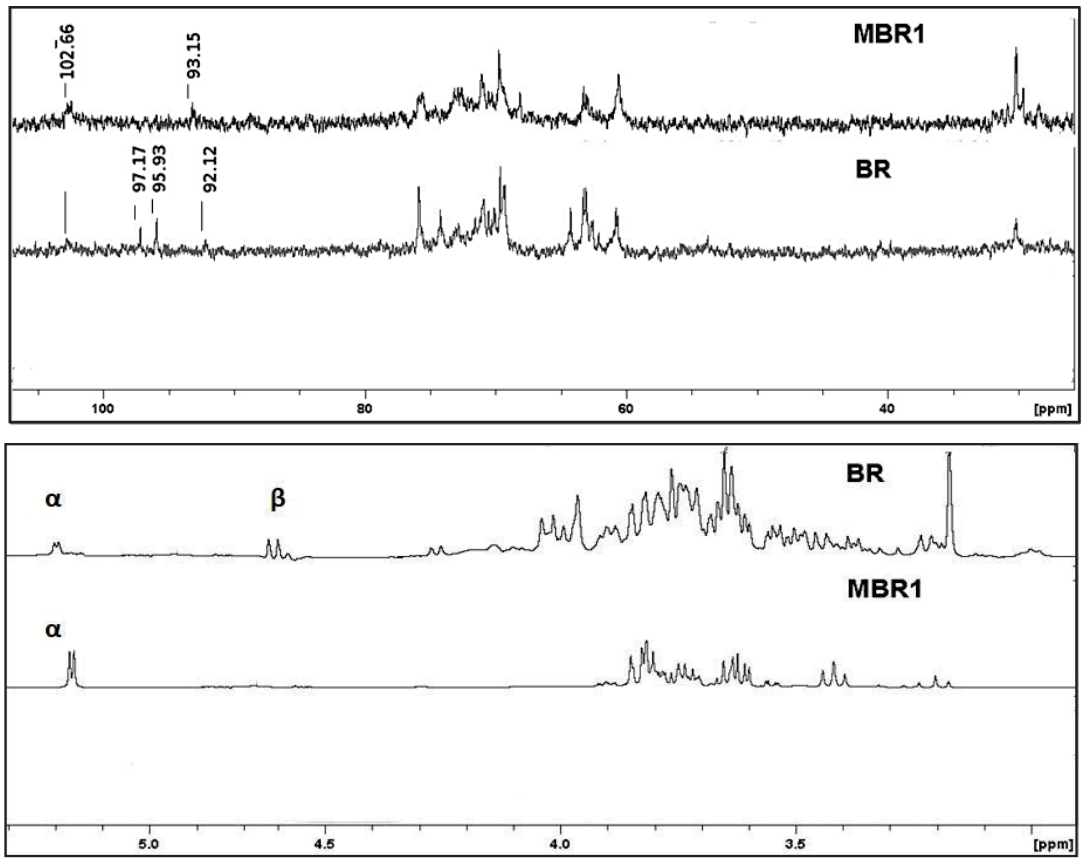


FIGURE. 24 - NMR SPECTRUM OF ^{1}H AND ^{13}C FROM THE BR AND MBR1 EXTRACTS.
Top image, ^{13}C spectrum with different signals for the MBR1 and BR extracts. bottom image. ^{1}H spectrum with different signals for BR and MBR1 extract. standard reference with acetone (^{1}H = 2.20 ppm and ^{13}C = 30.20 ppm).

5.2.5. HPSEC-MALLS-RID OF THE PURIFIED FRACTIONS

The MBR1 fraction (FIGURE. 25) was polydisperse and heterogenous because despite having a peak at 40 min there was a gradual decrease in molecular size between 43 to 60 min making the extract heterogeneous (blue line, MALLS / laser 90 °). Molecular concentrations were low throughout the extract (black line, RID). The highest concentration was at 55 min and decreased to 45 and 40 min. The ultraviolet (red line, UV) did not reveal noticeable peaks.

The MBR2 fraction (FIGURE. 25) was heterogeneous for RID (black line) with a higher signal in 70 min where salts are detected, followed by 65 min and 60 min. The spectrum of malls (laser 90 °) did not show significant signals but could have molecules of very low molecular weight between 60 and 80 min. The ultraviolet (UV) spectrum has no visible signal on the graph.

BR1 is a heterogeneous fraction (FIGURE. 25), larger molecules appeared at 40 min followed by 45 min. There may be small molecules between 60 and 80 min (MALLS / laser 90 °). In RID the highest molecular concentration was at 45 min, followed by 50 and 60 min. The lowest molecular concentration was at 40 min. In the ultraviolet there was only a small signal between 55 and 60 min.

The BR2 fraction is heterogeneous (FIGURE. 25). RID showed a higher molecular concentration at 60 min, followed by 65 and 75 min. In MALLS (90 ° laser) all molecules present had very low molecular weight. For ultraviolet (UV) there was only one signal at 60 min.

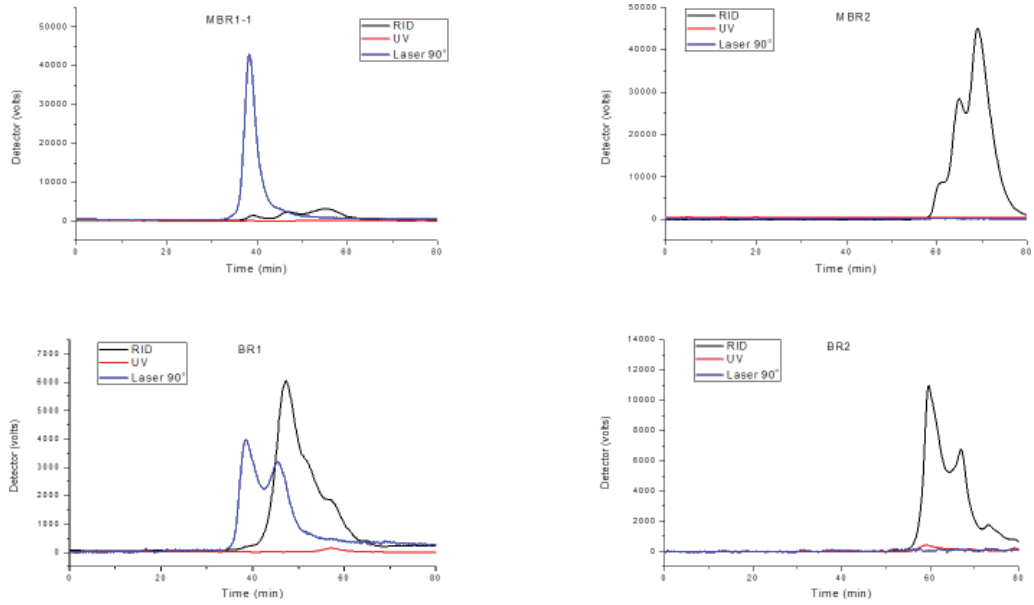


FIGURE. 25 - PSEC-MALLS-RID OF THE FRACTIONS MBR1, MBR2, BR1 AND BR2.
RID- BLACK LINE SHOWS THE CONCENTRATION IN THE FRACTIONS OF EACH MOLECULAR SIZE. UV- LINE IN RED SHOWS THE PRESENCE OF PROTEINS. LASER 90 °- MALLS, BLUE LINE SHOWS THE MOLECULAR SIZES WITHIN THE FRACTIONS. MEASURING RANGE FROM 0 TO 80 MIN.

5.2.6. MONOSACCHARIDE COMPOSITION OF PURIFIED FRACTIONS

The analysis of monosaccharides in the BR1 fraction showed an important content of glucose (75.83 %), mannose (10.88 %) and galactose (11.70 %). Fucose, arabinose and xylose had only traces. The BR2 fraction had 100% glucose monosaccharides. The MBR1 fraction had 46.94 % glucose, 28.74 % mannose, 21.02 % arabinose, 2.18% galactose and 1.11 % xylose. In MBR2 53.33 % glucose, 20.22 % mannose, 11.28 % arabinose and 15.17 % galactose were found. The high glucose content was common among all the fractions analyzed (TABLE. 19).

TABLE. 19 - MONOSACCHARIDE COMPOSITION DETECTED IN THE BR1, BR2, MBR1 AND MBR2 FRACTIONS.

MONOSACCHARIDES	BR1 %	BR2%	MBR1%	MBR2%
Rhamnose	n.d.	n.d.	n.d.	n.d.
Fucose	1.19	n.d.	n.d.	n.d.
Arabinose	0.16	n.d.	21.02	11.28
Xylose	0.24	n.d.	1.11	n.d.
Mannose	10.88	n.d.	28.74	20.22
Glucose	75.83	100	46.94	53.33
Galactose	11.70	n.d.	2.18	15.17

n.d.- not determined. for each extract, the sum of the monosaccharide percentages was 100 %.

5.2.7. HSQC OF THE PURIFIED FRACTIONS

By HSQC only anomeric ^{1}H / ^{13}C were described. For the BR1 fraction the anomeric 4.52 / 102.6 ppm were found. For the BR2 fraction the anomeric 4.6 / 96.3 and 5.19 / 92.2 ppm. In the MBR1 fraction the anomeric were found at 5.17 / 93.3 and 4.5 / 102.8 ppm. The MBR2 fraction had anomeric at 5.17 / 93.2 and 5.42 / 101.39 ppm (Figure. 26).

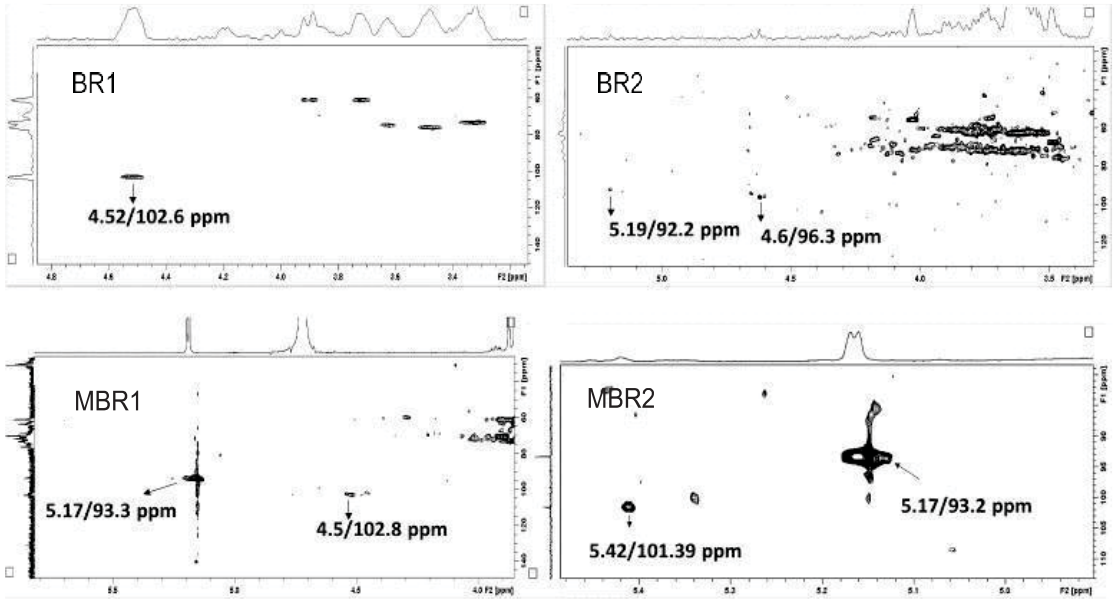


FIGURE. 26 - HSQC OF THE FRACTIONS MBR1, MBR2, BR1 AND BR2.

TOP LEFT IMAGE: 2D SPECTRUM OF THE BR1 FRACTION. TOP RIGHT IMAGE: 2D SPECTRUM OF THE BR2 FRACTION. BOTTOM LEFT IMAGE: 2D SPECTRUM OF THE MBR1 FRACTION. BOTTOM RIGHT IMAGE: 2D SPECTRUM OF THE MBR2 FRACTION. REFERENCE SIGNAL: ACETONE (^{1}H = 2.20 PPM, ^{13}C = 30.20 PPM).

5.3. DISCUSSION

5.3.1. DESCRIPTION AND SELECTION OF THE EXTRACTS CONTROL, RM, BR AND MBR1

Although there was no structural characterization of proteins the study carried out by SDS PAGE revealed the protein content of *G. lucidum* extracts. The BR extract distinguished itself among the extracts by the largest number of protein bands. Data found by other authors confirm that the presence of protein bands between 10 and 225 kDa are related to molecules with bioactive properties (LI et al., 2018). For example, the antitumour action was related to protein bands from 67 to 72.9 and 13 kDa by ZHANG et al., 2013 and LI et al., 2018 repectively; anti-inflammatory and anti-vasoconstrictive action between the 53.5 to 37.1 kDa bands (NURHUDA et al., 2013);

fibrinolytic activity in the band at 33.2 kDa, (SEKAR et al., 2011); antioxidant activity in protein bands between 2.8 to 3.35 kDa (SARNTHIMA et al., 2017) and antifungal activity in the protein band near 15 kDa (LI et al., 2018). Based on these data, extracts BR, RM and Control may have bands of proteins related to anti-inflammatory activity and fibrinolytic activity. The 225, 150, and 75 kDa bands found only in the BR extract need some further exploration because there are no reports in the literature.

In the analysis by HPSEC-MALLS-RID all extracts were heterogeneous, however, two extracts (MBR1 and BR) showed notable differences in their composition. The MBR1 extract may present low molecular weight carbohydrates and salts that may be related to antioxidant activity in cell culture medium, while the BR extract may present complex structures among proteins and carbohydrates. In the literature, low molecular weight carbohydrates are related to heteropolysaccharides composed by glucose, galactose and mannose (CHANG; LU, 2004) (WU et al., 2017) and in many cases may participate in complexes of proteoglycans or glycopeptides (YE et al., 2011; XIE et al., 2012; FERREIRA et al., 2015). This type of structures has been extracted using hot water and various authors associate them with antioxidant action. The results of this study confirm that MBR1 and BR extracts may have molecules related to antioxidant activity.

The RM and Control extracts presented larger carbohydrates than in the literature are related to antitumor activity (WU et al., 2017). However, as the objective was to identify molecules with antioxidant properties, The RM and Control extracts were not the focus if this study. It can be concluded that MBR1 and BR extracts are of interest for their antioxidant action and further purification of these extracts is necessary.

5.3.2. STRUCTURAL ANALYSIS BETWEEN BR AND MBR1 EXTRACTS

NMR analysis for BR extract in the ^{13}C spectrum shows dislocation for α-D-Glc*p* at 92.2 ppm and β-D-Glc*p* at 95.9 ppm (BREITMAIER; BAUER, 1984). These displacements are part of the glucose monomer. The same extract has a dislocation at 97.17 ppm related to →1)-α-D-Glc*p*-(3→ (SYNYTSYA; NOVÁK, 2013). The dislocation found in the MBR1 and BR extracts at 102.66 ppm is related to →1)-α/β-D-Glc*p*-(4→ y →1)-β-D-Glc*p*-(3→ (CARBONERO et al., 2005; SYNYTSYA; NOVÁK, 2013). For MBR1 extract, in addition to glucan the terminal →1)-β-D-Glc*p* was found at 93.15 ppm (BUBB, 2003). For ^{1}H spectra a difference between glucans was

found after observing the release of a polymer in the MBR1 extract because it has only the α dislocation of glucans, while the BR extract has the dislocation α and β. It may be argued that breaking of spores and exposure to a temperature of 30 °C for 24 h of BR extract released β glucans of type 1.3, the 10 min. microwave exposure of the MBR1 extract released the α glucan type 1,4 polymer. This is due to the lack of dislocation β in the ^{1}H spectrum. It can be concluded that BR and MBR1 have different glucan and protein structures. It would be necessary to obtain a greater amount of extraction of protein and carbohydrate fractions from the extracts before determining their biological activity.

5.3.3. PURIFICATION OF BR AND MBR1 EXTRACTS

The HPSEC-MALLS-RID analysis shows that the MBR1 and MBR2 fractions separated the high molecular weight carbohydrates from the proteins and salts of the initial crude extract by dialysis. The MBR1 fraction possesses high molecular weight carbohydrates in low concentration. The MBR2 fraction has a high salt content at minute 70 and MBR2 has an intermediate stretch between amino acids. In the BR1 and BR2 fractions there is also a clear separation between carbohydrates and proteins. The BR1 fraction has a high content of carbohydrates with a high molecular mass. Meanwhile, the BR2 fraction has a high content of low molecular weight proteins and a stretching between 65 and 70 min. The MBR2 and BR2 fractions show a common stretching between minerals and proteins. This stretching may occur by the release of a low molecular weight polymer. Also, the stretching could be due to the type of breaking and extraction at different temperatures. In the analysis of carbohydrate composition in all fractions the main monomer was glucose. Authors describe that there are carbohydrates and degraded carbohydrates of very low molecular weight. These have high biological activity because they have greater availability for the cell membrane (DESBRIÈRES et al., 2014)(SURAYOT et al., 2014). α glucans of type (1-3,4) are of low molecular weight and these stimulate cytokines and activate macrophages (SURAYOT et al., 2014). Likewise, the use of microwaves has been shown to handle temperatures between 100 and 121 °C (WANG, Q. et al., 2002) and acts on beta-glucans breaking chemical structure bonds that improve cellular metabolism protecting gastrointestinal cells (HARASYM; OLĘDZKI, 2018). In the case of the MBR2 fraction a very low molecular weight carbohydrate can be related. In

contrast, the BR2 fraction may have a complex between proteins and carbohydrates with possible biological activity for the cell membrane.

In the NMR analysis of the MBR1, MBR2, BR1 and BR2 fractions, the BR1 fraction showed high molecular weight molecules. These molecules showed displacements at 4.52 / 102.6 ppm for the ^{1}H / ^{13}C spectra (SYNYTSYA; NOVÁK, 2013). This type of structure is described for glucans of type: →4)-β-D-Glc*p*-(1→ (SYNYTSYA; NOVÁK, 2013). In the MBR1 fraction were present the fractions 4.5 / 102.8 ppm related to glucan type →4)-α-D-Glc*p*-(1→ y →3)-β-D-Glc*p*-(1→ (CARBONERO et al., 2005; SYNYTSYA; NOVÁK, 2013). Also MBR1 presented a dislocation at 5.17 / 93.3 ppm describing the glucan terminal type: β-D-Glc*p*-(→1 (BUBB, 2003). For the BR2 fraction the terminal α could be distinguished at 5.19 / 92.2 ppm (BREITMAIER; BAUER, 1984) and the terminal β at 4.6 / 96.3 ppm (MABEL et al., 2008) from the glucose monomer. For MBR2 extract a dislocation was found at 5.42 / 101.39 ppm type: →4)-α-D-Glc*p*-(1→ (TSAI et al., 2012) and the glucan terminal in the 5.17 / 93.2 ppm dislocation type: β-D-Glc*p*-(→1 (BUBB, 2003).

Analyses showed that fractions exposed to microwaves and different temperatures released very low molecular weight carbohydrates. The related carbohydrates are glucans →4)-α-D-Glcp-(1→ with its β-D-Glc*p*-(→1 terminal. The BR2 fraction has free Glucose that may be interacting with the UV detected proteins by HPSEC-MALLS-RID. The large number of protein bands can be related to the SDS PAGE result for the BR extract. It can be deduced that the four fractions have different types of glucans. These glucans have different molecular sizes. These glucans may be associated with proteins or interact with a high mineral content.

5.4. CONCLUSION

BR2 and MBR2 extracts may have glucans isolated or associated in complexes. Extracts may have proteins that increase antioxidant action in vitro cell cultures. Likewise, the extracts may have minerals that can be adjuvant in reproductive cells.

BR1 and MBR1 extracts have high molecular mass carbohydrates. These carbohydrates could act against cancer cells, fibrinolytic and antifungal action.

STEP 6: ROUTINE TESTING IN CELLS LINES

Fertility in men and women can be affected by several diseases because, the fertilizing capacity of the reproductive cells decreases in inflammatory processes and pharmacological treatments (KWAK-KIM et al., 2014). Consequently the demand for alternative medicines motivates new research that promotes cell viability (PERDICHIZZI et al., 2007; ARAÚJO et al., 2014).

Mushrooms of the family *Ganodermataceae* are investigated for their antioxidant action in several diseases (CHENG et al., 2010; SOCCOL et al., 2016). *G. lucidum* spores have antioxidant effect related to its polysaccharides and ganoderic acids (HELENO; BARROS; MARTINS; JOÃO; et al., 2012; HELENO et al., 2013; SOCCOL et al., 2016). This type of fungus has been used against cervical cancer cell lines in humans (EL-MEKKAWY et al., 1998; LIU et al., 2012). In *Ganoderma* polysaccharides and aminopolysaccharides with carbohydrates of type 1,3,6-, 1,3 and 1,6- linkages have receptors in cell membranes (BAO et al., 2001; TSAI et al., 2012). Many of these carbohydrates have low molecular weight and highest antioxidant activity (WU; WANG, 2010; TSAI et al., 2012).

The aim of this research is test different extracts of *G. lucidum* spores in cell cultures of preanthral follicles and sperm. The addition of the extracts was intended to evaluate the effects on cell viability.

6.1. MATERIALS AND METHOS

6.1.1. OVARIAN PROVENANCE

Ovaries were collected in a slaughterhouse in Fortaleza-Ceará, Brazil. Ten complete ovaries from ten prepubescent Yorkshire gilts (*Sus scrofa domestica*) were removed. The ovaries were washed once in 70 % ethanol and twice in Minimum Essential Medium (MEM) containing 100 µg/mL penicillin, 100 µg/mL streptomycin, and transported to the laboratory in MEM at 37 °C (MAO et al., 2004). From each ovaries pair were obtained 11 pieces to compose the three treatments (control group and *G. lucidum* extract treated group) during cultivation for 7 days.

6.1.1.1. EXPERIMENTAL DESIGN IN OVARIAN FOLLICLE

The ovarian cortex tissue in situ cultivation was according to MAO et al., 2004 methodology. The cultivation medium used was alpha MEM (pH 7.2 - 7.4), 100 mg/mL ascorbic acid, 1000 mg/1 mL BSA, 29,200 µg/mL hypoxanthine, 10 µg/mL transferrin, 1 mM pyruvate, 100 µg/mL penicillin and 100 µg/mL streptomycin. The organ culture system used herein has been previously described (CELESTINO et al., 2009; MAGALHÃES-PADILHA et al., 2012). Ovarian cortex tissue from each ovarian pair from the same animal was cut into 11 slices (approximate size: 5 mm × 5 mm x 1 mm) using a scalpel under sterile conditions. One slice was immediately fixed for histological and ultrastructural analysis (fresh control) and the other 10 were placed in culture for 7 days in individual wells. The cortex tissue samples were transferred to 24-well culture dishes containing 1 mL of culture medium and 10 µL of aqueous extracts of *G. lucidum*. Four extract concentrations were tested (10, 25, 50 and 100 %). In the control group was medium extracts free. Subsequently, was performed histological analyses.

6.1.1.2. HISTOLOGICAL ANALYSIS

Samples were fixed in 4 % paraformaldehyde solution in PBS at pH 7.2 for 12 h at 4 °C. They were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Histological samples were cut into 7 µm sections and stained with periodic acid – Schiff (PAS) – hematoxylin. Each section was evaluated using an optical microscope (Nikon, Tokyo, Japan) at 400x magnification. In reading were considered the living follicles, based on oocyte and granulosa cells integrity, presence or absence of pyknotic bodies, ooplasmic retraction and organization of the granulosa cells (Fig. 4.2).

The developmental stages of follicles have been defined previously (TELFER, 1996) as primordial (one layer of flattened) or growing follicles (primary – one layer of cuboidal granulosa cells, and secondary– two or more layers of cuboidal granulosa cells around the oocyte). Follicles were classified histologically as: normal, when an intact oocyte was observed; surrounded by granulosa cells, that were well arranged in one or more layers, and lacked a pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. The percentage of

morphologically normal follicles, before (day 0) and after culture, indicated the percentage of follicle survival.

6.1.2. EXPERIMENTAL DESIGN IN SPERMATOZOA

This study was reviewed and approved by: "Comitê de Ética em Pesquisa em Seres Humanos do Setor de Ciências da Saúde - Universidade Federal do Paraná (UFPR)", Curitiba-Brazil. CEP/SD-PB number 2813175 August 10th 2018CEP/SD-PB. Experimental phase was performed at the "Engenharia de Bioprocessos e Biotecnologia - Centro de Biotecnologia Agroindustrial do Paraná CENBAPAR" - UFPR. After signed the consent form by 12 male participants between 29 and 40 age, semen was collected and considered as a concession. Donors had prior preparation consisted of sexual abstinence for two to five days. Semen collections were analyzed and evaluated following the guidelines WHO (WORLD HEALTH ORGANIZATION, 2010). The parameters considered when semen collected were: sperm concentration (> 60 x 10^6 / mL), motility (> 50 %), progressive motility (> 20 %), number of motile sperm (> 40 x 10^6) and morphological abnormalities (< 10 %).

After collected, sample was liquefied, semen was washed with Dulbecco's medium (PBS) and diluted in Ham's F-10 culture medium. Then 1 mL of culture medium was placed in cell culture dishes and 120 mM sodium peroxide (H_2O_2) was added in each one. Each sample collected was divided into 6 wells where 600 μg of the extrats of *G. lucidum* spores (BR1, BR2, MBR1 and MBR2) were added. One well was used for negative control with 600 mM ascorbic acid and a positive control well where onlyH_2O_2. All groups composed of 6 wells were incubated for 45 min in a CO_2 cell culture incubator (5 %) at 37 °C (model ***SM-BPN-80CRH (UV)/SM-BPN-150CRH***). The reaction was stopped and washed with 1mL PBS (centrifugation at 800 *g* for 5 min). The cell pellet was suspended in PBS for further microscopic evaluation (CHAKI; MISRO, 2002; CHEN et al., 2014; FANAEI et al., 2014; WU et al., 2015).

6.1.2.1. VIABILITY AND MOTILITY OF SPERMATOZOA

All samples were evaluated following the standard guidelines of WORLD HEALTH ORGANIZATION (WHO(WORLD HEALTH ORGANIZATION, 2010). Samples were processed after the end of the incubation. 10 μL of each semen sample were placed on slide

microscope and were covered with a coverslip (22 x 22 mm coverslip). Parameter reading was performed on 200 cells per sample using a light microscope with 400X magnification lens (Nikon Eclipse E-200 binocular biological microscope). Concluded in vitro culture, total motility sample was observed, and viability was assessed by mixing 20 µL eosin staining and 20 µL sample of semen.

6.1.2.2. MEASURING DEOXYRIBONUCLEIC ACID (DNA) STAND BREAKS: COMET ASSAY

The alkaline gel electrophoresis, COMET assay, was performed to determine the fragmentation of the DNA during the in vitro experiment. Three replicates were used per sample. Slides were prepared by bathing in solution with 1 % multipurpose agarose and then letting them dry overnight at room temperature. A concentration of 10 µL of semen suspension (30 x 10^6 spermatozoa/mL) in Ham's F-10 medium was mixed with 90 µL of 0.5 % (w/v) low-melt agarose solution in PBS. Then, 20 µl of the mixture was placed on the slides and allowed to dry for 10 minutes at 4 °C. A thin layer of low-melting agarose was then placed on the slide and dried for 1 h at 4 °C (always covered by a coverslip). Then, a thin layer of low melting point agarose was placed on the sheet and dried for 1 h at 4 °C (always covering with a coverslip). The coverslip was removed, and the slides were immersed in a lysis buffer solution for 1 h at 4 °C (2.5 mol/L NaCl 100 mmol/L EDTA, 10 mmol/L Tris-HCl, 10 % v/v dimethyl sulfoxide, and 1 % v/v Triton X-100, pH 10). The lysis solution was replaced by a new lysis solution with proteinase K (60 µg/mL proteinase K, 2.5 mol/L NaCl, 10 mmol/L Tris-HCl, 100 mmol/L EDTA, pH 10) which was incubated overnight at 37 °C. The slides were then placed in a horizontal electrophoresis tank and covered with an alkaline buffer solution (300 mmol/mL NaOH, 1 mmol/L EDTA, pH 12.5). Electrophoresis was performed for 5 min at 20 V and once finished the slides were washed thrice with 0.4 mol/L of Tris-HCl, pH 7.4. Once the process was finished, the slides dried overnight. All samples were stained using silver stain and reserved for later reading (SINGH et al., 1989; HUGHES et al., 1996; ROBERT et al., 2008). In each sample, the size of the cellular DNA fragmentation was classified without considering the tail of the sperm. There are four categories to measure the type of DNA damage using the expansion diameter: A for 20 µm, B for 20.01 to 30 µm, C for 30.01 to 40 µm and D for more than 40.01 µM (SHAMSI et al., 2010). However, only category A was considered because the objective of the

analysis was to evaluate the antioxidant effects on the lowest DNA damage among the different extracts of the *G. lucidum* spores. Table. 20.

6.1.3. STATISTICAL ANALYSIS OF PREANTHRAL FOLLICLE CULTURE

For each extract, data analysis contemplates a control sample plus four concentrations groups. From each sample 30 follicles were counted, and the population was divided into three groups (primordial follicles, developing follicles and dead follicles). An average of each of the animals for each concentration was obtained. Note that the 30 oocytes read represent 100 % of each sample. Average values for each concentration were those used for statistical analysis. Control samples on days 0 and 7 were carried out to know the development of the oocytes in the culture medium in vitro.

The Shapiro-Wilk and Bartlett tests were used to confirm the data normal distribution and homoscedasticity. In this study, we considered a level of significance of 5 %. For analysis of dependent data, we use the methodology proposed by BONAT and JØRGENSEN, (2016). The regression models are specified using only second-moments assumptions and method used for parameter estimation using estimating functions approach. After adjustment of the regression models, we compare treatments and days of culture between the variable's survival, activation and follicular development and follicular and oocyte diameter by the Bonferroni multiple comparisons test. All data analysis was performed in R statistical software (TEAM, 2018) version 3.4.4 with software lattice (SARKAR, 2008) and mcglm (BONAT, 2018).

6.1.4. STATISTICAL ANALYSIS OF THE ANTIOXIDANT TEST IN SPERMATOZOA

The results of the in vitro sperm culture were presented using the mean ± SD. Because the data were not of normal distribution, the Kruskal Wallis nonparametric test was performed to evaluate all the treatments and the variances were compared to find the differences when the data were statistically significant ($p < 0.05$).

6.2. RESULTS

6.2.1. PREANTHRAL FOLLICLES MORPHOLOGICALLY NORMAL IN THE NON-CULTIVATED (DAY 0) AND CULTURED (DAY 7)

An example of morphologically degenerate follicle and a follicle morphologically normal on days 0 and 7 of in vitro culture are shown in FIGURE. 27. In morphologically degenerate follicles we can see shrunken oocytes, pyknotic nucleus and disorganized granulosa cells. In FIGURE. 28 in A, the percentage value of the morphologically normal follicles can be seen while, in B, the change in the percentage before and after the addition of *Ganoderma* extract in the concentrations of 0, 10, 25, 50 and 100 %. Usually the percentage of morphologically normal follicles decreases between days 0 and 7 of culture in vitro culture, going from 74.00 ± 00.07 to 56.00 ± 00.10 %.

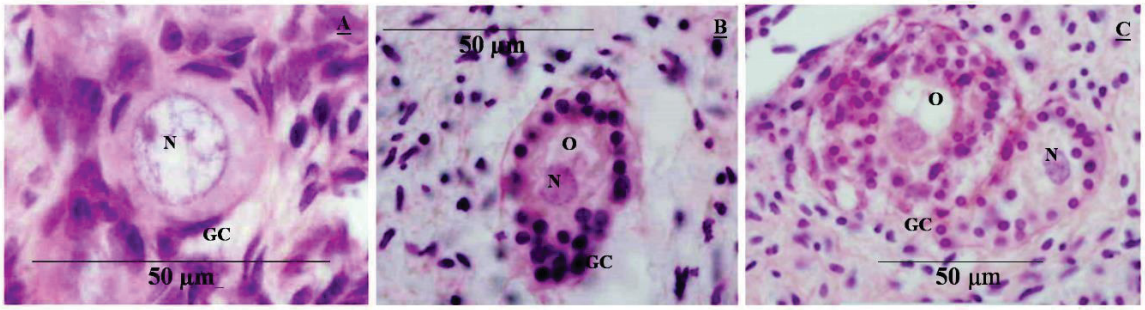


FIGURE. 27 - PREANTHRAL FOLLICLES.
A) MORPHOLOGICALLY NORMAL PRIMORDIAL FOLLICLE, B) MORPHOLOGICALLY NORMAL PRIMARY FOLLICLE AT 7 DAYS OF CULTURE, C) MORPHOLOGICALLY DEGENERATE PREANTHRAL FOLLICLE. (GC) GRANULOSA CELLS, (O) OOCYTE, (N) NUCLEUS. SCALE: 50 µM.

A.

Ganoderma extract	Follicle morphologically normal	
[%]	**Day 0 (%)**	**Day 7 (%)**
0	74.00 ± 00.07^{A}	$56.00\pm00.10^{B*}$
10		$42.66\pm00.07^{B**}$
25		$44.00\pm00.05^{B**}$
50		$56.66\pm00.07^{B*}$
100		$60.00\pm00.02^{B*}$

B.

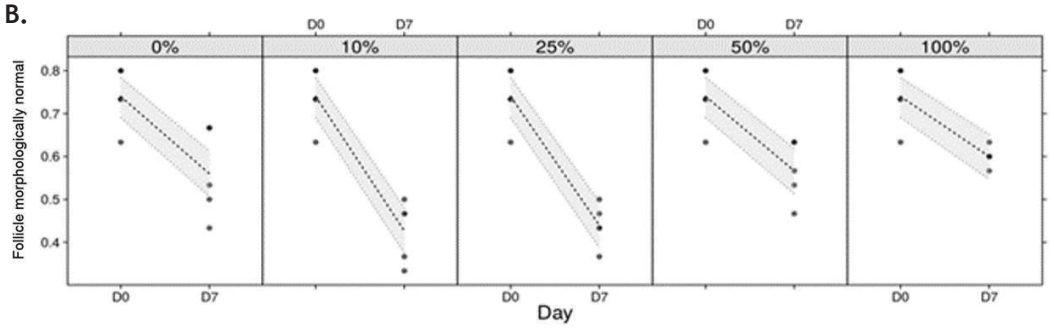


FIGURE. 28 - PERCENTAGE MORPHOLOGICAL NORMAL FOLLICLES.

A. PERCENTAGE OF MORPHOLOGICALLY NORMAL FOLLICLES, THE LETTERS ^{A} AND ^{B} SHOW THE DIFFERENCE BETWEEN DAYS WHILE * SHOWS THE DIFFERENCE BETWEEN CONCENTRATIONS AT DAY 7 IN VITRO CULTURE. **B**. PLOT OF PERCENTAGES OF MORPHOLOGICALLY NORMAL FOLLICLES BETWEEN DAYS 0 AND 7 OF IN VITRO CULTURE.

6.2.2. PRIMORDIAL FOLLICLE ACTIVATION IN VITRO

The percentage of primordial follicles (activated) and follicles developed at day 0 were 29.48 ± 12.57 and 70.52 ± 12.57 %, respectively, showing a low percentage of primordial follicles and a high percentage in developed follicles. In the 7th day, the difference between follicular activation and its development became more significant ($p < 0.05$) with 03.33 ± 04.71 and 96.67 ± 04.7 % respectively. A showing a greater number of follicles developed after 7 days of cultivation. Comparing the addition of 10, 25, 50 and 100 % *Ganoderma* extract, follicular activation and preanthral follicle development remained like day 0 in vitro culture ($p < 0.05$). FIGURE. 29.

A.

Ganoderma extract	Primordial follicle activation		Developing follicles	
[%]	**Day 0 (%)**	**Day 7 (%)**	**Day 0 (%)**	**Day 7 (%)**
0	29.48±12.57^{A}	03.33±04.71^{B*}	70.52±12.57^{A}	96.67±04.71^{B*}
10		27.16±07.83^{A**}		72.84±07.83^{A**}
25		27.92±18.40^{A**}		72.08±18.41^{A**}
50		25.44±10.03^{A**}		74.56±10.03^{A**}
100		24.08±07.25^{A**}		75.92±07.25^{A**}

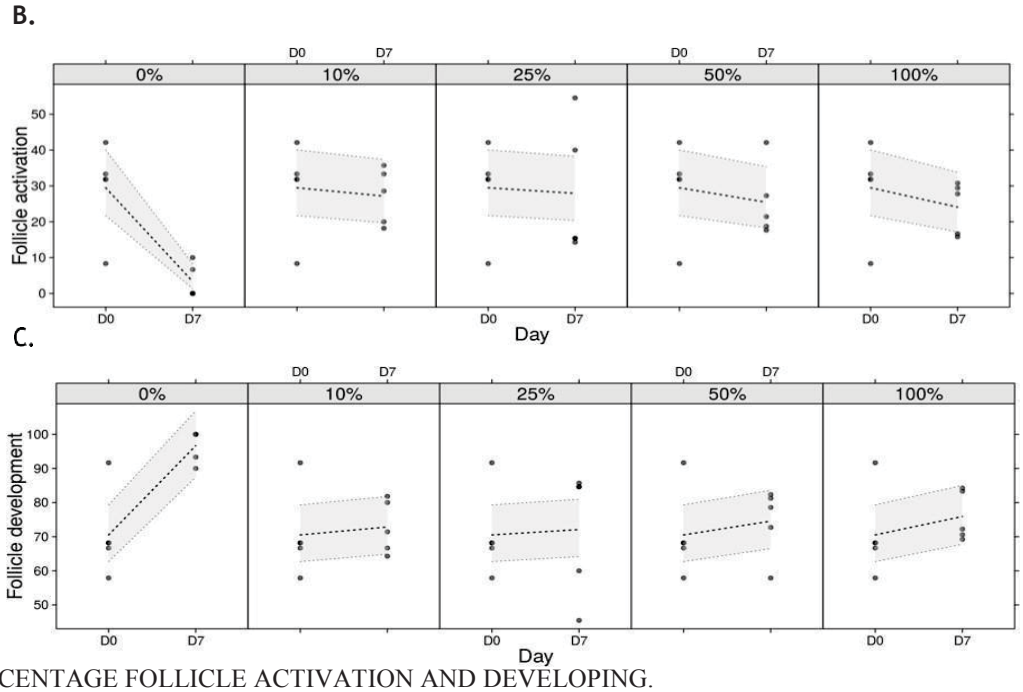


FIGURE. 29 - PERCENTAGE FOLLICLE ACTIVATION AND DEVELOPING.

A. PERCENTAGE OF PRIMORDIAL FOLLICLE ACTIVATION AND DEVELOPING FOLLICLES, THE LETTERS [A] AND [B] SHOW THE DIFFERENCE BETWEEN DAYS, WHILE * SHOWS THE DIFFERENCE BETWEEN CONCENTRATIONS AT DAY 7 IN VITRO CULTURE. **B**. PLOT OF PRIMORDIAL FOLLICLE ACTIVATION BETWEEN DAYS 0 AND 7 IN VITRO CULTURE. **C**. CHART OF DEVELOPING FOLLICLES BETWEEN DAYS 0 AND 7 OF CULTURE.

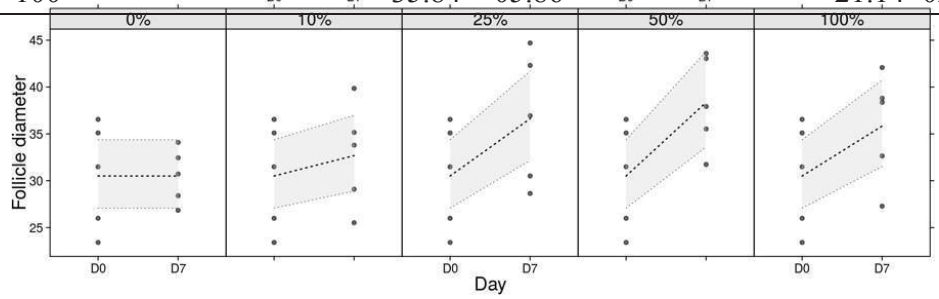
6.2.3. FOLLICLE DIAMETER IN NON-CULTURED AND CULTURED TISSUE

Cortical tissue cultured between day 0 and 7 showed no significant differences ($p < 0.05$) among follicular and oocyte diameters. Meanwhile, the 50 % concentration of *Ganoderma* extract shows a significant increase ($p < 0.05$) in oocyte diameter (24.60 ± 03.48 µm) and follicular diameter (38.36 ± 05.03 µm) at 7th day. Similarly, statistically correlating the increase in follicular and oocyte diameter, the 50 % concentration of the extract produced an overall increase in preanthral follicle diameter. The increase of the follicular diameter within the correlation is appreciated by the lack of linearity. FIGURE. 30.

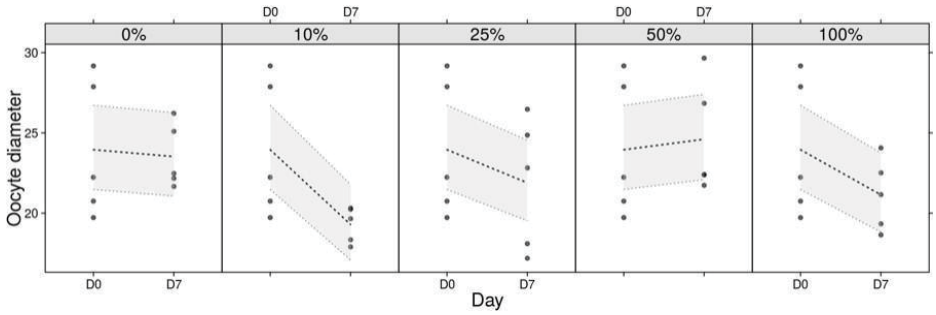
A.

Ganoderma extract	Follicle diameter		Oocyte diameter	
	[%]	**Day 0 (µm)**	**Day 7 (µm)**	**Day 0 (µm)**	**Day 7 (µm)**
	0	30.51±05.68^{A}	30.50 ± 02.94^{A*}	23.95±04.29^{A}	23.53±02.01^{A*}
	10		32.68 ± 05.55^{A*}		19.29±01.11^{B***}
	25		36.62 ± 07.05^{B*}		21.89±04.09^{A*}
	50		38.36 ± 05.03^{B**}		24.60±03.48^{A**}
	100		35.84 ± 05.86^{B*}		21.14±02.23^{A*}

B.



C.



D.

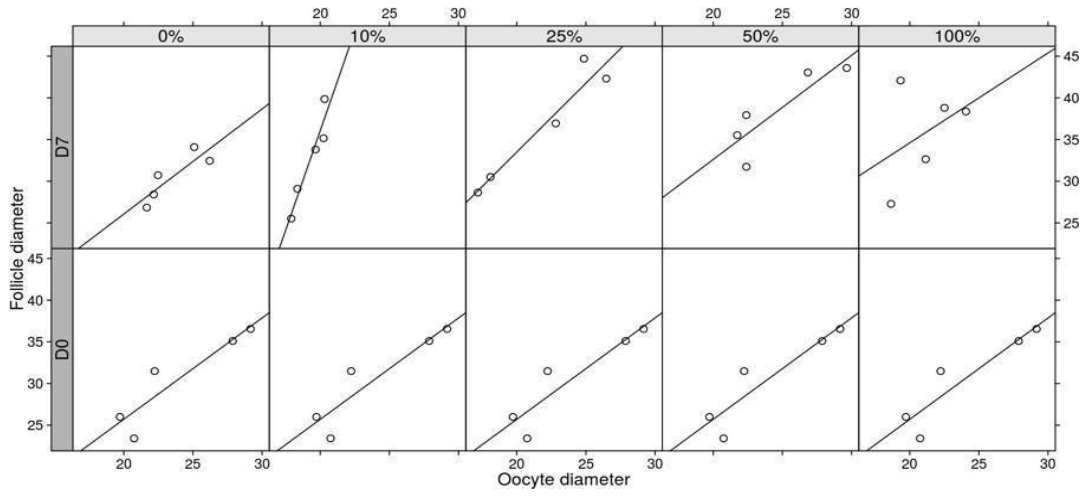


FIGURE. 30 – FOLLICLE DIAMETER AND OOCYTE DIAMETER.

A. FOLLICLE DIAMETER AND OOCYTE DIAMETER. FOLLICLE DIAMETER AND OOCYTE DIAMETER, THE LETTERS [A] AND [B] SHOW THE DIFFERENCE BETWEEN DAYS WHILE * SHOWS THE DIFFERENCE BETWEEN CONCENTRATIONS ON DAY 7 OF IN VITRO CULTURE. **B**. PLOT OF OOCYTE DIAMETER BETWEEN DAYS 0 AND 7 IN VITRO CULTURE. **C**. FOLD FOLLICLE DIAMETER BETWEEN DAYS 0 AND 7 IN VITRO CULTURE. **D**. CORRELATION AMONG OOCYTE DIAMETER AND FOLLICLE DIAMETER. LOSS OF ALIGNMENT WITHIN CORRELATION OF 50 AND 100 % CONCENTRATIONS OF *G. lucidum* ON DAY 7 OF CULTURE SHOWS INCREASED FOLLICULAR SIZE.

6.2.4. VIABILITY AND MOTILITY OF SPERMATOZOA

The MBR2 extract conserved significantly ($p < 0.05$) the viability of the spermatozoa with 19.20 ± 00.13 %. This value was the maximum compared between the treatment groups and the negative control with ascorbic acid treatment (12.40 ± 00.11 %). Furthermore, motility did not change significantly ($p < 0.05$) when the others extracts studied were added. Table. 20.

6.2.5. EFFECTS ON SPERMATIC DNA (DEOXYRINONUCLEIC ACID)

The semen samples prepared with the comet assay presented 4 categories of DNA integrity. FIGURE. 31 shows the circular halos of spermatozoa with high integrity of DNA (category A), while in categories B and C a stretched halo or a halo with small protruding threads is visible, indicating a medium degree of break in the DNA. Category D shows a large spot that moves away from the cell nucleus, revealing sperm with lower DNA integrity.

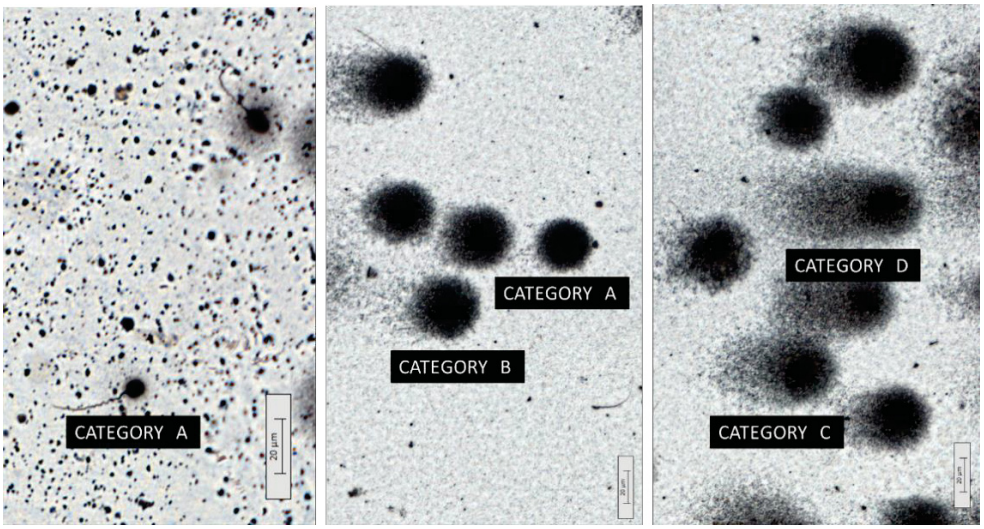


FIGURE. 31 - CATEGORIES COMET **A, B, C** AND **D** SHOWING DIFFERENT LEVELS OF DNA INTEGRITY IN THE EVALUATED SPERMATOZOA.

THERE ARE FOUR CATEGORIES TO MEASURE THE TYPE OF DNA DAMAGE: **A** FOR 20 μm, **B** FOR 20.01 - 30 μm, **C** FOR 30.01 - 40 μm AND **D** FOR MORE THAN 40.01 μm (SHAMSI et al., 2010). HOWEVER, ONLY CATEGORY A WAS CONSIDERED BECAUSE THE OBJECTIVE OF THE ANALYSIS WAS TO EVALUATE THE ANTIOXIDANT PROPERTIES ON THE LOWEST DNA DAMAGE AMONG THE DIFFERENT EXTRACTS OF THE *G. lucidum* SPORES. THE SPERM TAIL WAS NOT CONSIDERED IN THE MEASUREMENTS.

Category A had the best expected percentage, because it had the least DNA fragmentation among the sperm, the addition of the extracts did not produce any significant statistical difference ($p < 0.05$). However, there was a trend between negative control with ascorbic acid (64.86 ± 00.15 %) and MBR2 extract (77.14 ± 00.19 %) because it retained a higher sperm percentage in category A. See Table. 20 and FIGURE. 32. and FIGURE. 31.

TABLE. 20 - STATISTICAL PERCENTAGE OF MOTILITY, VIABILITY AND CATEGORY A IN COMET ASSAY OF THE SPERMATOZOA EVALUATED IN THE CULTURE IN VITRO.

Extract	% Motility	% Viability	% Comet (A)
AA, (-) Control	09.55±0.101	12.40±0.11	64.86±00.15
BR1	05.23±0.09	08.10±0.08	50.62±00.31
BR2	08.36±0.14	11.50±0.10	58.12±00.29
H2O2, (+) Control	05.22±0.11	05.04±0.07	48.17±00.22
MBR1	03.22±0.07	08.41±0.12	56.25±00.32
MBR2	09.36±0.13	19.20±0.13*	77.14±00.19
KRUSKAL-WALLIS	(0.3195)	(0.0508)	(0.362)

Note: The * in the data highlights the statistically significant value *($p < 0.05$)*. (-) Negative control. (+) Positive control.

6.2.6. CORRELATION BETWEEN THE VARIABLES: MOTILITY, VIABILITY AND COMET ASSAY

Correlations among motility, viability and A-category of comet assay did not show any significant relationship between them. Survivor was the only one with normal distribution.

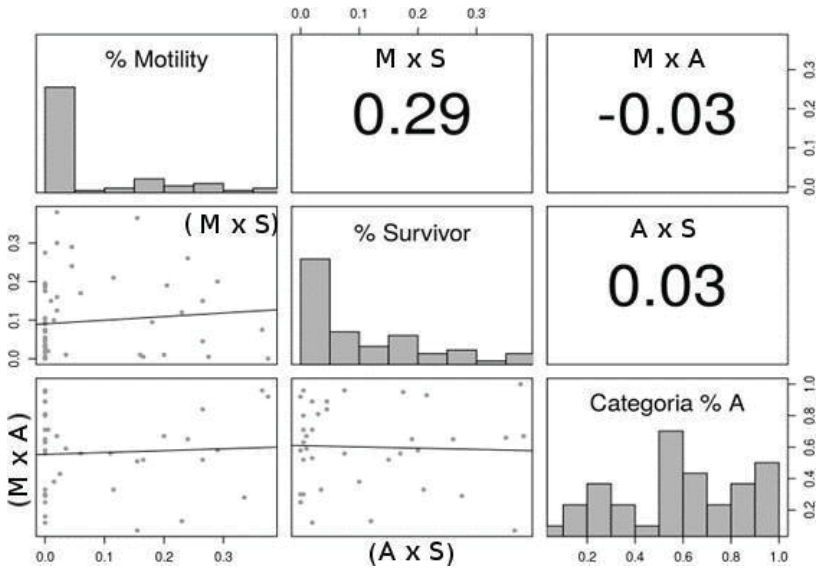


FIGURE. 32 - TABLE OF CORRELATIONS BETWEEN VARIABLES ANALYZED IN SPERMATOZOA. THE VARIABLES WERE MOTILITY PERCENTAGE, SURVIVOR AND CATEGORY A OF COMET ASSAY.

% MOTILITY – SPERM MOTILITY HISTOGRAM, DATA WITHOUT NORMAL DISTRIBUTION. **%**

SURVIVOR – SPERM SURVIVAL HISTOGRAM, NO NORMAL DISTRIBUTION. **CATEGORY % A OF COMET ASSAY** – HISTOGRAM OF EVALUATION CATEGORY A COMET, DATA WITHOUT NORMAL

DISTRIBUTION. **M X S** – CORRELATION BETWEEN MOTILITY AND SURVIVOR (0.29). **M X A** – CORRELATION BETWEEN MOTILITY AND KITE CATEGORY A (-0.03). **A X S** – CORRELATION BETWEEN COMET CATEGORY A AND SURVIVOR (0.03). **(M X S)**- DISPERSION BETWEEN VARIABLE MOTILITY AND SURVIVOR. **(M X A)**- DISPERSION BETWEEN VARIABLE MOTILITY AND CATEGORY A. **(A X S)**- DISPERSION BETWEEN VARIABLE CATEGORY A OF COMET ASSAY AND SURVIVOR.

6.3. DISCUSSION

6.3.1. APPLICATION OF CRUDE EXTRACT OF *G. lucidum* IN SWINE OVARIES

Survival is defined by the normal morphology of the preanthral follicle. Concentrations of 50 and 100 % *G. lucidum* spore extract had a follicular survival equal to the control group during culture. In the literature several soluble extracts of *G. lucidum* are antioxidants and stimulate the secretion of hormones, growth factors and cytokines (OKTEM; URMAN, 2010; CJ; GF, 2012). As example the interleukin 1β (IL- 1β) in bovine ovaries and rats is associated with the suppression of cellular apoptosis and the stimulation of follicular growth because cytokine inhibits tumor necrosis factor α (TNF- α), (PASSOS et al., 2016). Equally, there are several carbohydrates of *G. lucidum* can stimulate the secretion of IL-1β (WANG et al., 2017). Therefore, it is possible to say that carbohydrate-associated structures in *G. lucidum* contribute to maintain the population of preanthral follicles.

Follicular survival may also be related to the antioxidant properties of the *G. lucidum* spore extract. These extracts can regulate several cell death proteins (Bax, caspase 3, Bcl-xl) and decrease free radicals (ROS), (SOCCOL et al., 2016). ROS are reported to cause damage to the cell membrane of granulosa and theca (RODGERS; IRVING RODGERS, 2002; MAZOOCHI et al., 2009). These damages activate several apoptotic genes (p53, Bcl-2, caspase 3, Bax, Fas and Fas-ligand) that activate cell death and follicular atresia (MAZOOCHI et al., 2009; HE et al.,2016). In this case, the spore extract of *G. lucidum* may have properties to decrease ROS and indirectly block the mechanisms of cell death.

Follicular growth was observed in the cortical tissue of the ovary during the 7 days of culture. This growth, of the primary follicles, was evidenced by an increase in follicular and oocyte diameter ($p < 0.05$). The addition of *G. lucidum* spore extract 50 % concentrated, was responsible for the mentioned activity. Granulose cells have been described as having a complex paracrine

signaling system for follicular growth (DIAS et al., 2014). They also produce IL-1β for follicle growth (PASSOS et al., 2016). In addition, these cells have intercellular connections with the oocyte (gap junction) for the transport of ions, metabolites and molecules (HUIE; DI, 2004). Compounds of the spore extract of *G. lucidum* stimulated the proliferation of granulose cells because the extract is an adjuvant to the antioxidant system. This allows the correct secretion of substances for follicular development.

Although follicular development was normal, activation of primordial follicles was poor for all evaluated concentrations of *G. lucidum* extract. In ovarian physiology it is explained that granulosa and theca cells are not yet developed in primordial follicles. This explains the lack of factors that stimulate follicular growth (PASSOS et al., 2016). For this test it can be deduced that *G. lucidum* extracts do not stimulate the activation of primordial follicles.

6.3.2. POSSIBLE MECHANISM OF ACTION IN ACTIVATED FOLLICLES

According to the results of the cell culture the *G. lucidum* spores stimulate the production of antioxidant enzymes in the mitochondria and assist eliminate free radicals. This makes it possible to maintain an extracellular environment indicated for cell development and survival. Likewise, according to the literature carbohydrates of *G. lucidum* can stimulate the production of IL-1β associated with cell membrane receptors forming the complex IL-1-IL-1RI-IL-RIA (KARAKJI; TSANG, 1995; PASSOS et al., 2016; SILVA et al., 2017). The complex may stimulate the proliferation of granulose cells and indirectly the production of growth factors for oocyte maturation. Furthermore, IL-1β may also stimulate the phosphatidylinositol 3-kinase pathway (PK13) that regulates the survival of primordial follicles in bovine, porcine and human (KARAKJI; TSANG, 1995; PASSOS et al., 2016; SILVA et al., 2017).

6.3.3. ANTIOXIDANT PROPERTIES OF THE BR1, BR2, MBR1 AND MBR2 FRACTIONS OF *G. lucidum* SPORES

Among the fractions evaluated, MBR2 was the best because it maintained sperm survival despite the addition of H_2O_2. Studies in sperm show that this cells do not have repair mechanisms in their DNA (PERDICHIZZI et al., 2007). In addition, it is described that the cell membrane is rich in polyunsaturated fatty acids and proteins (MARTÍNEZ et al., 2007). These two molecules

must be considered since the mechanism of cellular apoptosis begins in the membrane (GOTTLIEB, 2001; TAYLOR et al., 2004). The presence of free radicals (ROS) in the extracellular environment are responsible for initiating lipid peroxidation (MARTÍNEZ et al., 2007). This process begins because in the membrane the FAS-ligand binds to its receptor. After stimulation, mitochondria activates cytochrome C and caspase 8, thus activating the mechanisms of cell death (GOTTLIEB, 2001; TAYLOR et al., 2004). It is therefore possible that MBR2 extract improves the conditions of the extracellular medium. This means that the extract can neutralize free radicals that activate the lipid peroxidation of the sperm membrane.

After adding MBR2 to the cell culture, there was a statistical tendency in the sperm to maintain motility and DNA integrity. It should be noted that the calcium content was important in the micro- and macronutrient analyses for the *G. lucidum* extracts studied (obtained data by comparing extraction methods). The physiology of the sperm membrane describes the existence of ion exchange channels. These channels maintain osmotic balance and calcium intake which helps maintain the flagellar movement (PARODI, 2014; MANNOWETZ et al., 2017). When the extracellular medium is toxic, the membrane closes these channels to prevent the entry of calcium, ROS, and other metabolites. Thus, mitochondrial metabolism and motility are deactivated (PARODI, 2014; MANNOWETZ et al., 2017). Calcium content (Table 7), anti-ROS power and the presence of other molecules in the MBR2 fraction maintain the extracellular medium. A suitable medium allows the motility and integrity of sperm structures to be maintained.

6.3.4. ANTIOXIDANT MECHANISMS OF *G. lucidum* EXTRACTS STUDIED

Although no measurements of free radicals or antioxidant enzymes were made, chemical analyses show a significant glucose content in the extracts. *G. lucidum* has β low molecular weight glucans and heteropolysaccharides with antioxidant action (WANG et al., 2016; VELJOVIĆ et al., 2017). The extracts studied have carbohydrate structures that demonstrated cell viability in spermatozoa and preanthral follicles.

Macro and micronutrient analysis show that *G. lucidum* extracts have important concentrations of Ca, K, Na, P, Mg and Mn. Studies about mineral supplements in animal diets show improvement in sperm quality(ABDEL-RAHMAN et al., 2000; PREEDY et al., 2018). The presence of these minerals can provide greater support for culture media.

Another antioxidant mechanism for *G. lucidum* is the stimulation in the production of SOD, and CAT in the mitochondria (CÖR et al., 2018b). In preanthral follicles, the production of these antioxidant enzymes blocks the activation of cell death caused by ROS.

6.4. CONCLUSIONS

It can be concluded that, *G. lucidum* spores extract at 50 % concentration, stimulated growth and maintained the survival of preanthral follicles. However, it did not stimulate primordial follicle activation.

The MBR2 fraction added to the sperm culture media improved the cell medium. In this way, survival and motility were maintained.

G. lucidum spore extracts maintain survival in reproductive cells since extracts have antioxidant action for the cell culture medium.

Further studies on reproductive cells that identify markers of cell death (Bax and caspase 3), cell proliferation markers (IL-1β) and antioxidant enzymes (SOD y CAT) would be necessary. This could confirm that *G. lucidum* spore extracts indirectly intervene in the processes of cell death and proliferation in granulosa cells.

STEP 7: CHARACTERIZATION OF THE FRACTION WITH BIOLOGICAL ACTIVITY (MBR2)

Characterization by NMR, FTIR and amino acid analysis was important because the MBR2 fraction demonstrated antioxidant properties in sperm culture. Likewise, in this part of the work, a discussion of the most relevant research data is developed.

7.1. MATERIALS AND METHODS

7.1.1. AMINO ACID ANALYSIS

A 6 mg sample of MBR2 extract was dissolved in 1 mL solution of 6 M HCL and TFA, in relation 4:1. Right after, the mixture was heated at 140 °C for 3 h. The solution was concentrated to give a dry residue and dissolved in 100 µl citrate buffer. The amino acid composition was analyzed from an aliquot of 4 µL, using SYKAM S7130 amino acid analyzer , (WANG, Y. et al., 2002).

7.1.2. FUNCTIONAL GROUPS FOR MIDDLE INFRARED FOR FOURIER TRANSFORM (FTIR)

The assignment of the different wave stretches was based on those already described for *G. lucidum* by PAN et al., (2012). The functional groups present in the spore samples and aqueous extracts of *G. lucidum* were determined by means of the Medium Infrared Spectroscopy, on a VERTEX 70 (Bruker) equipment, with the DRIFT accessory with 64 scans, 4 cm^{-1} resolution, without losing atmospheric compensation in the region between 4000 and 400 cm^{-1}. Samples were crushed, pulverized and oven dried. At determination, about 20 mg were mixed and homogenized with 100 mg of spectroscopic potassium bromide (KBr) to carry out the measures. The analysis for regions of lipids, carbohydrates and proteins was performed with methodology of (CHEN et al., 2012; WANG et al., 2012).

7.1.2. NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

The extracts were solubilized in deuterated water (D_2O). The samples were analyzed on the Bruker model DRX 400, Avance® series spectrometer. The extracts were placed in 5 mm diameter tubes at 30 °C. The chemical shifts (δ) were expressed in ppm using acetone for the ^{13}C experiments (δ = 30.20 ppm) and the HOD signal for ^{1}H experiments (δ = 2.20 ppm).

For magnetic resonance of carbon 13, the samples were concentrated from 15 to 30 $mg.mL^{-1}$. The coupled and decoupled NMR ^{-13}C spectra were obtained at the base frequency of 100.61 MHz, with a signal acquisition interval of 0.6 s to a measure of 6.600 acquisitions in 0.1 s intervals between pulses.

For the hydrogen resonance, NMR-1H, the samples were worked in concentrations between 10 to 20 $mg.mL^{-1}$. The spectra were obtained at the base frequency of 400.12 MHz. In two-dimensional methods, the heteronuclear single quantum coherence (HSQC) was used to determine which protons were bound to the carbons from signals already described in the literature. The homonuclear techniques Cation Spectroscopy (COSY) and Total Correlation Spectroscopy (TOCSY) were used to observe the interactions between protons. The COSY technique was used to correlate the chemical displacements of the coupled proton nuclei, for example: H1 with H2, thus observing the interaction in the form of a cross-peak between the signals. With the TOCSY technique the cross-peak were observed, which are generated by neighboring protons of the whole coupled spin system. In this way it was possible to read the interactions between H1 with H2, H1 with H3 and so on.

7.2. RESULTS OF THE ANALYSIS OF THE MBR2 FRACTION

7.2.1. COMPOSITION ANALYSIS OF MBR2 EXTRACT

The analysis by SEC/MALS shows that the extract is heterogeneous, the small molecules are in higher concentration and can correspond to amino acids, minerals and carbohydrates less than 12 kDa. The monosaccharide analysis showed a main content of glucose (53.33 %), mannose (20.22 %), galactose (15.17 %) and arabinose (11.28 %). For the amino acid analysis only, histidine was found in a concentration of 21.77 $nMol.mg^{-1}$ in 6 mg of the dry extract (Figure. 33).

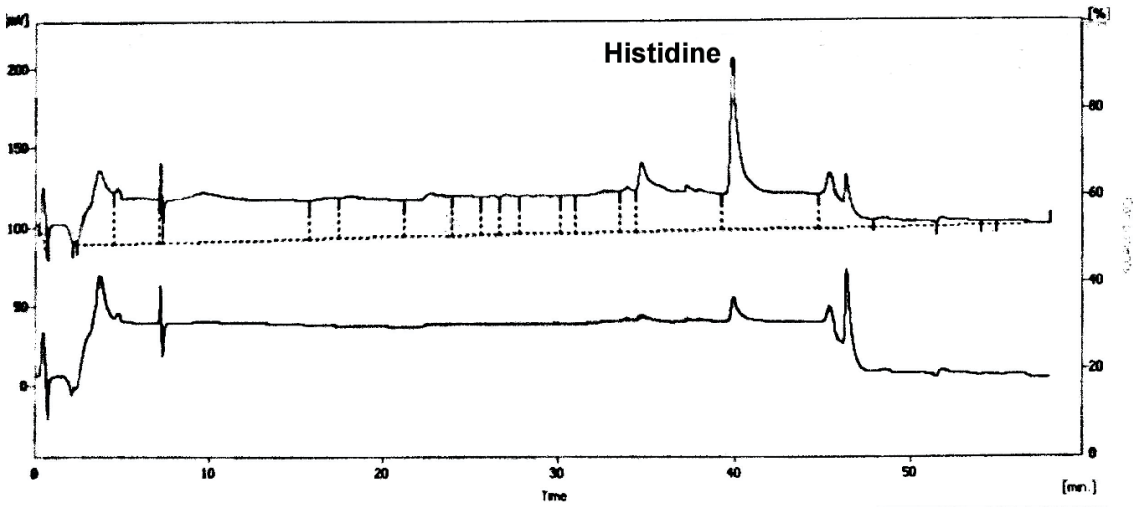


FIGURE. 33 – ANALYSIS OF AMINO ACIDS FOR THE MBR2 FRACTION.
THE HIGHEST PEAK CONCENTRATION FOR AMINO ACIDS CORRESPONDED FOR HISTIDINE WITH 21.77 NMOL.MG^{-1} IN 6 MG OF THE DRY EXTRACT.

7.2.2. ANALYSIS BY FTIR

The FTIR spectrum of the MBR2 extract exhibited absorption bands at 3360, 2929, 1660, 1597, 1462, 1402, 1230, 1049, 929 and 875 cm^{-1} (Figure. 34). Assignments for each stretching were: 3360 cm^{-1} stretching a hydroxyl group, 2929 cm^{-1} of a C-H stretching vibration, 1660 cm^{-1} stretch to amide I, 1597 cm^{-1} stretch to amide II, 1462 was Attributed with CH3, 1402 cm^{-1} was attributed to stretching vibration C-O-C group, 1230 cm^{-1} assigned for amide III, 1049 cm^{-1} stretch for pyranose-type sugars, 929 cm^{-1} indicated a configuration α-, 875 cm^{-1} was ascribed for ring structure as pyranose sugars.

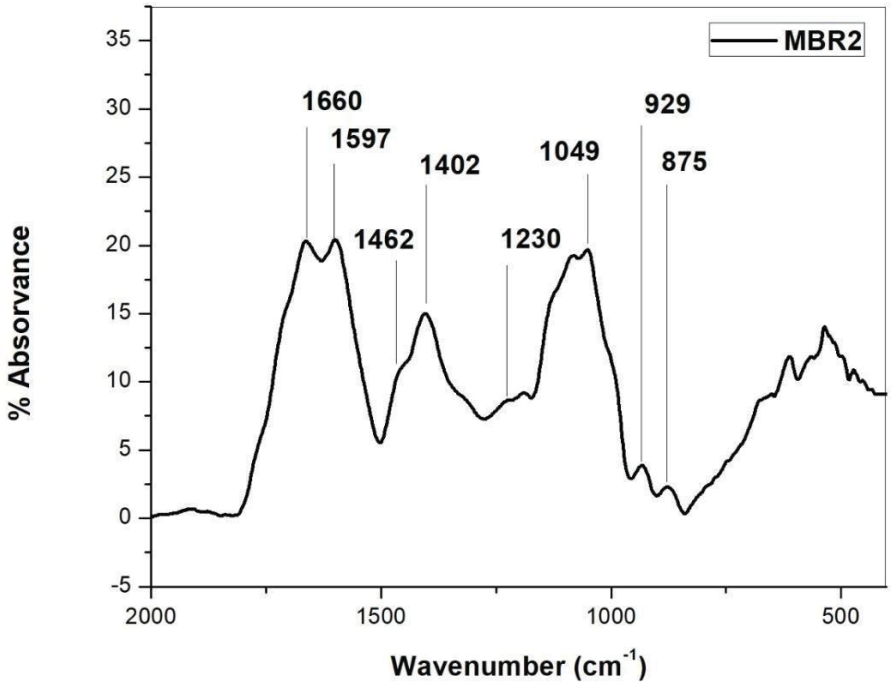


FIGURE. 34 - FTIR SPECTRUM OF MBR2 EXTRACT. ANALYSIS BETWEEN 2000 – 500 cm^{-1}.

7.2.3. RMN DEL EXTRACTO MBR2

According to the TOCSY and COSY experiments, the anomeric protons were assigned at 5.4 ppm and 5.2 ppm. The downfield proton belonged to the glucan, responsible for the α configuration (5.4 ppm). In the HSQC experiment, the proton and carbon anomeric were found for 5.17 / 93.2 ppm and 5.42 / 101.39 ppm (^{1}H / ^{13}C). In general, the MBR2 extract presented several overlapping bands that made it difficult to identify its structure. However, it was possible for anomeric to show two possible structures. A glucan structure: →4)-α-D-Glc*p*-(1→. Followed by the glucan terminal α-D-Glc*p*-(1→ (FIGURE. 35).

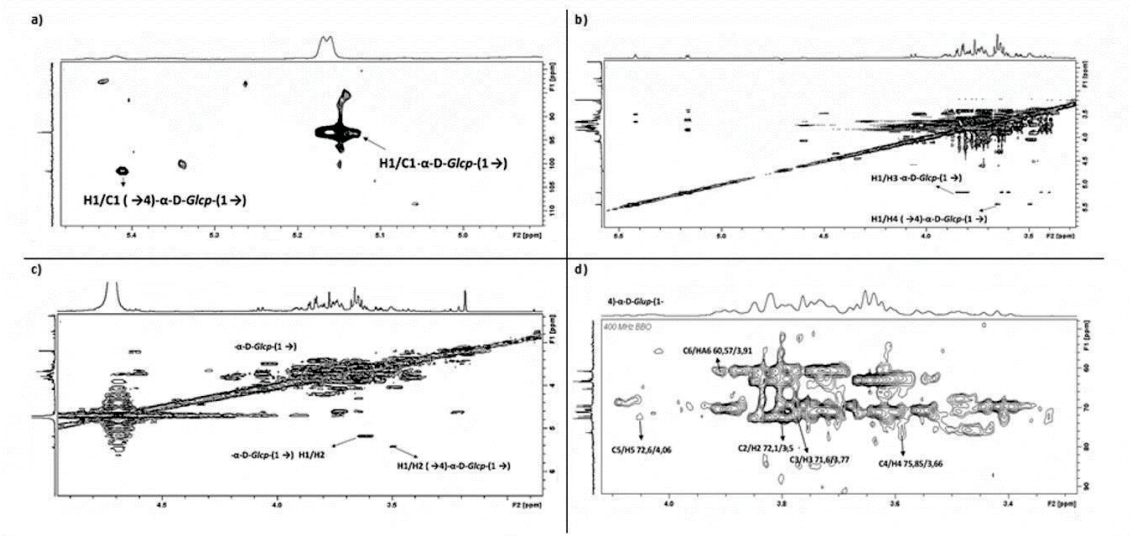


FIGURE. 35 – NMR OF THE MBR2 FRACTION.

A)- HSQC, ANOMERIC CAN BE OBSERVED FOR GLUCAN →4)-A-D-GLC*P*-(1→ IN: 5.42/101.39 PPM. THE GLUCAN TERMINAL A-D-GLC*P*-(1→ IN: 5.17/93.2 PPM ($^{1}H/^{13}C$). **B)-** TOCSY, CAN BE SEE THE PROTON H3 AT 3.65 PPM FOR THE GLUCAN TERMINAL AND THE PROTON H4 AT 3.66 FOR THE GLUCAN A GLUCAN. **C)-** COSY, CAN BE SEEN THE PROTON H2 FOR THE GLUCAN AT 3.5 PPM AND FOR THE GLUCAN TERMINAL AT 3.63 PPM. **D)-** HSQC, THE PROTONS AND CARBONS OF THE 1,4 A-GLUCAN ARE OBSERVED. H2 / C2: 72.1 / 3.5 PPM. H3 / C3: 71.6 / 3-77 PPM. H4 / C4: 75.85 / 3.66 PPM. H5 / C5: 72.6 / 3.82 PPM. H6 / C6: 60.57 / 3.91 PPM. STANDARD REFERENCE WITH ACETONE (^{1}H = 2.20 PPM; ^{13}C = 30.20 PPM).

7.3. GENERAL DISCUSSION

Returning several results for the MBR2 fraction of *G. lucidum* spores extract, the analysis by HPSEC-MALLS-RID showed three molecular stretches at 60, 65 and 70 min. These stretches are related to amino acids, small molecular mass carbohydrates, and minerals. MBR2 fraction had not significant content of proteins. The fraction contains mainlyhistidine. This amino acid content can be seen by FTIR in amide I, II and III stretchings. Moreover, MBR2 can have several minerals, because before dialysis in the microwave extract calcium, Fe, K, Al, Mg, Na, P and Zn were found. Likewise, analysis of monosaccharides showed that the MBR2 fraction had mainly glucose (53.33 %). In the FTIR analysis this type of glucose shows carbohydrates pyranose type with α configuration. This configuration is confirmed by NMR due to the absence of the stretch β. Similarly, NMR confirmed the presence of glucans, because anomeric of →4)-α-D-Glc*p*-(1→ at 5.42 / 101.39 ppm and its terminal α-D-Glc*p*-(1→ at 5.17 / 93.2 ppm was found. It has been described that the spore walls may have different types of polysaccharides, heteropolysaccharides,

mannoglucan and glycopeptide (ZHANG et al., 2007; SYNYTSYA; NOVÁK, 2013). Glucans 1,3 and 1,4 are reported to have cell surface recognition function, thus biological activity (ZHANG et al., 2007; KONO et al., 2017). Using microwave expands particles and contracts molecules (LI, Y. et al., 2013). The use of elevated and sustained temperatures can degrade glucans and increase their cellular bioavailability. Consequently, antioxidant power can be significantly increased (SURAYOT et al., 2014). In MBR2 the use ofmicrowaves and different temperatures allowed the extraction of carbohydrates from the spore wall. It also made available α antioxidant glucans for the sperm cell membrane by increasing cell survival and the tendency to retain motility. On the other hand, the rupture of the spore walls released several amino acids and minerals that are important to maintaining the conditions of the cell culture medium. It is possible to state that it would be necessaryperform a greater purification of the carbohydrates to elucidate their structure, characterize the amino acids and the minerals of the MBR2 fraction.

Overall, the extracts of *G. lucidum* spores were antioxidants maintaining survival in preanthral follicles and sperm. Also, because there was an increase in follicular diameter. The literature describes that aqueous extracts increase the production of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), (HELENO; BARROS; MARTINS; QUEIROZ; et al., 2012; WANG et al., 2017). The neutralization of free radicals is also described (CÖR et al., 2018a). Low molecular weight glucans have the best antioxidant power (TSAI et al., 2012; VELJOVIĆ et al., 2017). In addition, extracts of *G. lucidum* spores are related to mechanisms of cellular apoptosis such as: Bax, caspase and Fas (SOCCOL et al., 2016). Both preanthral follicles and sperm share the mechanism of programmed death Fas and its ligand that activate caspases 3, 6 and 7 (GOTTLIEB, 2001; TAYLOR et al., 2004). Likewise, granulosa cells produce growth factors and hormones for follicular maturation (DIAS et al., 2014; PASSOS et al., 2016). Extracts of *G. lucidum* spores showed antioxidant activity because they can serve as adjuvants to the cell culture medium. Since they can reduce the presence of free radicals, stimulate antioxidant enzymes in the mitochondria and above all offer an extracellular means of maintenance. In preanthral follicles this anti-radical support allows the proliferation of granulose cells and the consequent maturation of the oocyte.

7.4. GENERAL CONCLUSIONS

G. lucidum spores are adjuvants in cell cultures. *G. lucidum* spores have an important antioxidant function because theymaintain cell survival in vitro cultures. This function is found in low molecular weight molecules. These molecules can be extracted by rupture of the spores and exposure to various temperatures.

It can be concluded that low molecular weight carbohydrates, minerals and the presence of amino acids, such as histidine, offer to the cellular environment a better osmotic balance.

Minerals, carbohydrates and possible amino acids need to be purified and separated in order to characterize and identify them the antioxidant molecules involved in this process.

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STEP 8: STATISTICAL ANNEX

Análise Descritiva

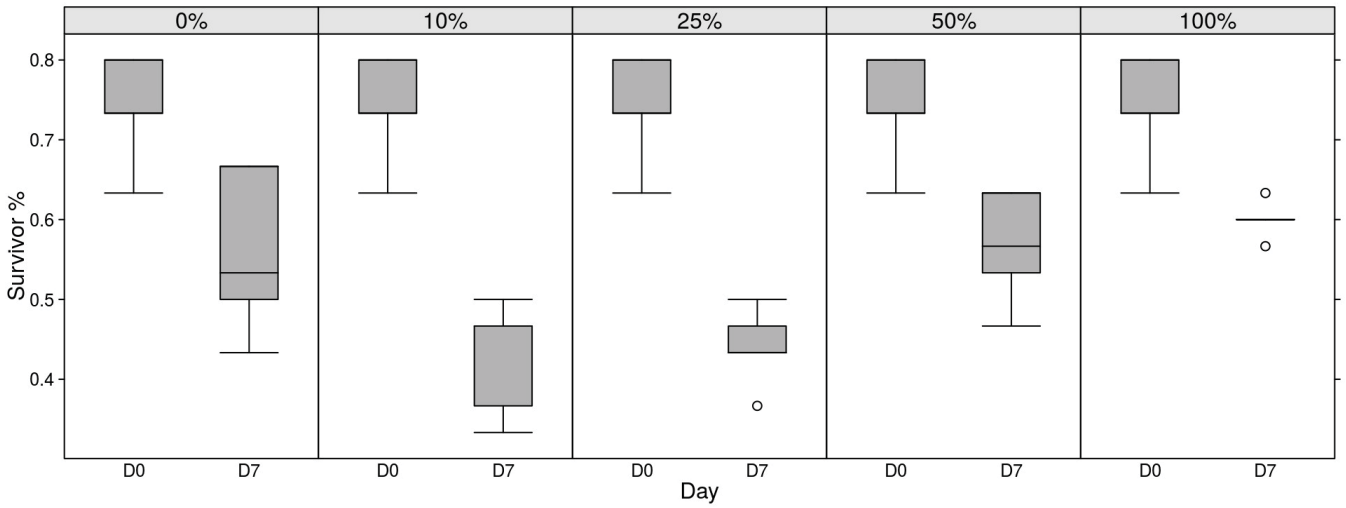
Sobrevivência

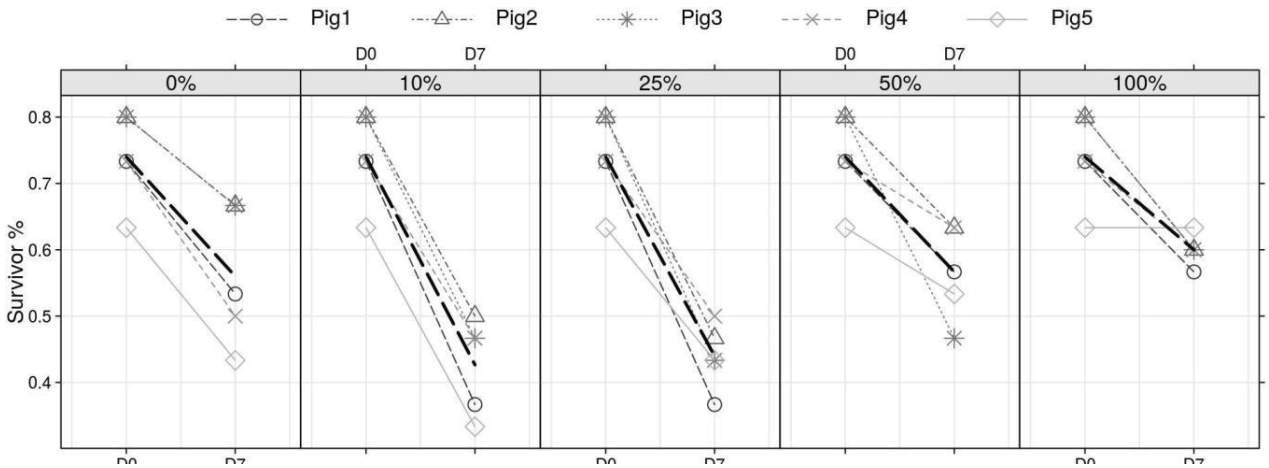
Média da sobrevivência por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##       0%        10%   25%        50% 100%
## D0 0.74  0.7400000 0.74  0.7400000 0.74
## D7 0.56  0.4266667 0.44  0.5666667 0.60
```

Desvio padrão da sobrevivência por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##             0%         10%         25%         50%        100%
## D0 0.06831301 0.06831301 0.06831301 0.06831301 0.06831301
## D7 0.10381608 0.07226494 0.04944132 0.07071068 0.02357023
```





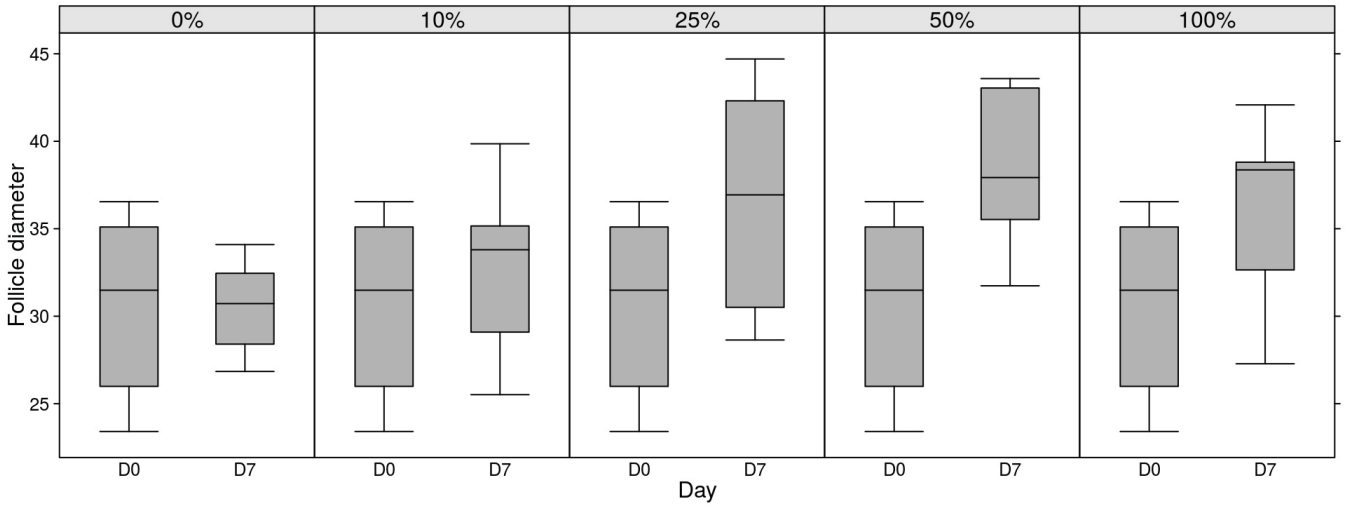
Diâmetro folicular

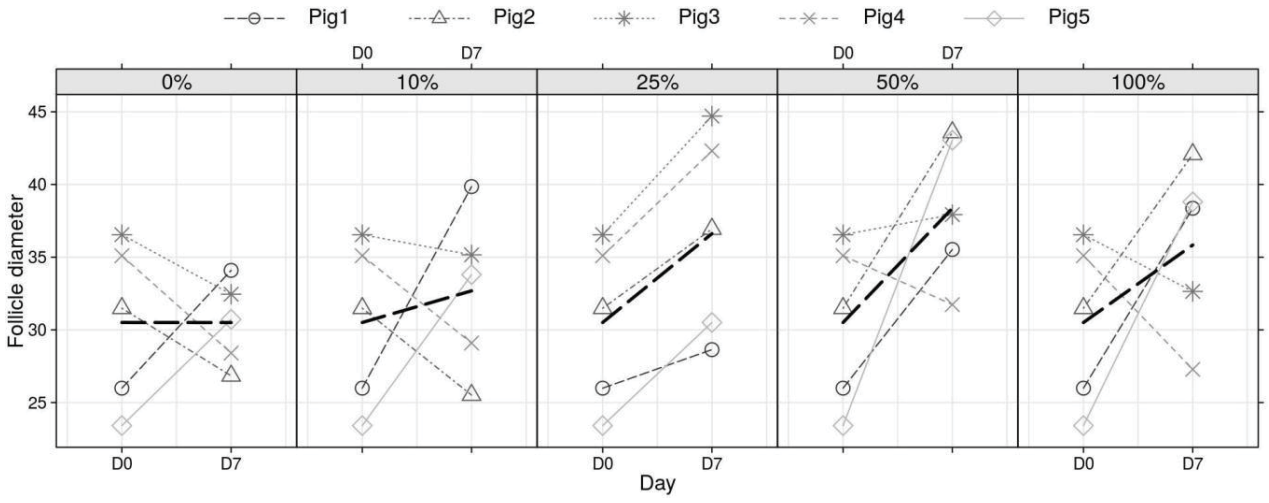
Média do diâmetro folicular por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##         0%      10%      25%      50%    100%
## D0 30.507  30.5070  30.5070  30.5070 30.507
## D7 30.502  32.6835  36.6195  38.3635 35.835
```

Desvio padrão do diâmetro folicular por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##           0%       10%       25%       50%      100%
## D0 5.684402 5.684402 5.684402 5.684402 5.684402
## D7 2.939018 5.546953 7.050652 5.031597 5.863671
```





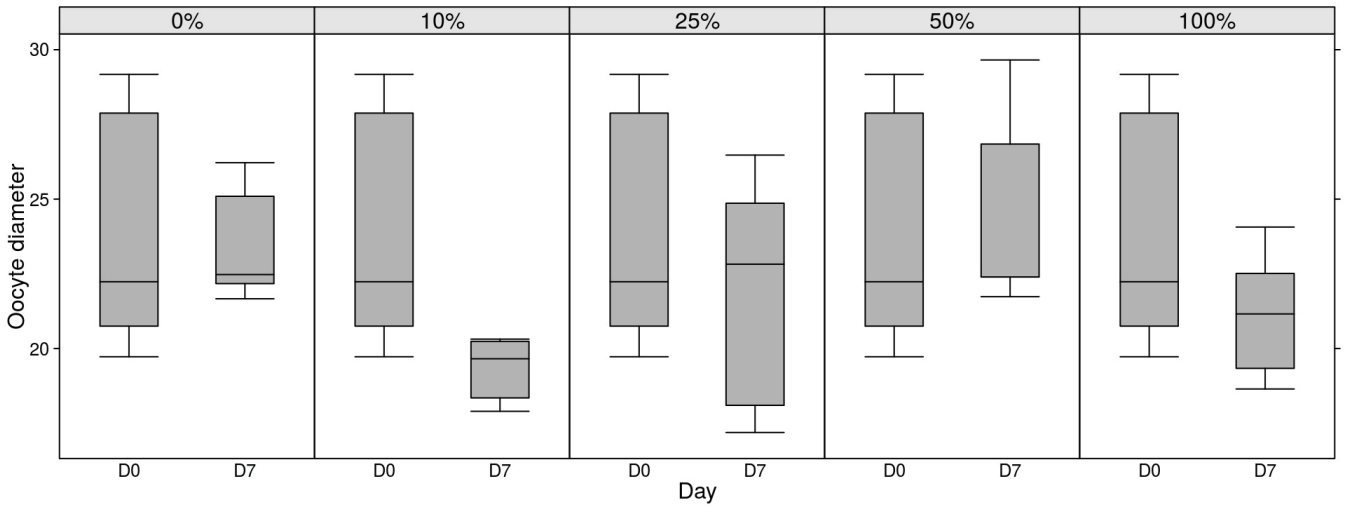
Diâmetro ocitário

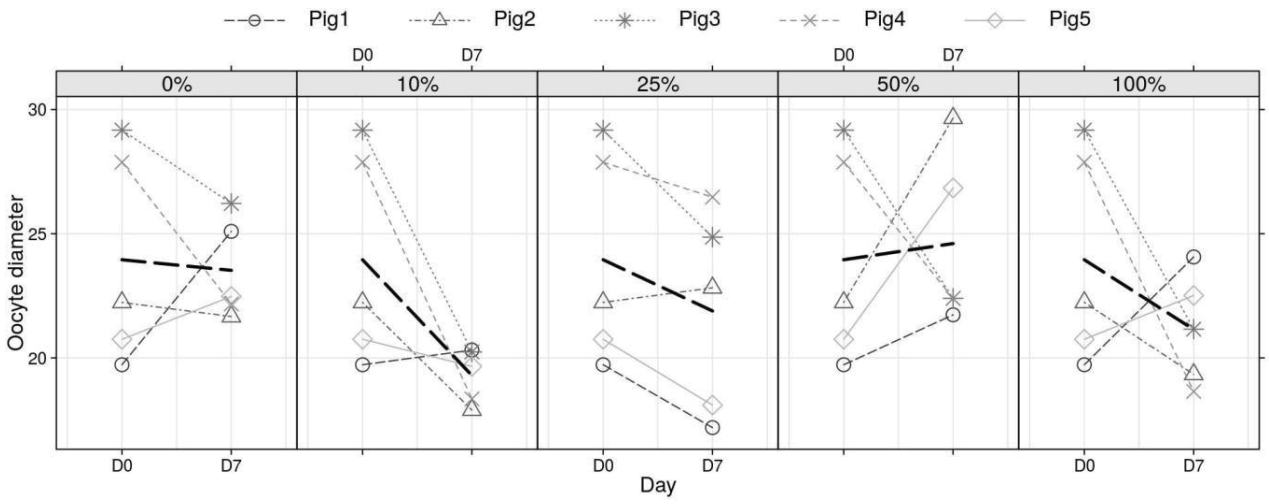
Média do diâmetro ocitário por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##         0%     10%     25%      50%   100%
## D0 23.952 23.952 23.952  23.9520 23.952
## D7 23.525 19.292 21.890  24.6045 21.144
```

Desvio padrão do diâmetro ocitário por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##           0%       10%       25%       50%      100%
## D0 4.293355 4.293355 4.293355 4.293355 4.293355
## D7 2.006602 1.107764 4.096804 3.482600 2.230536
```





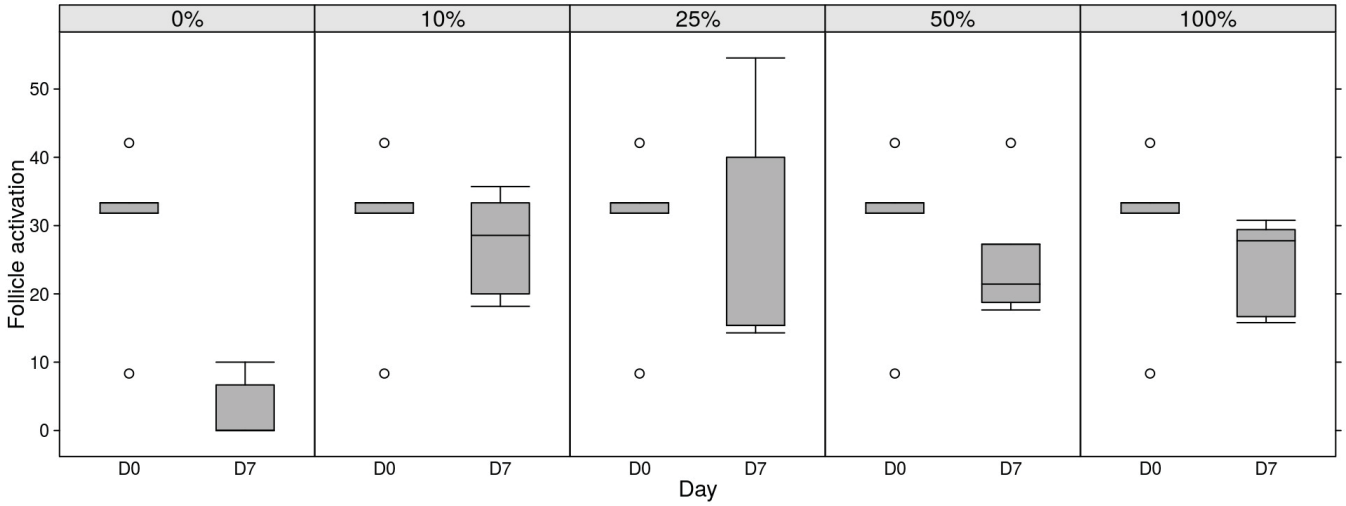
Ativação folicular

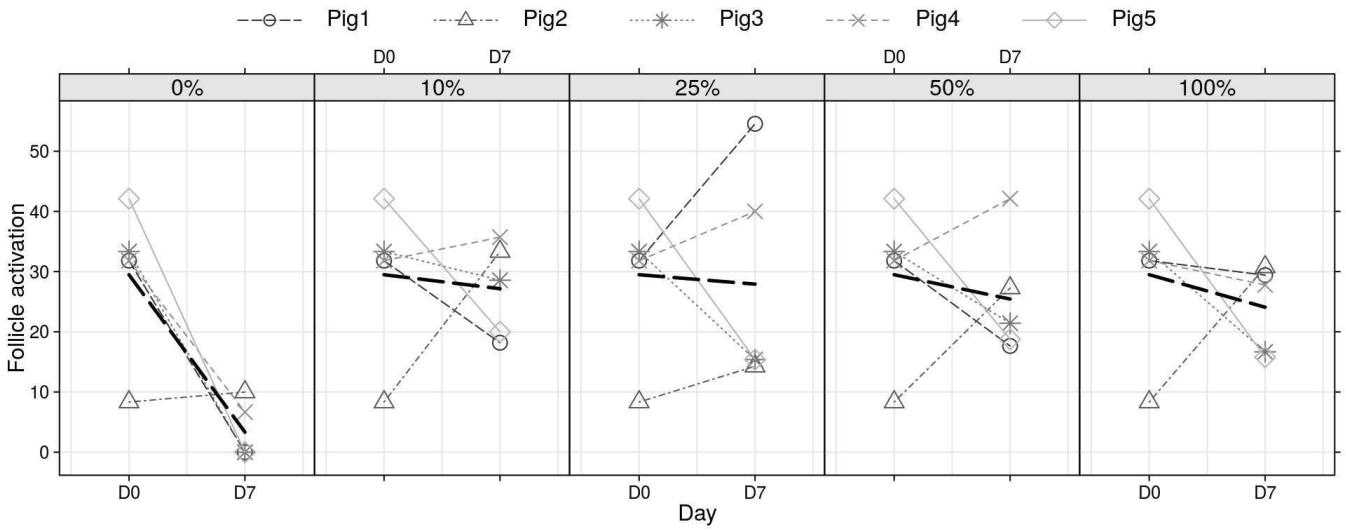
Média da Ativação folicular por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##            0%       10%       25%       50%      100%
## D0 29.481659 29.48166 29.48166 29.48166 29.48166
##  D7  3.333333 27.16017 27.92008 25.44072 24.08298
```

Desvio padrão da Ativação folicular por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##            0%       10%       25%       50%       100%
## D0 12.573396  12.573396 12.57340 12.57340  12.573396
##  D7  4.714045  7.828655 18.40521 10.03329  7.254953
```





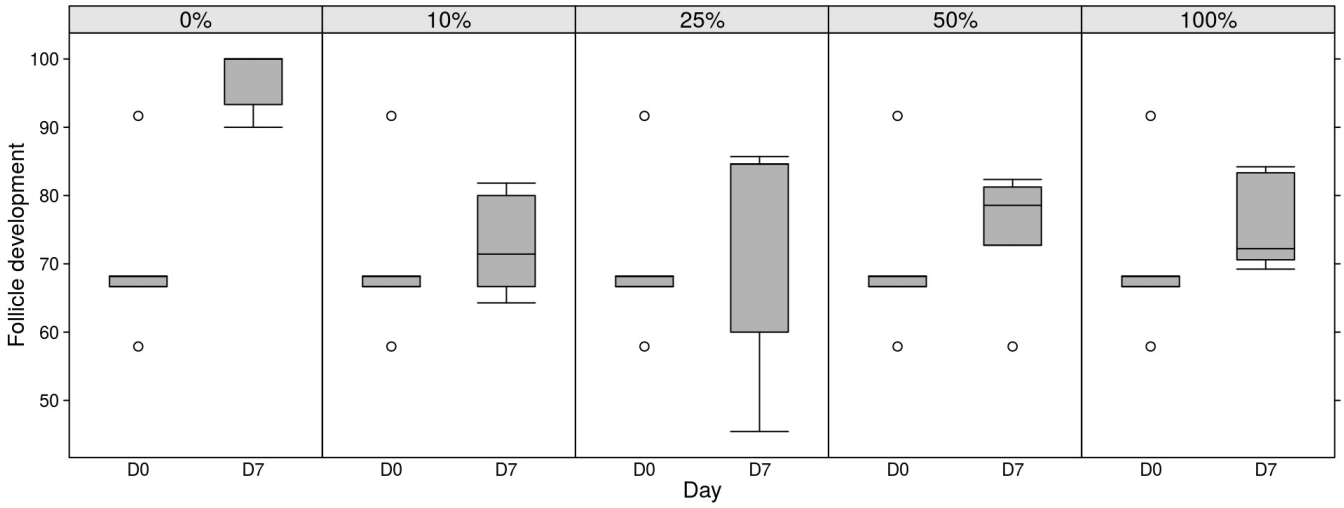
Desenvolvimento folicular

Média do Desenvolvimento folicular por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##           0%       10%       25%       50%      100%
## D0 70.51834  70.51834  70.51834  70.51834  70.51834
## D7 96.66667  72.83983  72.07992  74.55928  75.91702
```

Desvio padrão do Desenvolvimento folicular por tempo (D0 ou D7) e concentração do extrato (0, 10, 25, 50 ou 100%)

```
##            0%        10%       25%       50%       100%
## D0 12.573396  12.573396 12.57340 12.57340  12.573396
## D7  4.714045   7.828655 18.40521 10.03329   7.254953
```



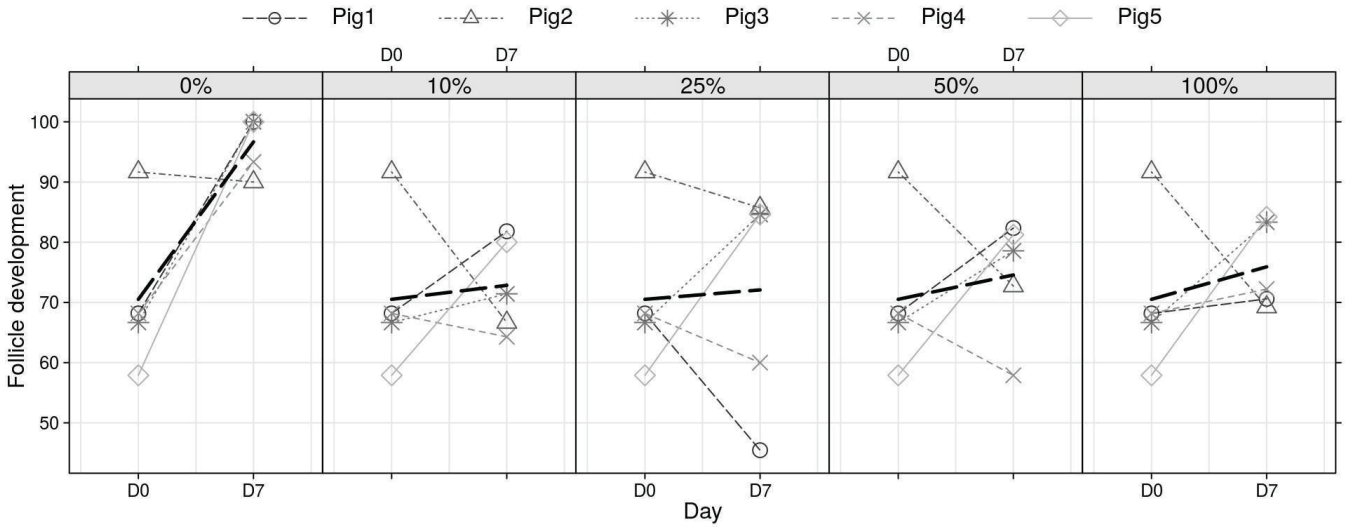
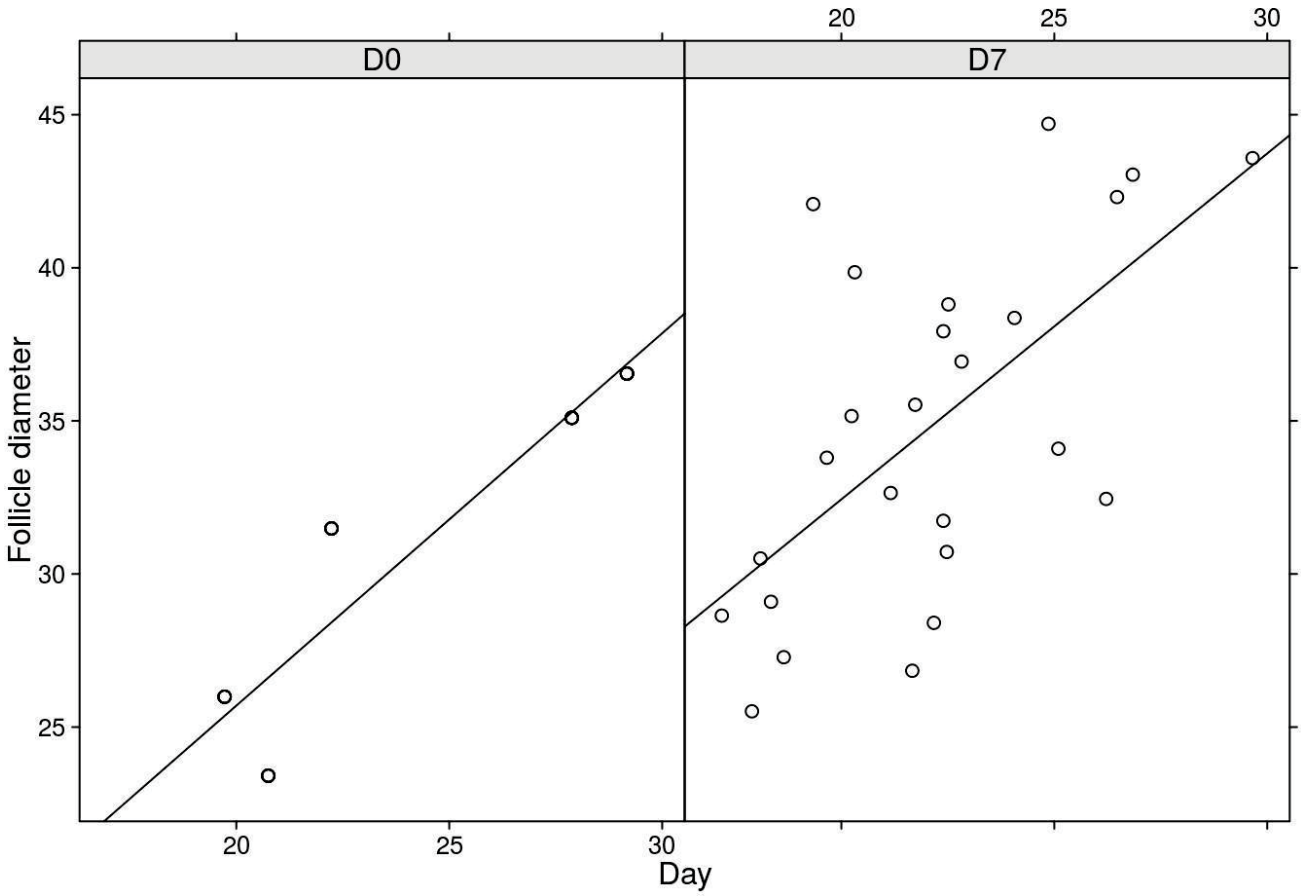
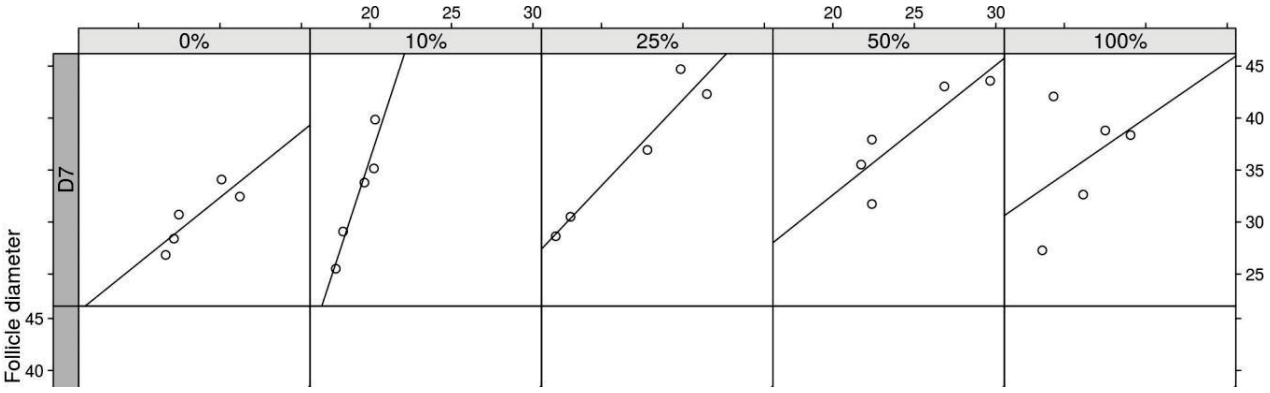


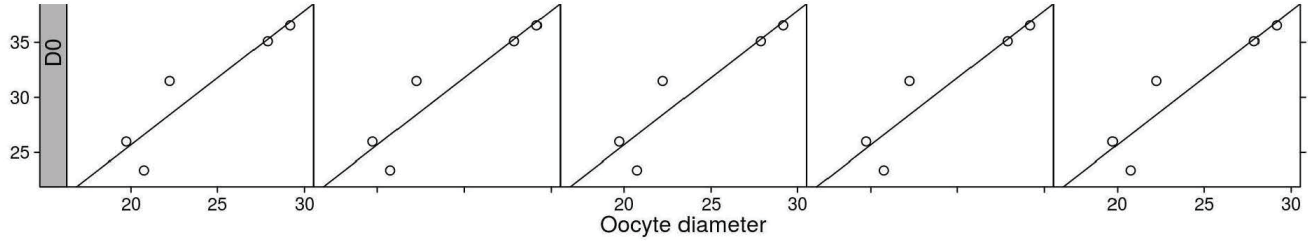
Gráfico para correlação entre diâmetro folicular e diâmetro ocitário

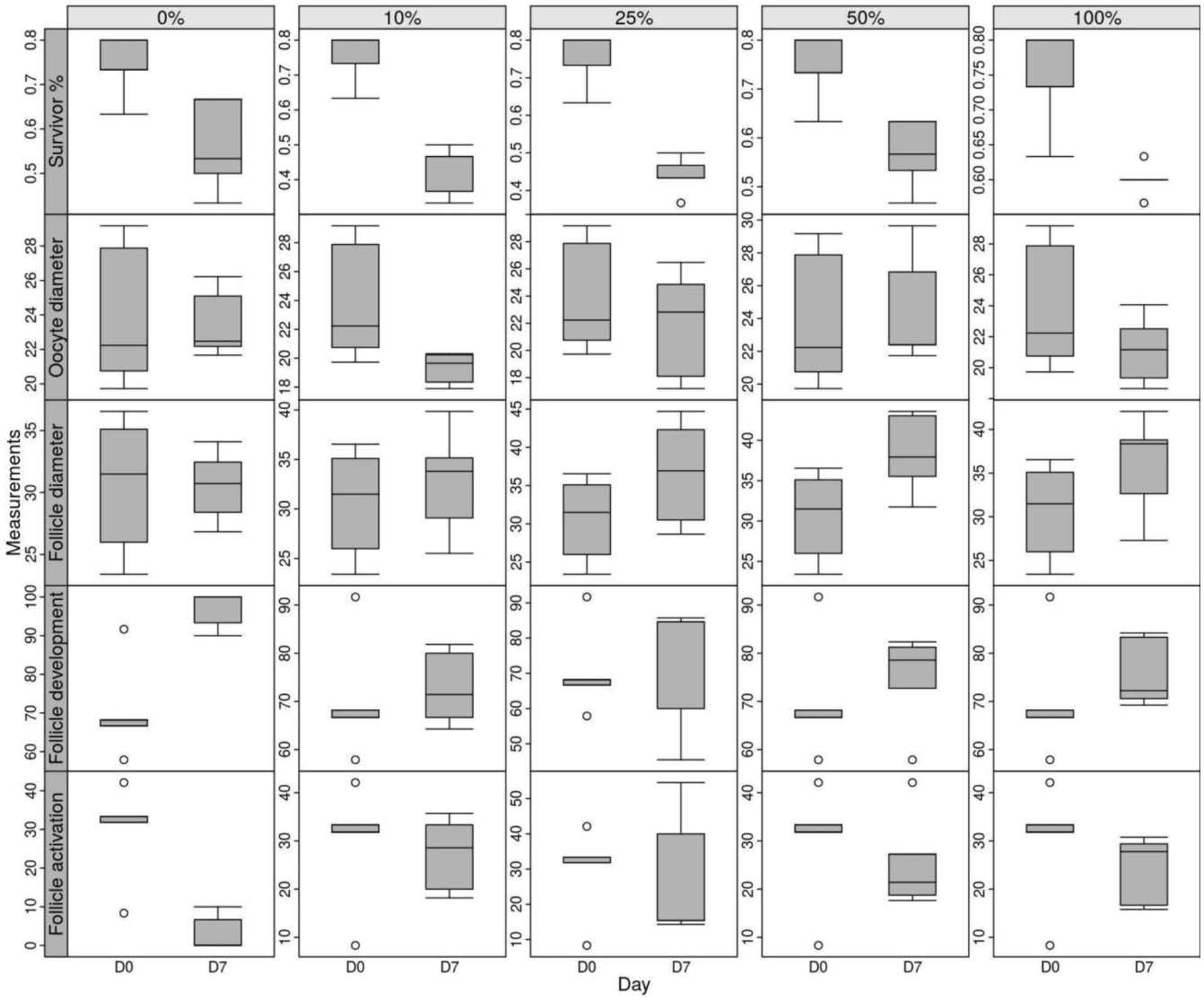


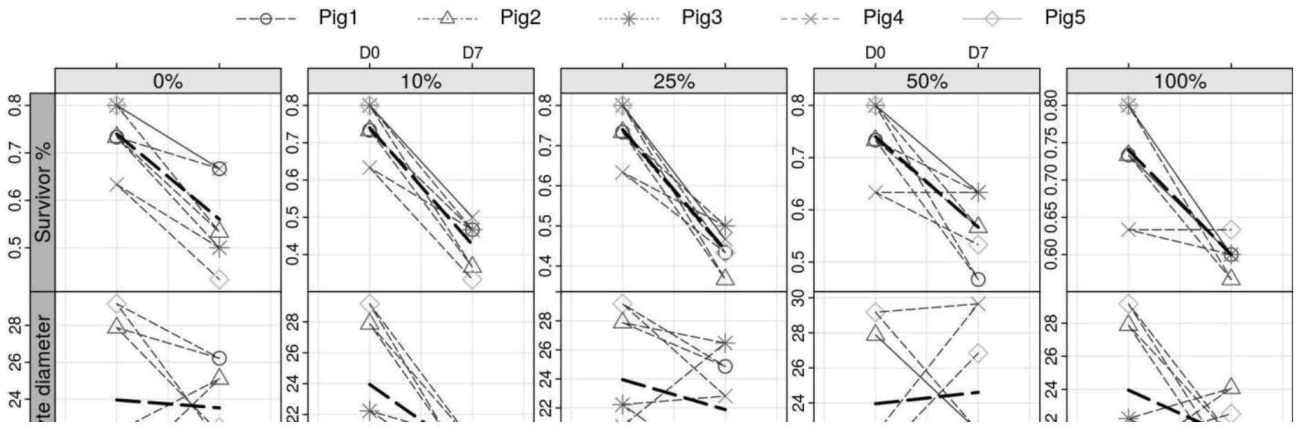
Correlação entre diâmetro folicular e diâmetro ocitario para os tempos D0 e D7

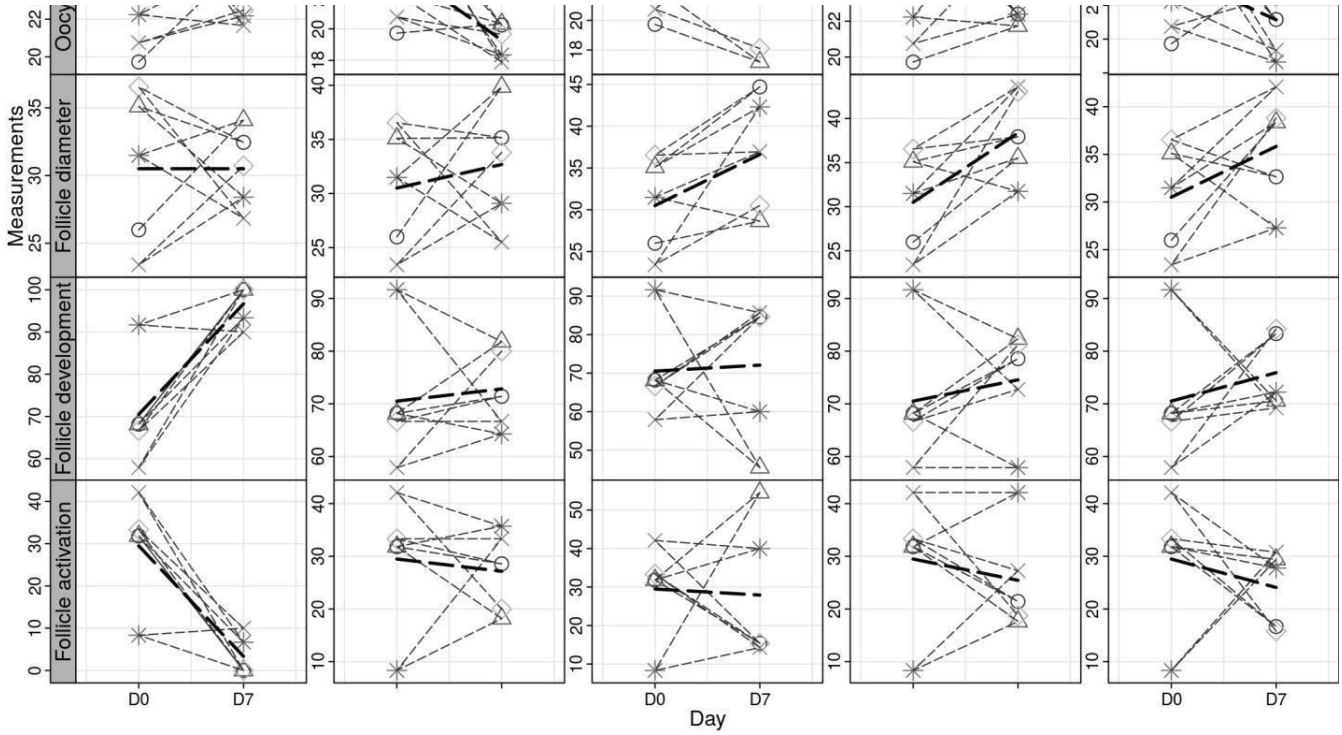
```
##    Coeficiente p-valor
## D0       0.919   0.000
## D7       0.623   0.001
```




D0
Oocyte diameter


0%
10%
25%
50%
100%
Survivor %
Oocyte diameter
Follicle diameter
Follicle development
Follicle activation
Measurements
D0
D7
Day


Pig1
Pig2
Pig3
Pig4
Pig5
0%
10%
25%
50%
100%
Survivor %



Modelos de regressão para dados longitudinais

Variável resposta: Sobrevivência (%)

Preditor linear: Sobrevivência = tempo * grupos (efeito de interação)

Função de variância: binomialP

Função de ligação: logit

Matriz de correlação de trabalho: Compoundsymmetry

Resumo com os coeficientes estimados pelomodelo:

```
## Call: sobre ~ tempo * grupos
##
## Link function: logit
## Variance function: binomialP
## Covariance function: identity
## Regression:
##                         Estimates  Std.error  Z  value  ##
(Intercept)  1.045969e+00 0.1233573  8.479179e+00 ##  tempoD7
                     -8.048065e-01  0.1121434  -7.176583e+00
##   grupos10%   -2.178867e-16   0.1183168   -1.841553e-15   ##
grupos25%           -2.308044e-16 0.1183168 -1.950732e-15 ##
grupos50%           -2.443684e-16 0.1183168 -2.065373e-15 ##
grupos100%    -3.866734e-16    0.1183168    -3.268118e-15    ##
tempoD7:grupos10%  -5.366263e-01  0.1580178  -3.395987e+00  ##
tempoD7:grupos25%  -4.823241e-01  0.1578918  -3.054777e+00  ##
tempoD7:grupos50%  2.710193e-02  0.1579513  1.715840e-01  ##
tempoD7:grupos100% 1.643031e-01 0.1583562 1.037554e+00 ##
## Dispersion:
##     Estimates    Std.error   Z value
## 1 0.006733459 0.001759042  3.827913
## 2 0.007905316 0.005427479 1.456536
##
## Algorithm: chaser
## Correction: TRUE
## Number iterations: 12
```

Teste de comparações múltiplas com correção de bonferroni

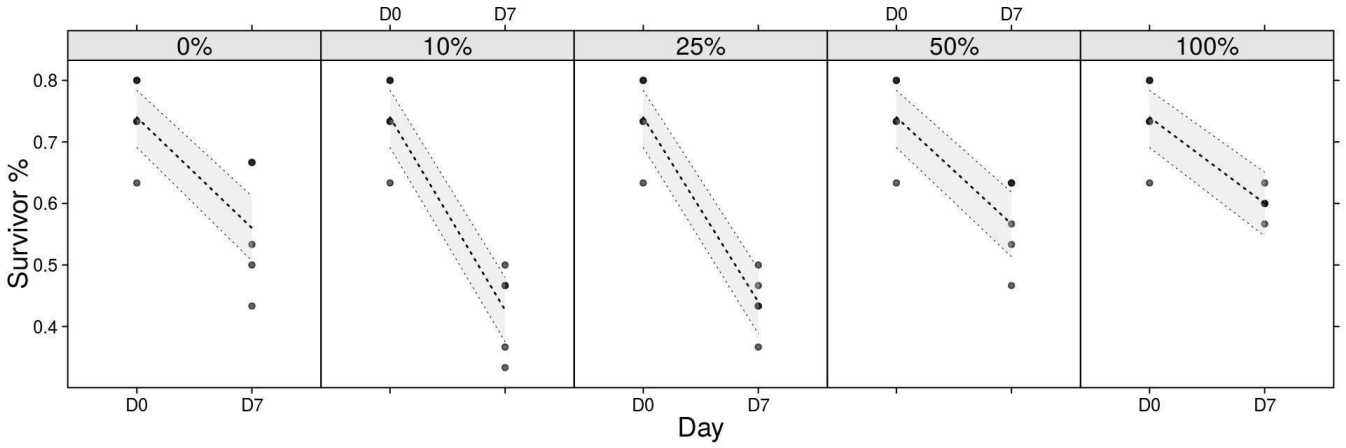
Grupos-tempos

```
## $`0%`
##       Est   EP    z pval
## D0-D7 0.8 0.11 7.18    0
##
## $`10%`
##        Est   EP     z pval
## D0-D7 1.34 0.11 11.95    0
##
## $`100%`
##        Est   EP    z pval
## D0-D7 0.64 0.11 5.68    0
##
## $`25%`
##        Est   EP     z pval
## D0-D7 1.29 0.11 11.48    0
##
## $`50%`
##        Est   EP    z pval
## D0-D7 0.78 0.11 6.93    0
```

Tempos-grupos

```
## $D0
##           Est   EP z pval
## 0%-10%      0 0.12 0    1
## 0%-25%      0 0.12 0    1
## 0%-50%      0 0.12 0    1
## 0%-100%     0 0.12 0    1
## 10%-25%     0 0.12 0    1
## 10%-50%     0 0.12 0    1
## 10%-100%    0 0.12 0    1
## 25%-50%     0 0.12 0    1
## 25%-100%    0 0.12 0    1
## 50%-100%    0 0.12 0    1
##
## $D7
##             Est   EP     z pval
## 0%-10%     0.54 0.10  5.12    0
## 0%-25%     0.48 0.10  4.61    0
## 0%-50%    -0.03 0.10 -0.26    1
## 0%-100%   -0.16 0.11 -1.56    1
## 10%-25%   -0.05 0.10 -0.52    1
## 10%-50%   -0.56 0.10 -5.38    0
## 10%-100%  -0.70 0.11 -6.65    0
## 25%-50%   -0.51 0.10 -4.87    0
## 25%-100%  -0.65 0.11 -6.14    0
## 50%-100%  -0.14 0.11 -1.30    1
```

Curvas de predição com bandas de confiança



Variável resposta: Diâmetro folicular

Preditor linear: Diâmetro folicular = tempo * grupos (efeito de interação)

Função de variância: tweedie

Função de ligação: log

Matriz de correlação de trabalho: Compound symmetry

Teste de comparações múltiplas com correção de bonferroni

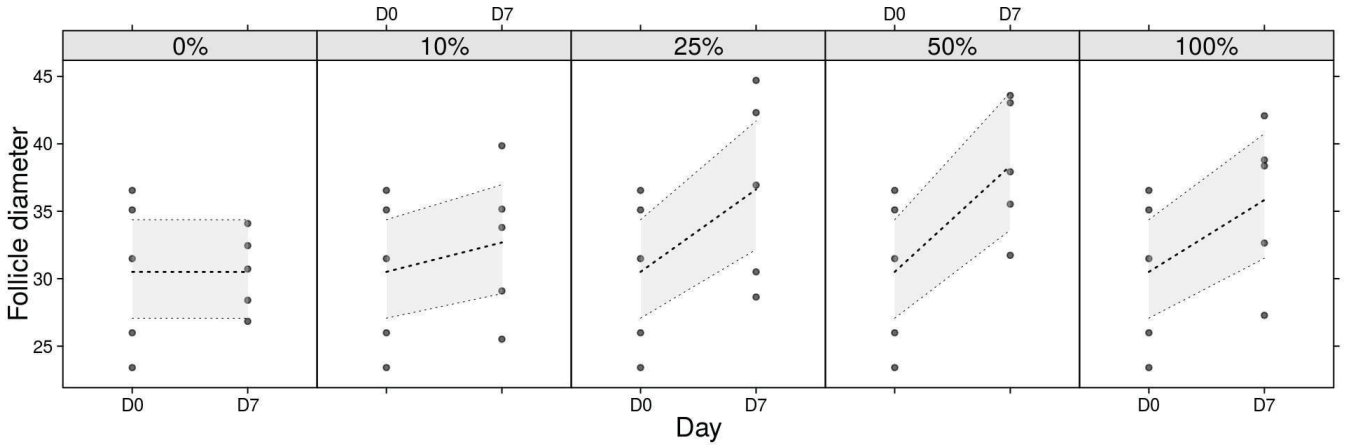
Grupos-tempos

```
## $`0%`
##       Est   EP z pval
## D0-D7   0 0.08 0    1
##
## $`10%`
##          Est   EP     z pval
## D0-D7 -0.07 0.08 -0.89 0.38
##
## $`100%`
##          Est   EP     z pval
## D0-D7 -0.16 0.08 -2.02 0.04
##
## $`25%`
##          Est   EP     z pval
## D0-D7 -0.18 0.08 -2.28 0.02
##
## $`50%`
##          Est   EP     z pval
## D0-D7 -0.23 0.08 -2.83    0
```

Tempos-Grupos

```
## $D0
##           Est   EP z pval
## 0%-10%      0 0.08 0    1
## 0%-25%      0 0.08 0    1
## 0%-50%      0 0.08 0    1
## 0%-100%     0 0.08 0    1
## 10%-25%     0 0.08 0    1
## 10%-50%     0 0.08 0    1
## 10%-100%    0 0.08 0    1
## 25%-50%     0 0.08 0    1
## 25%-100%    0 0.08 0    1
## 50%-100%    0 0.08 0    1
##
## $D7
##             Est   EP     z pval
## 0%-10%    -0.07 0.08 -0.89 1.00
## 0%-25%    -0.18 0.08 -2.29 0.22
## 0%-50%    -0.23 0.08 -2.83 0.05
## 0%-100%   -0.16 0.08 -2.03 0.43
## 10%-25%   -0.11 0.08 -1.40 1.00
## 10%-50%   -0.16 0.08 -1.95 0.51
## 10%-100%  -0.09 0.08 -1.14 1.00
## 25%-50%   -0.05 0.08 -0.55 1.00
## 25%-100%   0.02 0.08  0.26 1.00
## 50%-100%   0.07 0.08  0.81 1.00
```

Curvas de predição com bandas de confiança



Variável resposta: Diâmetro ocitário

Preditor linear: Diâmetro ocitário = tempo * grupos (efeito de interação)

Função de variância: tweedie

Função de ligação: log

Matriz de correlação de trabalho: Compound symmetry

Teste de comparações múltiplas com correção de bonferroni

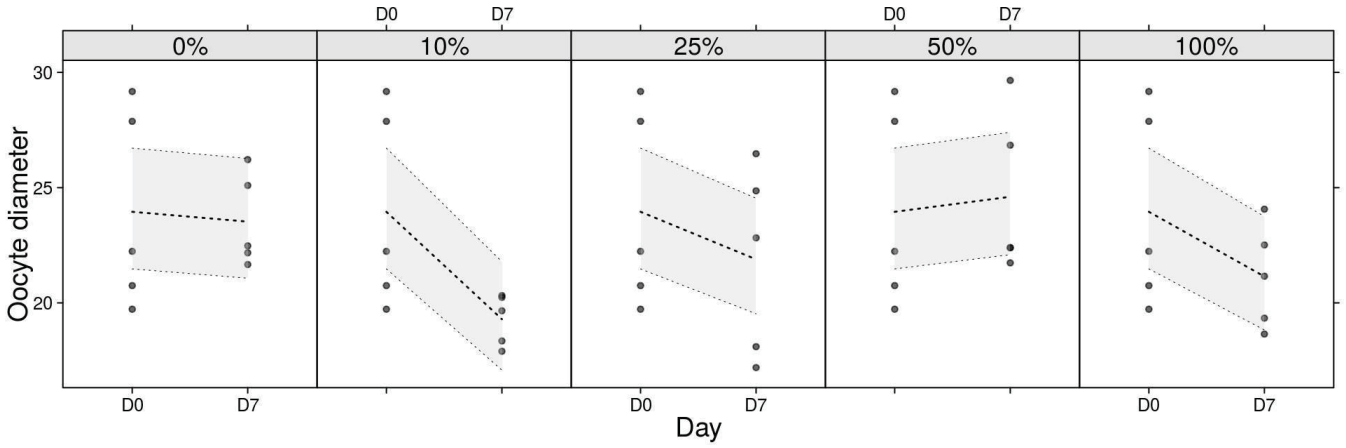
Grupos-tempos

```
## $`0%`
##         Est   EP    z pval
## D0-D7 0.02 0.06 0.28 0.78
##
## $`10%`
##         Est   EP    z pval
## D0-D7 0.22  0.07 3.17    0
##
## $`100%`
##      Est   EP    z pval ##
D0-D7 0.12 0.07 1.87 0.06 ##
## $`25%`
##      Est   EP    z pval ##
D0-D7 0.09 0.07 1.36 0.17 ##
## $`50%`
##       Est   EP     z pval ##
D0-D7 -0.03 0.06 -0.42 0.67
```

Tempos-Grupos

```
## $D0
##           Est   EP z pval
## 0%-10%      0 0.06 0    1
## 0%-25%      0 0.06 0    1
## 0%-50%      0 0.06 0    1
## 0%-100%     0 0.06 0    1
## 10%-25%     0 0.06 0    1
## 10%-50%     0 0.06 0    1
## 10%-100%    0 0.06 0    1
## 25%-50%     0 0.06 0    1
## 25%-100%    0 0.06 0    1
## 50%-100%    0 0.06 0    1
##
## $D7
##             Est   EP     z pval
## 0%-10%     0.20 0.07  2.89 0.04
## 0%-25%     0.07 0.07  1.09 1.00
## 0%-50%    -0.04 0.06 -0.70 1.00
## 0%-100%    0.11 0.07  1.60 1.00
## 10%-25%   -0.13 0.07 -1.81 0.70
## 10%-50%   -0.24 0.07 -3.58 0.00
## 10%-100% -0.09 0.07 -1.30 1.00
## 25%-50%   -0.12 0.07 -1.78 0.75
## 25%-100%   0.03 0.07  0.51 1.00
## 50%-100%   0.15 0.07  2.29 0.22
```

Curvas de predição com bandas de confiança



Variável resposta: Desenvolvimento folicular

Preditor linear: Desenvolvimento folicular = tempo * grupos (efeito de interação)

Função de variância: tweedie

Função de ligação: log

Matriz de correlação de trabalho: Compound symmetry

Teste de comparações múltiplas com correção de bonferroni

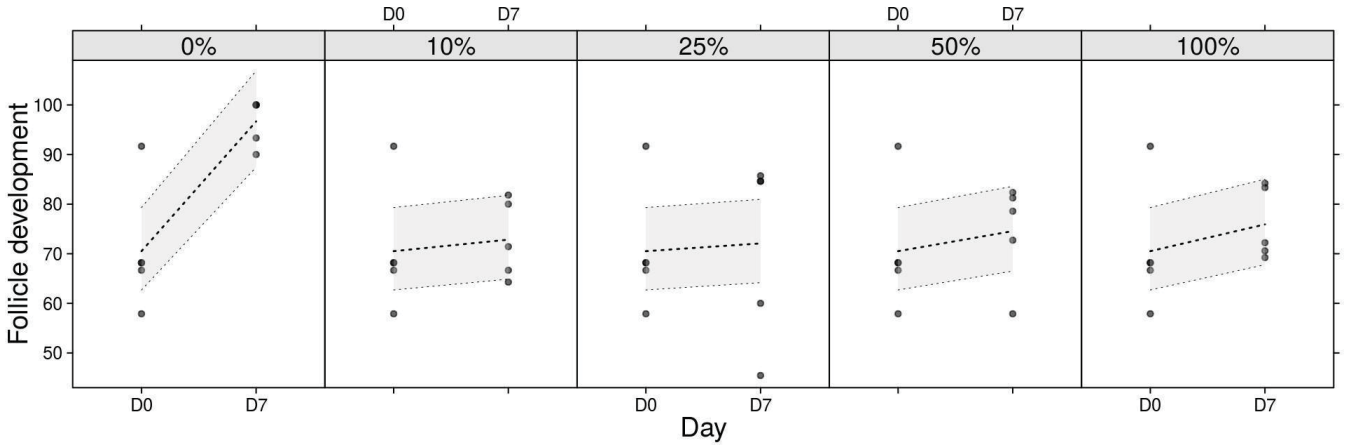
Grupos-tempos

```
## $`0%`
##          Est   EP     z pval
## D0-D7 -0.32  0.07  -4.41    0
##
## $`10%`
##          Est   EP     z pval
## D0-D7 -0.03  0.08 -0.43 0.67
##
## $`100%`
##          Est   EP     z pval
## D0-D7 -0.07  0.08 -0.98 0.33
##
## $`25%`
##          Est   EP     z pval
## D0-D7 -0.02  0.08 -0.29 0.77
##
## $`50%`
##          Est   EP     z pval
## D0-D7 -0.06  0.08 -0.74 0.46
```

Tempos-grupos

```
## $D0
##           Est   EP z pval
## 0%-10%      0 0.08 0    1
## 0%-25%      0 0.08 0    1
## 0%-50%      0 0.08 0    1
## 0%-100%     0 0.08 0    1
## 10%-25%     0 0.08 0    1
## 10%-50%     0 0.08 0    1
## 10%-100%    0 0.08 0    1
## 25%-50%     0 0.08 0    1
## 25%-100%    0 0.08 0    1
## 50%-100%    0 0.08 0    1
##
## $D7
##             Est   EP     z pval
## 0%-10%     0.28 0.07  4.00 0.00
## 0%-25%     0.29 0.07  4.13 0.00
## 0%-50%     0.26 0.07  3.69 0.00
## 0%-100%    0.24 0.07  3.46 0.01
## 10%-25%    0.01 0.08  0.14 1.00
## 10%-50%   -0.02 0.08 -0.31 1.00
## 10%-100%  -0.04 0.07 -0.55 1.00
## 25%-50%   -0.03 0.08 -0.45 1.00
## 25%-100%  -0.05 0.07 -0.69 1.00
## 50%-100%  -0.02 0.07 -0.24 1.00
```

Curvas de predição com bandas de confiança



Variável resposta: Ativação folicular

Preditor linear: Ativação folicular = tempo * grupos (efeito de interação)

Função de variância: tweedie

Função de ligação: log

Matriz de correlação de trabalho: Compound symmetry

Teste de comparações múltiplas com correção de bonferroni

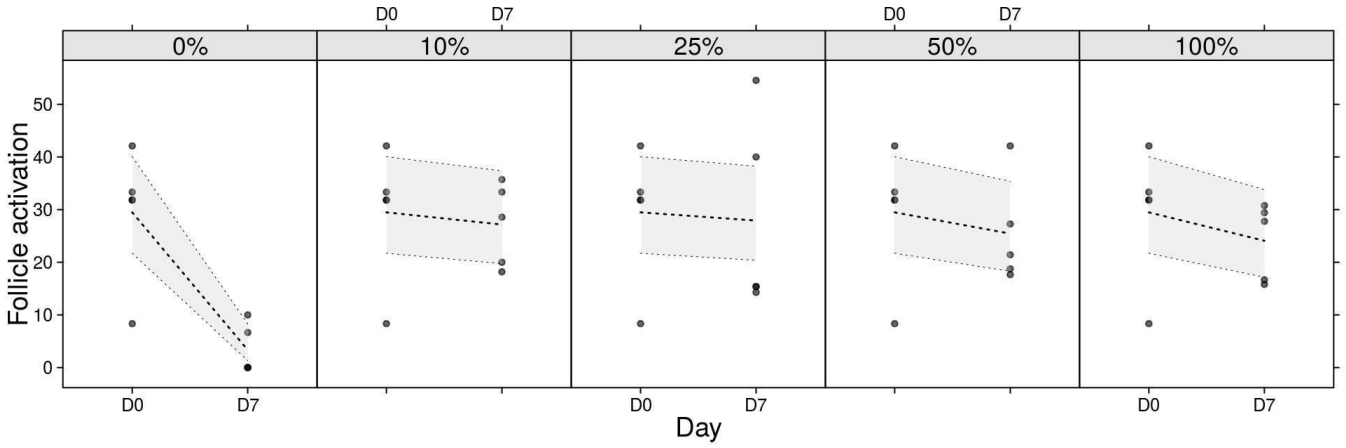
Grupos-tempos

```
## $`0%`
##         Est   EP    z pval
## D0-D7 2.18 0.48 4.59    0
##
## $`10%`
##         Est   EP    z pval
## D0-D7 0.08 0.21 0.38  0.7
##
## $`100%`
##     Est   EP    z pval ##
D0-D7 0.2 0.22 0.91 0.36 ##
## $`25%`
##         Est   EP    z pval
## D0-D7 0.05 0.21 0.26  0.8
##
## $`50%`
##         Est   EP    z pval
## D0-D7 0.15 0.22 0.68  0.5
```

Tempos-grupos

```
## $D0
##           Est   EP z pval
## 0%-10%      0 0.21 0    1
## 0%-25%      0 0.21 0    1
## 0%-50%      0 0.21 0    1
## 0%-100%     0 0.21 0    1
## 10%-25%     0 0.21 0    1
## 10%-50%     0 0.21 0    1
## 10%-100%    0 0.21 0    1
## 25%-50%     0 0.21 0    1
## 25%-100%    0 0.21 0    1
## 50%-100%    0 0.21 0    1
##
## $D7
##             Est   EP     z pval
## 0%-10%    -2.10 0.48 -4.40    0
## 0%-25%    -2.13 0.48 -4.46    0
## 0%-50%    -2.03 0.48 -4.25    0
## 0%-100%   -1.98 0.48 -4.12    0
## 10%-25%   -0.03 0.22 -0.13    1
## 10%-50%    0.07 0.22  0.29    1
## 10%-100%   0.12 0.23  0.53    1
## 25%-50%    0.09 0.22  0.42    1
## 25%-100%   0.15 0.22  0.66    1
## 50%-100%   0.05 0.23  0.24    1
```

Curvas de predição com bandas de confiança



Referências

- Bonat, W. H. mcglm: Multivariate Covariance Generalized Linear Models. GitLab, 2016. Http://git.leg.ufpr.br/wbonat/mcglm (Http://git.leg.ufpr.br/wbonat/mcglm). R package version 0.4.0.
- Bonat, W. H. and Jørgensen, B. (2016). Multivariate covariance generalized linear models. Journal of the Royal Statistical Society: Series C (Applied Statistics), 65(5):649–675.
- Bonat W. H. Multiple response variables regression models in R: The mcglm package. Journal of Statistical Software 2018; 84(4):1–30, doi: 10.18637/jss.v084.i04.
- Højsgaard, S., Halekoh, U., 2016. doBy: Groupwise Statistics, LSmeans, Linear Contrasts, Utilities. R package version 4.5-15. Available: http://CRAN.Rproject.org/package¼adoBy (http://CRAN.Rproject.org/package¼adoBy)
- R DEVELOPMENT CORE TEAM. 2017. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Sarkar, D. Lattice: Multivariate Data Visualization with R. Springer, New York, 2008. ISBN 978-0-387-75968-5.

Análise Descritiva

Movimentação (% Motility)

Mínimo por grupo

```
##   AA  BR1  BR2 H2O2 MBR1 MBR2
##    0    0    0    0    0    0
```

Máximo por grupo

```
##    AA   BR1   BR2  H2O2  MBR1  MBR2
## 0.290 0.265 0.375 0.275 0.200 0.365
```

Média por grupo

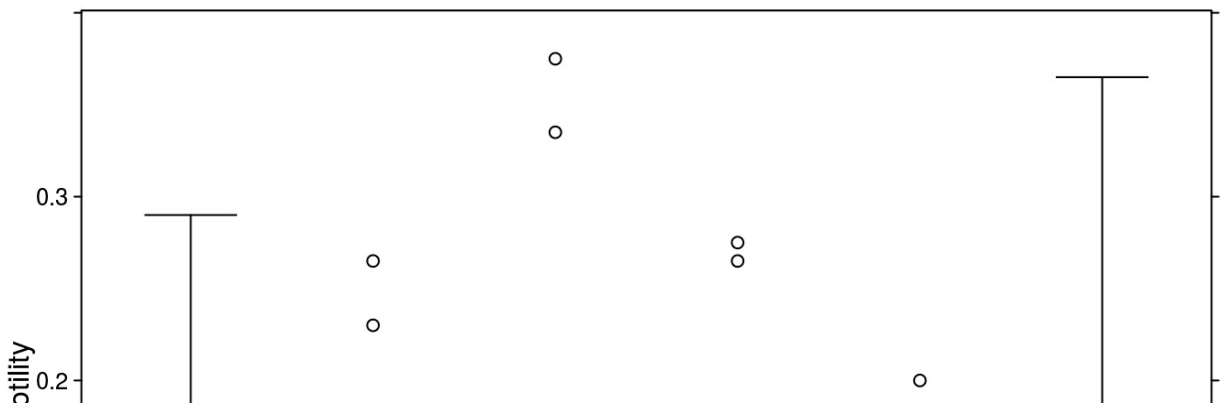
```
##          AA         BR1         BR2        H2O2        MBR1        MBR2
## 0.09550000 0.05227273 0.08363636 0.05181818 0.03227273 0.09363636
```

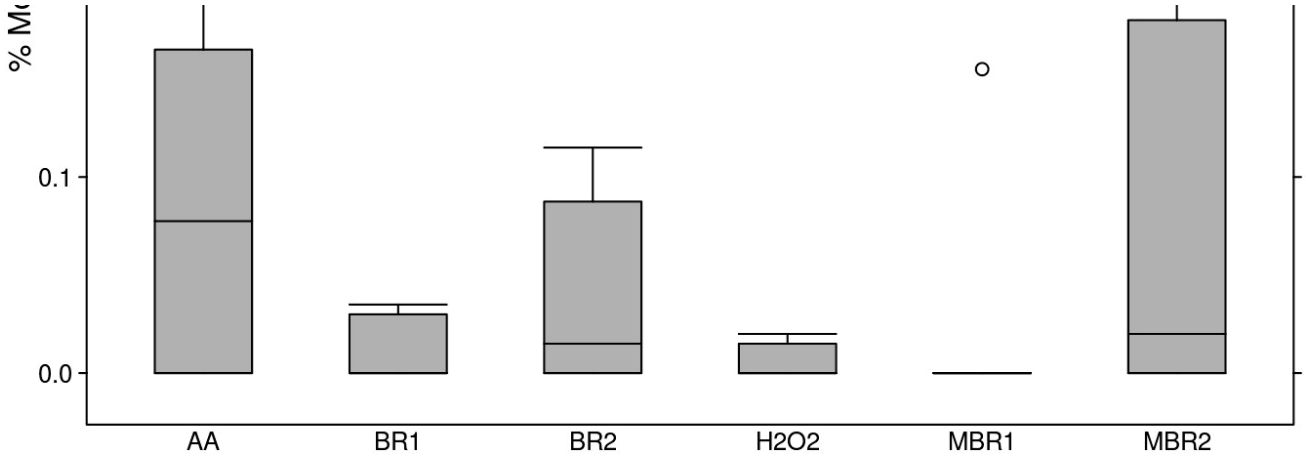
Mediana por grupo

```
##     AA    BR1    BR2   H2O2   MBR1   MBR2
## 0.0775 0.0000 0.0150 0.0000 0.0000  0.0200
```

Desvio padrão por grupo

```
##          AA         BR1         BR2        H2O2        MBR1        MBR2
## 0.10125463 0.09763103 0.13905199 0.10808036 0.07250392 0.12761448
```





Teste: Kruskal-Wallis

grupos: p = 0.3195

Vivos (% Survivor)

Mínimo por grupo

```
##    AA   BR1   BR2  H2O2  MBR1  MBR2
## 0.005 0.000 0.000 0.000 0.000 0.020
```

Máximo por grupo

```
##    AA   BR1   BR2  H2O2  MBR1  MBR2
## 0.350 0.215 0.300 0.160 0.365 0.380
```

Média por grupo

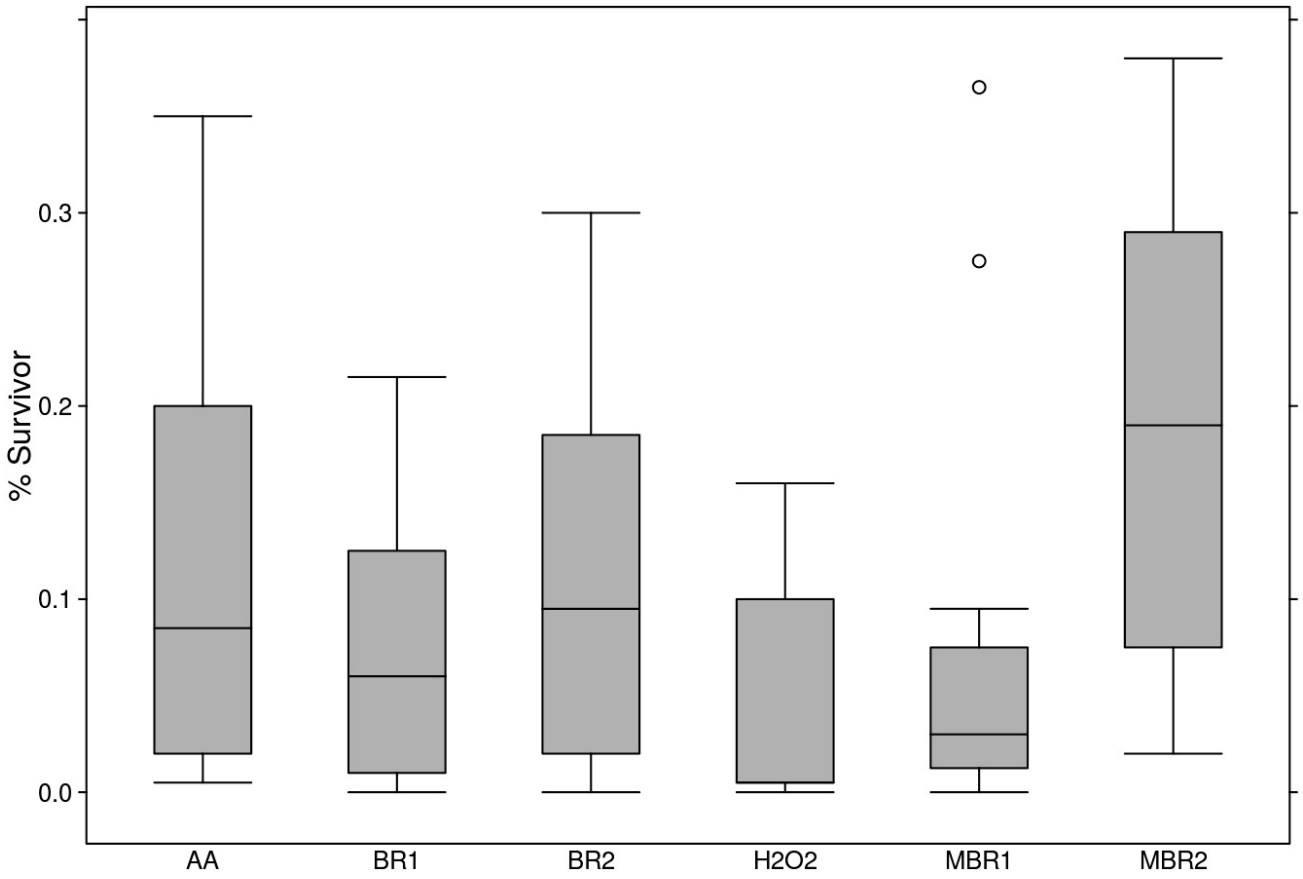
```
##          AA         BR1         BR2        H2O2        MBR1        MBR2
## 0.12400000 0.08100000 0.11150000 0.05045455 0.08409091 0.19200000
```

Mediana por grupo

```
##    AA   BR1   BR2  H2O2  MBR1  MBR2
## 0.085 0.060 0.095 0.005 0.030 0.190
```

Desvio padrão por grupo

```
##          AA         BR1         BR2        H2O2        MBR1        MBR2
## 0.11403703 0.07947047 0.10146291 0.06754460 0.12136347 0.13273198
```



Teste: Kruskal-Wallis

grupos: p = 0.05082

Teste de comparações múltiplas

```
##
##  Pairwise comparisons using Conover's-test for multiple
##                           comparisons of independent samples
##
## data:  vivos by grupos
##
##       AA    BR1   BR2   H2O2  MBR1
## BR1   1.000 -     -     -     -
## BR2   1.000 1.000 -     -     -
## H2O2  0.589 1.000 1.000 -     -
## MBR1  1.000 1.000 1.000 1.000 -
## MBR2  1.000 0.543 1.000 0.026 0.296
##
## P value adjustment method: holm
```

Note que, apenas MBR2 difere de H2O2 (p = 0.026).

Cometa (Categoria % A)

Mínimo por grupo

```
##   AA  BR1  BR2 H2O2 MBR1 MBR2
## 0.52 0.12 0.28 0.16 0.07  0.51
```

Máximo por grupo

```
##   AA  BR1  BR2 H2O2 MBR1 MBR2
## 0.95 0.93 0.96 0.80 0.89  1.00
```

Média por grupo

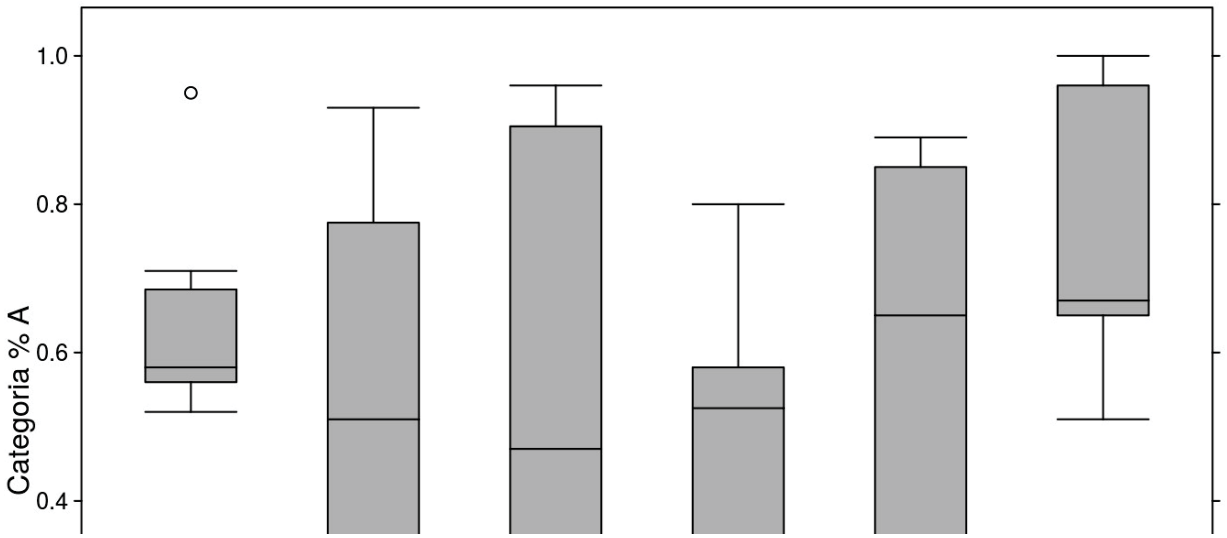
```
##        AA       BR1       BR2      H2O2      MBR1      MBR2
## 0.6485714 0.5062500 0.5812500 0.4816667 0.5625000 0.7714286
```

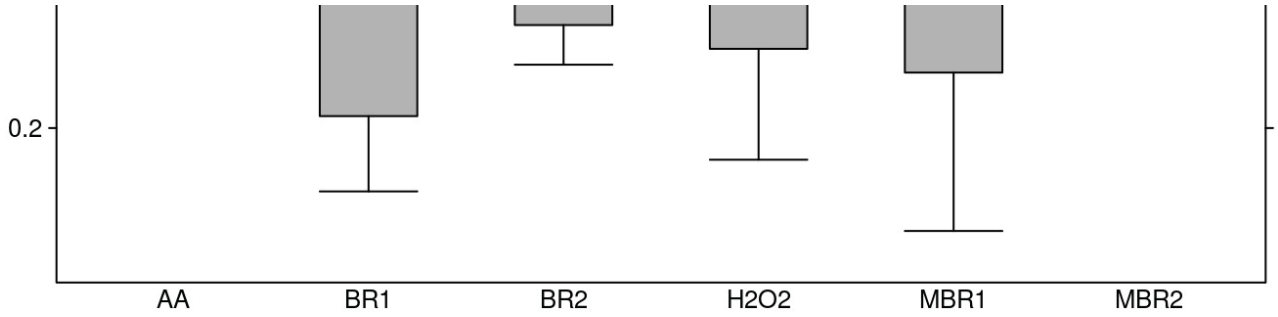
Mediana por grupo

```
##    AA   BR1   BR2  H2O2  MBR1  MBR2
## 0.580 0.510 0.470 0.525 0.650 0.670
```

Desvio padrão por grupo

```
##        AA       BR1       BR2      H2O2      MBR1      MBR2
## 0.1481473 0.3113536 0.2955594 0.2241800 0.3176588 0.1964203
```

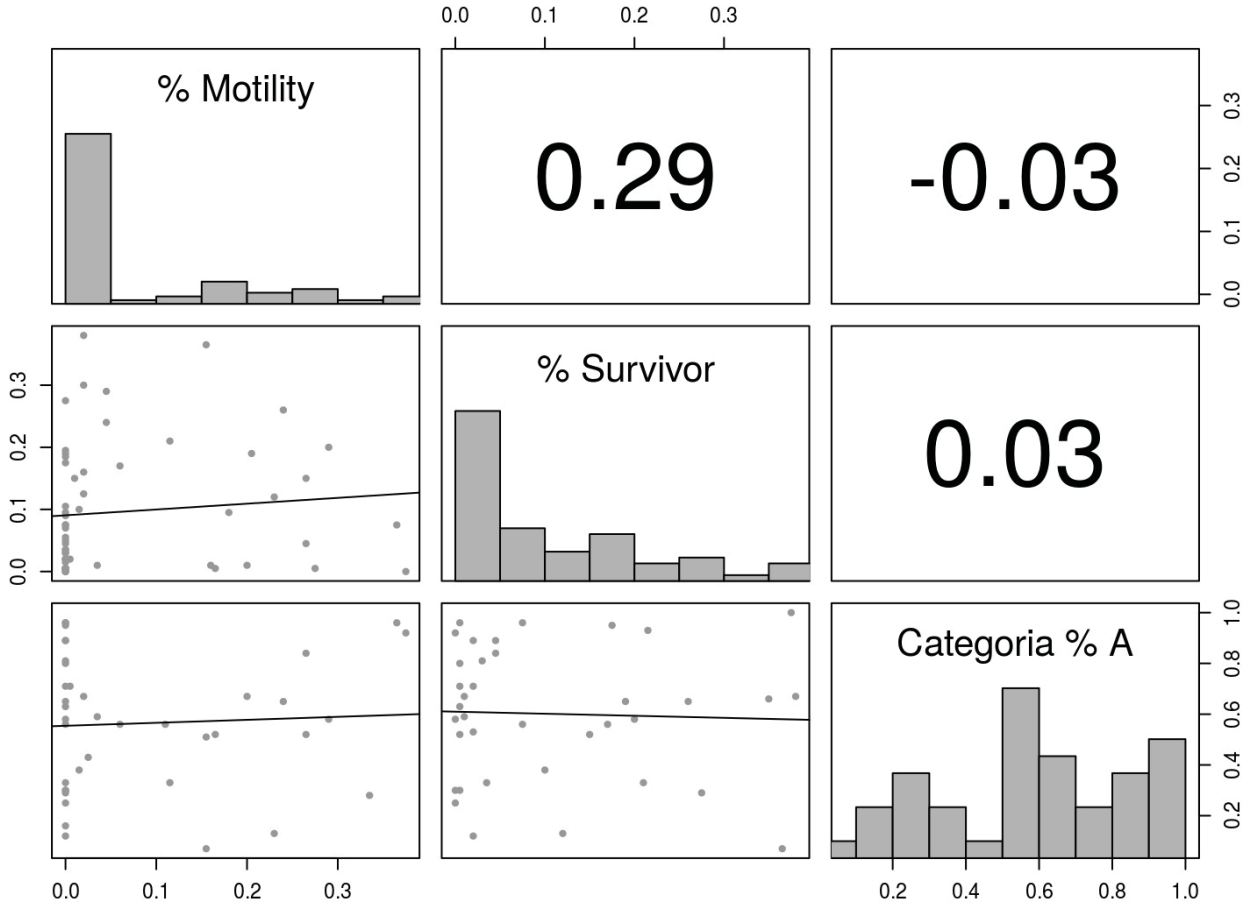




Teste: ANOVA

grupos: p = 0.362

Correlação entre a % de movimentação, vivos e cometa



Referência do software

- R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/ (https://www.R-project.org/).