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CAROLINA GIROTTO PRESSETE

Sclerotinia sclerotiorum (white mold): cytotoxic, mutagenic and antimalarial effects

Alfenas - MG
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CAROLINA GIROTTO PRESSETE

"SCLEROTINIA SCLEROTIORUM (WHITE MODE) CYTOTOXIC, MUTAGENIC AND ANTIMALARIAL EFFECTS..".

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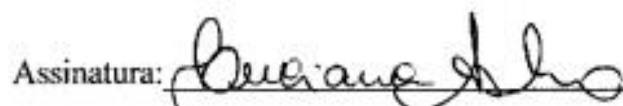
Assinatura: 

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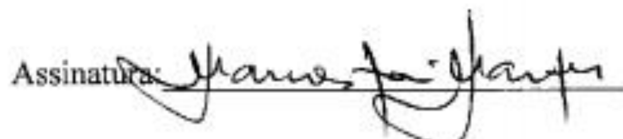
Assinatura: 

ABSTRACT

The fungi rejected in high amount *Sclerotinia sclerotiorum* infects economically important crops and vegetables. There is evidence that compounds from this fungus have mutagenic and cytotoxic effects against colon adenocarcinoma. Thus, this work aimed to evaluate the biological effects of acetate fraction (AcOET) and its fraction (F3) of sclerotia from *S. sclerotiorum* by exploring its activities on mutagenisis, oxidative stress, cancer and malaria. A chemical profile was determined by UHPLC-HRMS analysis identifying β-D-glucan, norditerpenoid dilactone, sclerolide and others compounds. For anti-proliferative assay the AcOET fraction (HCT8 19.8 µg/mL and A549 101.8 µg/mL) and F3 (HCT8 123.1 µg/mL and A549 277.6 µg/mL) exhibited higher GI50 values to cancer cells than IMR90 non-cancer cells (16.05 µg/mL to AcOET and 12.73 µg/mL to F3). Regards oxidative stress, the results showed that the all AcOET fraction concentrations tested on IMR90 non-cancer cell increased ROS production (by five-fold) in more intense way than in tested cancer cells. The *in vivo* study showed an increase of follow biomarkers: % DNA in comet tail in peripheral blood (129.00-212.00%) and liver cells (260.00-296.00%); micronucleated cells for erythrocytes (147.82%-239.13%) colon (173.68%-223.68%) and lipid peroxidation (149.00% and 200.00%). These results indicate the sclerotia as genotoxic and mutagenic agent, as possible due to their oxidative stress effects. The effects against IMR90 cells was observed with low IC50 values while against *Plasmodium falciparum* the IC50 values was higer than the cell, which indicates that our sample presented cytotoxic activities more so to the IMR90 cells than to *P. falciparum.* Furthermore, the GI50 in IMR90 value is low, which may be reached by human comsumption of sclerotia contaminated foods. This aspect warrants a more detailed investigation, since this contamination may lead to fungal toxic effects with a risk to human health.

Keywords: Fungus. *Sclerotinia sclerotiorum.* Sclerotia. Cytotoxicity. Oxidative stress. Mutagenicity. DNA-damage. Apoptosis. Anti-malarial activity.

RESUMO

Os fungos rejeitados em grande quantidade *Sclerotinia sclerotiorum* infectam culturas e vegetais economicamente importantes. Há evidências de que os compostos deste fungo têm efeitos mutagênicos e citotóxicos contra o adenocarcinoma do cólon. Assim, este trabalho teve como objetivo avaliar os efeitos biológicos da fração acetato (AcOET) e sua fração (F3) de escleródios de *S. sclerotiorum*, explorando suas atividades sobre mutagenisis, estresse oxidativo, câncer e malária. Um perfil químico foi determinado por análise UHPLC-HRMS identificando β-D-glucano, dilactona norditerpenóide, esclerolida e outros compostos. Para o ensaio antiproliferativo, a fração AcOET (HCT8 19,8 µg / mL e A549 101,8 µg / mL) e F3 (HCT8 123,1 µg / mL e A549 277,6 µg / mL) exibiram valores mais altos de Gl50 para células cancerígenas do que células não cancerígenas IMR90 (16,05 µg / mL para AcOET e 12,73 µg / mL para F3). Quanto ao estresse oxidativo, os resultados mostraram que todas as concentrações da fração AcOET testadas em células não cancerígenas IMR90 aumentaram a produção de ROS (em cinco vezes) de maneira mais intensa do que em células cancerosas testadas. O estudo *in vivo* mostrou um aumento dos seguintes biomarcadores: % DNA na cauda do cometa no sangue periférico (129.00-212.00%) e nas células do fígado (260.00-296.00%); células micronucleadas para eritrócitos (147,82% -239,13%) cólon (173,68% -223,68%) e peroxidação lipídica (149,00% e 200,00%). Esses resultados indicam que o escleródio é um agente genotóxico e mutagênico, tanto quanto possível devido aos seus efeitos de estresse oxidativo. Os efeitos contra as células IMR90 foram observados com baixos valores de IC50 e contra Plasmodium falciparum os valores de IC50 foram maiores que os da célula, o que indica que nossa amostra apresentou atividade citotóxica mais para as células IMR90 que para P. falciparum. Além disso, o valor de GI50 no IMR90 é baixo, o que pode ser alcançado pelo consumo humano de alimentos contaminados com escleródios. Esse aspecto justifica uma investigação mais detalhada, uma vez que essa contaminação pode levar a efeitos tóxicos fúngicos com risco à saúde humana.

Palavras-chave: Fungo. *Sclerotinia sclerotiorum*. Escleródio. Citotoxicidade. Estresse oxidativo. Mutagenicidade. Dano ao DNA. Apoptose. Atividade anti-malária.

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1 INTRODUCTION

Currently, it is known that not all-natural compounds are only beneficial to health, since that within our everyday life, we are confronted with a variety of toxic substances of natural or artificial origin (WANG; OUYANG; LIN, 2018). Toxins are hazardous substances, causing illness or damage to an exposed organism if inhaled, swallowed or absorbed through the skin (SCHILTER; CONSTABLE; PERRIN, 2014). Fungi, bacteria, yeasts, and moulds which are capable of producing toxic secondary metabolites or different mycotoxins that can contaminate food and cause adverse effects on human health (AICHINGER et al., 2018), and their properties include acute or chronic toxicity, genotoxicity, mutagenicity, carcinogenicity, neurotoxicity (TOURNAS, 2005), pulmonary infection, allergies, osteomyelitis, endocarditis, keratitis (FRĄC; JEZIERSKA-TYS; YAGUCHI, 2015), hepatotoxicity and teratogenicity upon consumption (KONG et al., 2014; SHIN et al., 2014). On the other hand, many of them causes a broad variety of biological activities, including, anticancer, anti-inflammatory, antimicrobial (CHOW; TING, 2015; LIU et al., 2018; SHIN et al., 2014), antibacterial, antiviral, antiprotozoal therapies (BHADURY; MOHAMMAD; WRIGHT, 2006). So, a large number of secondary metabolites from fungi occupy a significant position in the pharmaceutical industry and have been integrated into drug development (BHADURY; MOHAMMAD; WRIGHT, 2006). Compounds that are ultimately selected for development of new drugs must meet the requirements of safe drugs and that not show any overt toxicity to human health (FIDOCK et al., 2004), however the latter requirement is not always met. Sadorn et al., (2018) showed that crude extracts from fungus *Cytospora eugeniae* had cytotoxic activity against human breast adenocarcinoma MCF-7, papilloma carcinoma KB and lung NCI-H187 cancer cells, but also demonstrate toxic effect in non-cancerous (Vero, African green monkey kidney fibroblasts) cells.

The fungus *Sclerotinia sclerotiorum* is remarkable for its extremely broad host range and for its aggressive host tissue colonization (LIANG; ROLLINS, 2018). This nonspecific plant pathogen may infect economically important crops and vegetables, such as sunflower, bean, soybean, canola, cotton, potatoes, peas and tomatoes (DUAN et al., 2018; HEARD; BROWN; HAMMOND-KOSACK, 2015; XU et al., 2015). Their infection causes stem rot or white mold and respresents one of the major challenges for agricultural production (BOLTON; THOMMA; NELSON, 2006; MALENČIĆ et al., 2010). The food contamination with *S. sclerotiorum* may be derived from sclerotia, a hard melanized rind enclosing compact dark bodies, that are not always removed during harvest and post-harvest procedures and this may

lead to their human consumption (AZEVEDO et al., 2016). However, little is still known about the effects of human consumption of contaminated food with *S. sclerotiorum* since their compounds can be a potential health hazard to individuals. This fungus presented mutagenic and cytotoxic effects against colon adenocarcinoma (HT29) by the compounds from their sclerotia aqueous extract. The aqueous extract is recognized by carring mainly sugar components by their hydrophilic and polar behavior, which was observed on the analyses of ^{1}H NMR spectrum (AZEVEDO et al., 2016). Herein, we used ethyl acetate, which was able to extract compounds of intermediate polarity, as further highlighted in this study.

Thus, it is important to know this fungus chemical profile, since its composition may have many substances able to exhibit interesting biological properties for the treatment of human diseases, such cancer and malaria parasite (LEE et al., 2013). However, some compounds also may show severe toxicity (SCHILTER; CONSTABLE; PERRIN, 2014). A great example of compound with toxic activities are the alkaloids, where Ashfaq et al. (2018) reported that this compound presented in vitro toxicity on HepG2 cells at a concentration of >1 μg/mL.

2 OBJECTIVE

The following are the General objective and specific objectiver of the work.

2.1 General Objective

Hence, this work aimed at performing the chemical profile of sclerotia from S. sclerotiorum and to assay its toxicological activities combined with its possible antimalarial and anticancer potential.

2.2 Specific Objectives

a) Obtain and characterize the chemical profile of samples obtained from the sclerotia of the fungus *Sclerotinia sclerotiorum;*
b) To evaluate the antiproliferative activity and toxicity of samples of sclerotia from *Sclerotinia sclerotiorum* in cancer and normal cells *in vitro*;
c) To evaluate the effect of samples in the viability of chloroquine sensitive and resistant *Plasmodium falciparum* cultures *in vitro*;
d) To evaluate the sample effect on the DNA damage *in vivo*;
e) To evaluate the apoptotic pottencial of samples *in vivo*;
f) To analyze the sample effect on the oxidative stress *in vitro* and *in vivo*.

3 MATERIALS AND METHODS

The following are all developed matherials and methods in this work.

3.1 Sample preparation from sclerotia of *S. sclerotiorum* and chemical profile analysis

Sclerotia cultures were obtained from fungal isolates collected from soybeans infected by the fungus *S. sclerotiorum* from the collection of Seed Pathology Laboratory of the Federal University of Lavras (Lavras, Brazil). After isolating the sclerotia, the fungus material was homogenized and extracted with 2 L of ethanol. The solvent was evaporated to 500 mL. The resulting ethanol extract was diluted with 50 mL of H_2O and partitioned with acetate (3 x 500 mL). Being obtained the acetate (AcOET) fraction. The fraction was subjected to a by C18 reversed-phase column chromatography with a H_2O/MeOH gradient as the eluent. Three AcOET fraction were obtained: 100% H_2O (F1), 1:1 H_2O/MeOH (F2), and 100% MeOH (F3).

The UHPLC-HRMS analyze of AcOET fraction and its fraction F3 was performed using a Nexera XR instrument coupled with a photodiode array detector and mass spectrometry Bruker compact detector. The UHPLC-HRMS analyze was performed using a C_{18} column (2.1 × 100 mm, 2.2 μm particle size, Acclaim^{TM} RSLC120) with a flow rate of 0.4 mL/min and the following gradient elution: 5–99% of acetonitrile in water containing 0.1% of formic acid and 0,25% of sodium hydroxide through 20 min. The *in house* data base specific for *Sclerotinia* and sclerotia was used to putative identification of the compounds. The data was treated to decovolution, alignment and identification of the chemical features (*m/z* and retention time) on the softwares Target Analysis (Bruker) and MZmine 2.33. The venn diagram was done on the software Venny 2.1.

3.2 Cancer cell and *Plasmodium falciparum* culture

The following cancer cell lines were obtained from the Rio de Janeiro Cell Bank (BCRJ) were human lung adenocarcinoma epithelial (A549), ileocecal colorectal adenocarcinoma (HCT8) and normal lung cell (IMR90). IMR90 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/low glucose (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, São Paulo, Brazil); A549 and HCT8 cells were cultured in DMEM/Ham-F12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum. All culture medium were

added with 100 µg/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich). The cell lines were incubated in humidified atmosphere containing 5% CO_2, 5 % de O_2 and 95% N_2 at 37 °C (CARDONA; MERTENS-TALCOTT; TALCOTT, 2015).

Plasmodium falciparum resistante (W2) and chloroquine-sensitive (3D7) strains were obtained from the Oswaldo Cruz Foundation, René Rachou Research Center and were used for anti-malarial study. The parasites were maintained using erythrocytes type O+ from human healthy local donor. The culture media consisted of standard RPMI 1640 (Sigma-Aldrich) supplemented with 10 % Albumax II (Gibco), 100 µM hypoxanthine (Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), 12.5 µg/mL gentamicine (Sigma-Aldrich) and 25 mM $NaHCO_3$ (Sigma-Aldrich). Each culture was started by mixing uninfected and infected erythrocytes to achieve a 4 % haematocrit, 1% parasitemia and incubated in 5% CO_2 and 95% N_2 at 37°C in tissue culture flasks (DUTTA et al., 2017; MONERIZ et al., 2009, 2011; RADFAR et al., 2009). The progress of growth in the culture was determined by microscopy in thin blood smears stained with May Grünwald – *Giemsa* (Merck) (MONERIZ et al., 2011).

3.2.1 *In vitro* assays of cytotoxicity and proliferation

Cell viability was assessed as described by De Menezes et al., (2016), using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma), a yellow solution which is converted to blue formazan crystals by mitochondrial activity (GEIRNAERT et al., 2017). For the assay, the cells were seeded in 96-well plates at density of $5x10^3$ cells/well (A549 and HCT8) and $2x10^3$ cells/well (IMR90). After 24 hours, cells were treated serial concentrations of AcOET fraction and F3 fraction (10 to 500 µg/ml) of *S. sclerotiorum*. After incubation for 48 hours, 10 µL of solution of MTT (0.5 mg/ml in milliQ water) was added in each well and, after 4 hours incubation at 37 ° C, the supernatant was removed and the fomazan crystals that had formed were dissolved in 100 µL of dimethyl sulfoxide (DMSO) (AHMAD et al., 2018; DE MENEZES et al., 2016; THAKOR et al., 2017). The amount of the formazan dye generated by the activity of dehydrogenases within cells was directly proportional to the number of living cells (RAMIREZ-MARES; KOBAYASHI; DE MEJIA, 2016). The absorbance at 570 nm was read using a microplate reader (Synergy™ H1, Biotek) using Gen5TM data analysis software. In accordance with the method described by Ramirez-Mares; Kobayashi; De Mejia (2016) the following three parameters were used. Firstly, IC50: the concentration of the agent that inhibits growth by 50% is the concentration at which (T/C) × 100 = 50, where T = number of cells, at time t of treatment; C = control cells at time t of treatment. Secondly, GI50: the concentration of the agent that inhibits

growth by 50%, relative to untreated cells is the concentration at which $([T - T0]/ [C - T0]) \times 100 = 50$, where T and C are the number of treated and control cells, respectively, at time t of treatment and $T > T0$; T0 is the number of cells at time zero. Thirdly, LC50: the concentration of the agent that results in a net loss of 50% cells, relative to the number at the start of treatment, is the concentration at which $([T - T0]/ T0) \times 100 = -50$; $T < T0$. The experiments were conducted in quadruplicate measurements and the dose-response analysis was determined by non-linear regression using GraphPad Prism® (GraphPad Software, Inc., San Diego, CA, USA) software.

3.2.2 Generation of reactive oxygen species (ROS)

For the assay, the normal IMR90 cells and cancerous HCT-8 and A549 cells were seeded at a density of $6x10^4$ cells/well on a 96-well black plat. Twenty-four hours after seeding, cells were treated for 1 hour with different concentrations of *Sclerotinia sclerotiorum* AcOET fraction (5 to 100 µg/ml), added in DCFH-DA solution (25 mmol/L) at different concentrations, which the higher concentration was GI20 from cell viability. The procedure followed the method proposed by WOLF; LIU (2007), which was modified by using pre-treatment with H_2O_2 at 15 µmol/L for all the experiments. Subsequently, the cells were incubated at 37 °C for 1 h and washed with PBS. For the positive control, the cells were treated with 15 µmol/L H_2O_2 and for the negative control, the cells were treated with PBS, which was taken as reference to the percentage of protection against H_2O_2. The fluorescence intensity was measured at an excitation wavelength of 485 nm and at an emission wavelength of 538 nm using an ELISA luminescence spectrophotometer. The data were expressed as percentage of fluorescence intensity relative to the control wells.

3.2.3 Fluorimetric assays for anti-malarial activity

A SYBRGreen I® microfluorimetric DNA-based assay was used to monitor parasite growth inhibition. SYBRGreen I® was purchased to Invitrogen and diluted as indicated by the manufacturer in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Synchronized rings from stock cultures were used to test different amounts 50 to 1200 µg/mL of AcOET fraction and F3 fraction of *Sclerotinia sclerotiorum* and 0,01 to 3 µg/mL serial dilutions of chloroquine (Sigma-Aldrich) in 96-well culture microplates. Thus, 150 µL of parasites at 4% haematocrit, 0,15% parasitaemia chloroquine resistant strain (W2) and 0,25% parasitaemia chloroquine sensitive strain (3D7) were allowed to grow for 48-hour in 5% CO_2 and 95% N_2 at 37°C. The microplates

with parasites were then centrifuged at 600 g for 10 minutes and re-suspended in 150 μL of saponin (0.15%, w/v in phosphatebuffered saline) to lyse the erythrocytes and release the malaria parasites. The pellet was washed by the addition of 150 μL of PBS, to eliminate traces of haemoglobin and then followed by centrifugation at 600 g for 10 minutes. Finally, pellets were re-suspended in 100 μL of PBS and 100 μL of SYBRGreen I® diluted in TE were added to each well. Plates were incubated for 30 minutes in the dark and the fluorescence intensity was measured at 485 nm excitation and 528 nm emission (MONERIZ et al., 2009, 2011). Growth inhibition was calculated by Fluorescence Unit subtracting the control negative (fluorescence from uninfected erythrocytes). In accordance with the method described by Moneriz et al., (2009), the Fluorescence Unit was converted to percentage (%) of survival of parasites by the following equation: Survival (IC_{50}) = (Fd – Fcn)/(Fcp – Fcn) x 100%, where, Fd is the fluorescence from drug treated infected erythrocytes, Fcp is the fluorescence control positive: fluorescence from non-treated infected erythrocytes, and Fcn is fluorescence control negative: fluorescence from uninfected erythrocytes.

3.2.4 Selectivity index (SI)

The SI value allows the evaluation of the selective activity of the AcOET and F3 against the parasite compared to its toxicity for normal lung cell (IMR90). The SI value is calculated as the ratio between cytotoxic IC_{50} values of normal cell and 3D7 or W2 parasitic IC_{50} values (JANSEN et al., 2012).

3.3 *In vivo* genotoxicologic analyses of AcOET fraction

The toxicological analyses of acetate AcOEt were verified in sixty-six Swiss mice (weaned mice 15 ± 5g) obtained from Federal University of Alfenas (UNIFAL-MG), Brazil. All the experiments and procedures were approved by the Ethical Committee for Animal Research (Protocol 408/2012). The mice were fed *ad libitum* with a commercial pelleted diet (Nuvilab Produtos Agropecuarios Ltda, Curitiba, PA) and water and divided into six treatment groups containing eleven animals in each group (n = 11). The animals were kept in an air-conditioned room, under 50 ± 20% relative humidity, at a constant temperature of 22 ± 2 ° C, with a light dark cycle of 12 hours, for 14 days. The experiment was carried out with the following groups: groups 1, 2 and 3 received commercial pelleted diet, group 4 received crushed diet + 6 mg of AcOET fraction/100g diet (wich corresponds 0,06g AcOET/ kg diet and 4,50g

sclerotia/ kg diet) , group 5 received crushed diet + 60 mg of AcOET fraction/100g diet (wich corresponds 0,60g AcOET/ kg diet and 45,00g sclerotia/ kg diet) and , group 6 received crushed diet + 600 mg of AcOET fraction/100g diet (wich corresponds 6,00g AcOET/ kg diet and 450,00 g sclerotia/ kg diet). On the 14th day of the experiment, the animals were treated with: group 1 (negative control) received a physiological solution (PS, NaCl 0.9% w/v); group 2 (positive control) received doxorubicin chloridate (DXR, 30 mg/kg b.w.) via intraperitoneal injection; group 3 (positive control) received , N'-dimethylhydrazine via oral (DMH, 30 mg/kg b.w.) via gavage; and groups 4, 5 and 6 (AcOET fraction) received a physiological solution (PS, NaCl 0.9% w/v). At the end of the study (15th day) all animals were anesthetized with ketamine 10% (0,1 mg/100g b.w.) and xylazine (0.05 mg/100g b.w.) and then euthanized. At necropsy, blood, bone marrow cells, colon and liver were collected from all animals.

3.3.1 Bone marrow and colon micronucleus analysis and apoptosis analysis

The mutagenicity of the AcOET fraction was evaluated using the bone marrow micronucleus test, according to the protocol by Macgregor et al. (1987), with modifications by Azevedo et al. (2010). The bone marrow was flushed out from both femurs using 3 mL of fetal bovine serum, centrifuged, and the supernatant was discarded. The cells were scored using a light microscope at 1000× magnification. Two thousand polychromatic erythrocytes (PCEs) were counted per mouse to analyze the frequency of MNPCE (micronucleated PCEs) (AZEVEDO et al., 2016).

For the *in vivo* gut micronucleus test, the methods described by De Lima et al. (2015) were followed. The colon was excised, flushed with 0.9% NaCl to remove fecal debris, cut open longitudinally, and rolled from caecum to anus forming the “swiss rolls” and stained with Feulgen-fastgreen. These swiss rolls were fixed in 10% (v/v) neutral formalin, embedded in paraffin (Paraplast, CML, France) and sectioned (5 µm). For each animal, thousand colonic epithelial cells and the total number of crypts analyzed were scored using a light microscope at 1000× magnification.

For the identification of apoptotic cells, a total of twenty perpendicular well-oriented crypts were examined in each animal, counting the total number of epithelial cells in each crypt (CHANG; CHAPKIN; LUPTON, 1997). The apoptotic cells were identified as previously described by Risio et al. (1996). The apoptosis was estimated as the percentage of apoptotic cells in relation to the total number of cells.

3.3.2 Oxidative stress by Malondialdehyde (MDA) assay and Comet assay (peripheral blood and liver)

The peripheral blood and liver comet assay were executed, as described in Singh et al. (1988), with modifications by Azevedo et al. (2003) and Azevedo et al. (2007). The fluorescent labeled DNA was visualized using a Nikon Eclipse 80i fluorescence microscope at 200 X magnification, with a green filter. The parameters for DNA damage analysis include tail moment (TM), tail lenght and % DNA in comet tail. Fifty cells were counted from each slide for a total of one hundred cells per animal. Resulting images were captured and processed with image analysis software (TriTek CometScore™ v1.5, Sumerduck, VA).

The oxidative stress assay was carried out in accordance with the protocol by Uchiyama; Mihara (1978). The liver samples (0.5 g) was homogenized with 1.15% (w/v) potassium chloride (KCl) and centrifuged, and the supernatant was used to determine lipid peroxidation by MDA. The protein content was quantified by the Bradford method (BRADFORD, 1976) and the results were expressed in nmol mg protein $^{-1}$ (UCHIYAMA; MIHARA, 1978).

3.4 Statistical analysis

One-way analysis of variance (ANOVA) was used, followed by Tukey's test for the cytotoxicity and proliferation cellular tests, ROS, anti-malarial and anti-hemolytic activity, lipid peroxidation analysis and comet assay. T-test was performed for all *in vitro* analysis, and chi-square test ($\chi 2$) for the micronucleus and apoptosis assays (AZEVEDO et al., 2010; DRAGANO et al., 2010). All tests were considered statistically significant with p-values of $p \leq 0.05$.

4 RESULTS AND DISCUSSION

The following are all the results obtained in this work.

4.1 Chemical composition of AcOET fraction and F3 fraction from sclerotia

Using UHPLC-HRMS chemical profile analysis of AcOET fraction and F3 fraction, it was possible to noted that the main differences between the chemical composition of the F3 fraction and AcOET fraction was most pronounced in positive mode (Table 1). The literature lists some volatile compounds emitted by *S. sclerotiorum* sclerotia, such as: 2-methyl-2-bornene, 1-methylcamphene, 2-methylisoborneol, 2-methylenebornane and a diterpene with a molecular weight of 272. The characteristic musty earthy odor that fungus *S. sclerotiorum* exhale is from the compound 2-methylisoborneol (FRAVEL et al., 2002). Another compound identified as part of the chemical composition of this fungus is 5-*O*-(α-D-galactopyranosyl)-D-glycero-pent-2-enono-1,4-lactone that aid in the production of oxalic acid an acid that has a toxic effect, attacking and degrading the complex structure of the host plant cell wall (KEATS et al., 1998; KOMATSU et al., 2008). In addition to these compounds, steroids such as ergosterol, ergosterol peroxide and fatty acids were identified by Dembitsky (2015) from *S. sclerotiorum,* who highlight their possible antimalarial, antibacterial, cytotoxic, and other pharmacological activities as an important source of leads for drug discovery.

We dereplicated on AcOET fraction and F3 fraction (Figure 1 A and B) and the samples showed 122 substances in common to both samples and 211 only on the AcOET fraction and 770 only on the F3 fraction (Figure 2 C). The β-D-glucan, previously described by Ohno and Yamode (1987) was detected exclusively to the AcOET fraction samples (Table 1). This extracellular polysaccharide was previously described to the genus, in the *Sclerotinia libertiana*, and it is important to highlight that it was associated to antitumor properties (OHNO; YADOMAE, 1987).

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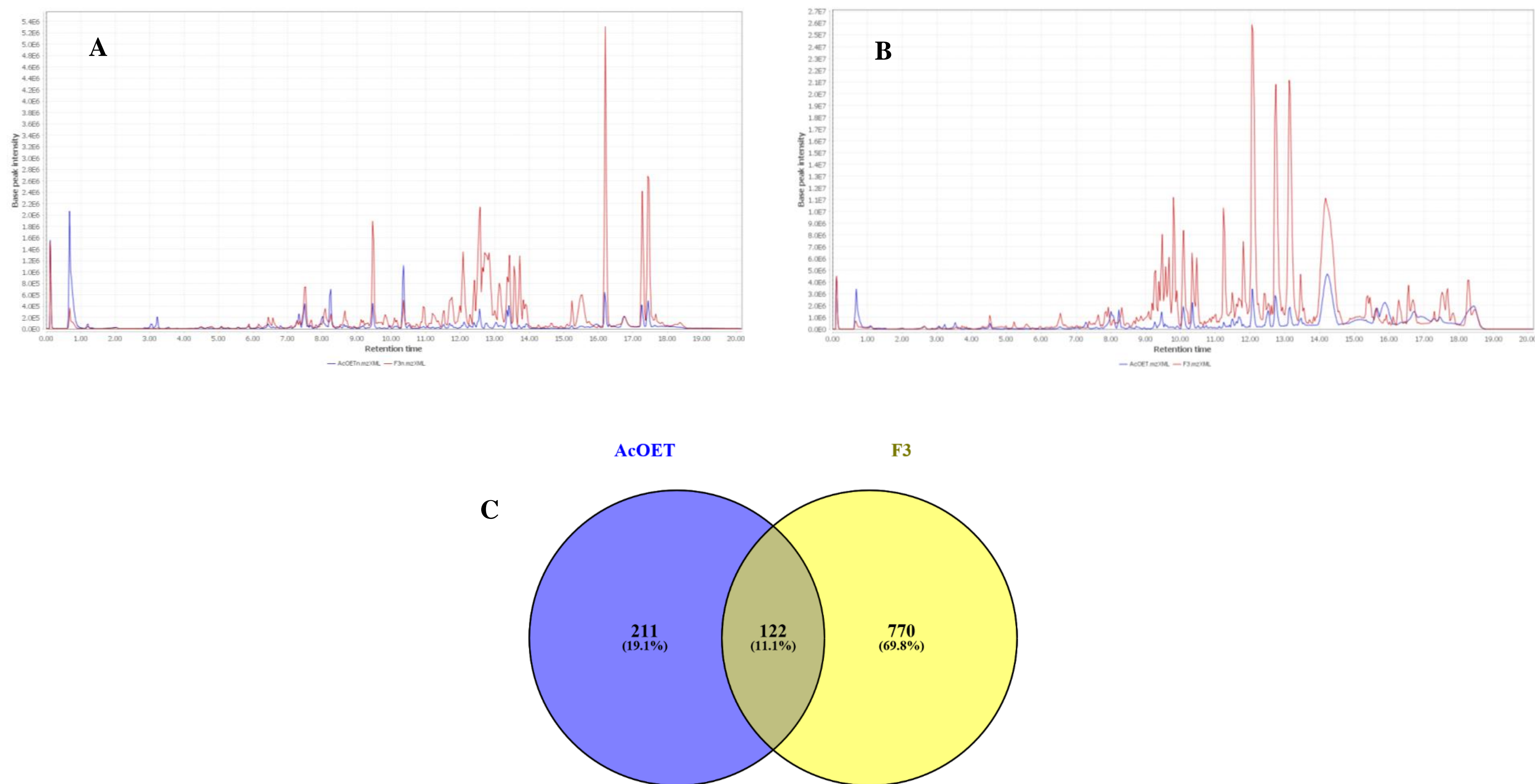

Figure 1- Base peak chromatogram of AcOET (blue line) and F3 (red line) detected on the UHPLC-HRMS in negative mode (A) and positive mode (B). (C) Number the compounds detected in UHPLC-HRMS. Some compounds are in both samples and others exclusively of AcOET or F3.
Source: by author

Table 1- Putative identification of the compounds using *in house* data base from the genus *Sclerotinia* and general sclerotia.

Retention time	Ion	Molecular formula	Error (mDa)	mSigma	Compound	Area in AcOET	Area in F3
0.1	$[M+H]^+$	C18H32O16	2.5	28.9	β-D-glucan	75776	-
1.3	$[M+H]^+$	C11H19N3O3S1	4.6	-	sclerothionine	19156	-
4.2	$[M+H]^+$	C13H14O4	-1.5	-	sclerin	17348	-
5.4	$[M-H]^-$	C14H20O6	0.1	15	colletodiol	1420	3674
5.4	$[M+H]^+$	C14H20O6	0.4	1.4	colletodiol	3027	13157
6.3	$[M+H]^+$	C18H16O5	4.7	-	sclerodione	-	11187
6.9	$[M-H]^-$	C21H23Cl1O5	1.9	-	sclerotiorin	517	1097
7.3	$[M-H]^-$	C13H16O5	-1.9	3.4	antibiotic LL-253 α	1191531	149251
7.6	$[M+H]^+$	C18H14N2O3	0.4	25.0	sclerominol	63989	23975
7.6	$[M-H]^-$	C12H14O4	4.0	37.9	sclerotin C	-	13675
7.0	$[M+H]^+$	C12H14O4	1.8	47.8	sclerotin C	-	11681
7.9	$[M-H]^-$	C18H16O6	3.7	34.1	scleroderolide	922	8633
8.4	$[M+H]^+$	C15H22O3	1.5	37.1	11-hydroxy-sclerosporin	6651	-
8.8	$[M+H]^+$		3.6	-	sclerotiorin	24743	14753
9.9	$[M+H]^+$	C15H22O2	0.4	36.9	sclerosporin	10650	-
10.3	$[M+H]^+$	C16H16O6	6.0	15.7	norditerpenoid dilactone	2025	2321
12	$[M+H]^+$	C23H36N4O4	3.2	26.6	sclerotiotide E	190158	-
12.5	$[M+H]^+$	C21H38O4	0.2	3.5	glycerol monolinolate	1462260	-
13.4	$[M+H]^+$	C12H14O4	-0.8	10.7	sclerolide	99576	-
15.6	$[M+H]^+$	C21H32N4O5	0.9	-	sclertotiotide F	-	41646

Source: by author

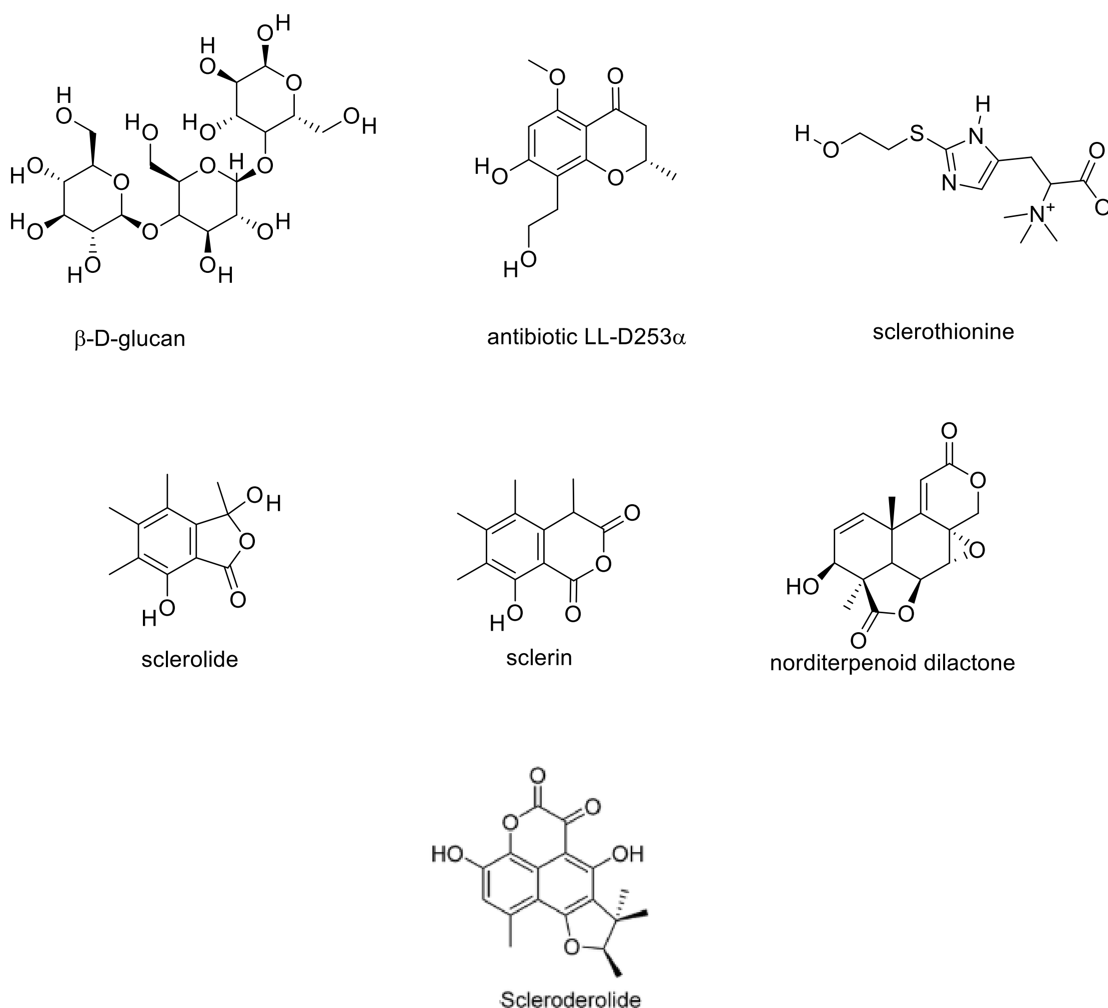

Figure 2- Some compounds from *Sclerotinia* detected on the samples according described on the Table1.
Source: by author

4.2 *In vitro* studies in non-cancer and cancer cell lines

Cytotoxicity is considered as the potential of a compound to induce cell death. *In vitro* cytotoxicity tests are necessary to define basal cytotoxicity and to establish the dose at which 50% of the cells are affected. By cytotoxicity tests, it is possible to compare quantitatively responses of single compounds in different systems or of several compounds in individual systems (EISENBRAND et al., 2002).

Regards the biological activity of sjamples from sclerotia (IC_{50}, GI_{50} and LC_{50}, Table 2), AcOET fraction showed low GI_{50} values against HCT8 cancer cell (19.8 µg/mL) and IMR90 non-cancer cells (16.05 µg/mL), while both AcOET fraction and F3 fraction were inneffective against cancer cell line A549 (101.8 µg/mL to AcOET and 277.6 µg/mL to F3). These results highlight that the cytotoxic activities were selective to the cell line, which indicates that low

concentrations of AcOET can kill some types of normal cell however may be usefull against some cancerous cell lines. In contrast, Azevedo et al. (2016) using sclerotia aqueous extract obtained that HT-29 cancer cells (0.01598 mg/L) had almost three times lower GI50 values than non-cancer cell CCD-18Co (0.04712 mg/L), which could be explained by variation between aqueous and AcOET fraction and F3 fraction composition. Which turn evident the importance of chemical profile and their interactions obtained from each extract.

Thus, this chemical difference on the composition between the two samples can explain the higher activity of AcOET fraction against the HCT8 cells (IC_{50} 48.0; GI_{50} 19.8 μg/mL) compared to F3 fraction (IC_{50} 250.5; GI_{50} 123.1 μg/mL). The norditerpenoid dilactone was detected on the samples, it is previously described to the genus *Sclerotinia* and also showed *in vitro* cytotoxicic effects against five different tumor cell lines (BANDARA HERATH et al., 2010). We also identified an isocoumarin-structure previously described from *S. sclerotiorum* named sclerolide (SUZUKI et al., 1968). Scleroderolide and sclerodione are other examples of cytotoxic compounds detected in this study that may regulates proliferation and cytotoxicity of cell. These compounds were already isolated from the fungi *Penicilium* and *Lophiostoma bipolare*, and both of them showed antiproliferative activity against glioma (LI et al., 2018). Among the compounds identified, Sclerotiorin was already pointed out as potent anti-proliferative agent against cancer and normal cell (GIRIDHARAN et al., 2012). Chen et al., (2017) assessed the cytotoxicity of sclerotiorin against HeLa cells, MCF-7 cells and macrophages and noted that the viability was over 90%, indicating that this compound did not extremely affect growth of mammalian cells. In this sense and taking the results together, AcOET fraction and F3 fraction samples seem be a good source of compounds with cytotoxic compounds.

Moreover, when we analyze the oxidative stress, it is clear that the fungus AcOET fraction inducted ROS generation, which may explain the cytotoxic and antiproliferative effects. The results showed that the all AcOET fraction concentrations tested on non-cancer cell increased ROS production (by five-fold) in more intense way than in studied cancer cells, where only the highest concentration inducted ROS generation in HCT8 (by two-fold) (Fig. 3). Healthy cells have developed specific adaptations to overcome the damaging effects of ROS, through the balanced generation of these species and sufficient antioxidant activity (MOLONEY; COTTER, 2018). However, in the present study, the increase of ROS production in IMR90 non-cancer cells showed that cell antioxidant repair systems, such as superoxide dismutase, glutathione reductase and glutathione peroxidase, have not neutralised the

overproduction of free radicals leadind these normal cells to death and lower GI/IC values than cancer ones. In contrast to our study, Azevedo et al. (2016) observed that sclerotia aqueous extract increased ROS production in more intensity in HT-29 cancer cells than non-cancer cell CCD-18Co, leading this cancer cell to triggering the intrinsic and extrinsic apoptotic pathways by ROS overproduction. We should consider that the treatments tested in our study are not pure compounds, but a complex matrix of several compounds, which can interact with each other with consequent variation in bioacessibility and biodisponibility, besides synergism and antagonism can occur (ROLEIRA et al., 2015). So, the main differences in these results may be the caused by chemical variation of aqueous and acetate fraction from ethanol extract composition and its biological behavior, such as β-D-glucan, sclerothionine, sclerin, norditerpenoid dilactone and others.

Foods contamination with sclerotia may lead to their human consumption. For this reason, the sclerotia from *S. sclerotiorum* should be more investigated about their chemical composition of bioactive constituents and toxicology, since these fungi present cytotoxic effets against some cancer cells associated with toxic effect against non-cancer cells.

Table 2- Cytotoxicity and inhibition of proliferation of human lung adenocarcinoma epithelial (A549), ileocecal colorectal adenocarcinoma (HCT8) and normal lung cell (IMR90) after 48 h exposure to AcOET fraction and F3 fraction:

Cell lines		AcOET fraction (µg/mL)	F3 fraction (µg/mL)
IMR90	IC_{50}	103.64 ± 0.59	127.63 ± 0.38
	GI_{50}	16.05 ± 2.94	12.73 ± 4.22
	LC_{50}	205.9 ± 2.67	177.3 ± 1.73
HCT8	IC_{50}	48.03 ± 0.85	250.50 ± 3.96
	GI_{50}	19.80 ± 1.12	123.10 ± 4.68
	LC_{50}	282.2 ± 9.77	384.1 ± 6.42
A549	IC_{50}	107.80 ± 7.98	259.40 ± 3.65
	GI_{50}	101.80 ± 2.11	277.60 ± 3.17
	LC_{50}	180.08± 0.5	>500

Source: by author
IC_{50}: the concentration of the agent that inhibits growth by 50%, is the concentration at which (T/C) × 100 = 50, where T = number of cells, at time t of treatment; C = control cells at time t of treatment. GI_{50}: the concentration of the agent that inhibits growth by 50%, relative to untreated cells, is the concentration at which ([T − T0]/[C − T0]) × 100 = 50, where T and C are the number of treated and control cells, respectively, at time t of treatment and T > T0; T0 is the number of cells at time zero. LC_{50}: the concentration of the agent that results in a net loss of 50% cells, relative

to the number at the start of treatment, is the concentration at which ([T − T0]/T0) × 100 = − 50; T < T0. Note: ND: Not determined by the statistical program; SD: Standard deviation.

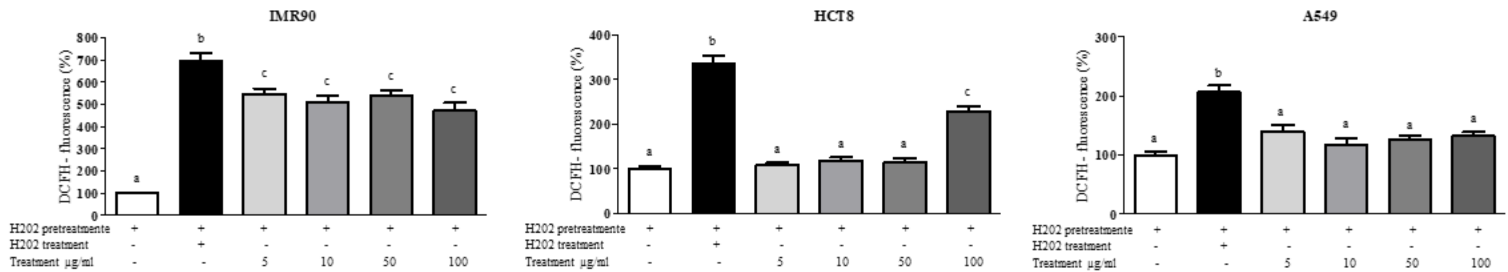

Figure 3- Results of intracelular ROS generation in human lung adenocarcinoma (A549), ileocecal colorectal adenocarcinoma (HCT8) and normal lung cell (IMR90) after treatment with AcOET fraction or 15 µmol/L H2O2 (positive control treatment) or medium (negative control) for 1 h in cancer cells. Quantitative data are the mean ± standard deviation. Different letters within same parameter indicate a significant difference ($p < 0.05$, Tukey test).

Source: by author

4.3 *In vitro* studies in Plasmodium falciparum strains

The following are the results obtained in *P. falciparum* cultures.

4.3.1 Anti-malarial activity of *S. sclerotiorum*

There is continuing need for new and improved drugs to tackle malaria, which remains a major public health problem, especially in tropical and subtropical regions of the world (ATEBA et al., 2018). In addition, fungus that widely colonize healthy tissues of plants have been shown to synthesize a great variety of secondary metabolites that might possess antiplasmodial benefits (ATEBA et al., 2018). However, compounds that are ultimately selected for development of new antimalarial drugs must meet the requirements of rapid efficacy to counter the spread of malaria parasites, safe drugs and that not show any overt toxicity to human health (FIDOCK et al., 2004). Thus, in line with WHO guidelines and basic criteria for antiparasitic drug discovery (FIDOCK et al., 2004; PINK et al., 2005), activities of extracts should be classified into four classes according to their $_{IC50}$ values: high activity (IC_{50} ≤5 µg/ml); promising activity (5 µg/ml < IC_{50} ≤15 µg/ml); moderate activity (15 µg/ml < IC_{50} ≤50 µg/ml); weak activity (IC_{50} >50 µg/ml) and a pure compound is defined as highly active when its IC_{50} ≤1 µg/ml (JANSEN et al., 2012). Regards the antimalarial activity of *S. sclerotiorum* (IC_{50}), herein the AcOET fraction and F3 fraction do not show effective activity against chloroquine resistant (W2) and chloroquine sensitive (3D7) *P. falciparum* strains, since

they presented high IC_{50} values (330.4 to 465.1 µg/ mL), suggesting that it is necessary to use high concentrations of the treatments to inhibit the growth of the parasite, meaning low cytotoxicity to this strain or weak activity (Table 3). This antimalarial behavior was different from other fungi extracts, where Ateba et al. (2018) found out that extracts from *Paecilomyces lilacinus*, *Penicillium*, and *Paecilomyces sp.* exerted highly potent activities with $IC_{50} < 1$ µg/mL, extracts from *Mucor falcatus* and *Aspergillus aculeatus* showed moderate potency with IC_{50} between 10 and 25 µg/mL, while the extract from *Aspergillus tamarri* with $IC_{50} > 100$ µg/mL was considered as inactive, all tested against chloroquine-resistant *P. falciparum.*

When dealing with antiplasmodial activities, one must be careful, due their possible toxic effect against human cells (SADORN et al., 2018). Herein this possible side effect of *S. sclerotiorum* samples were checked by selectivity index ($IC_{50\ (IMR90)}/IC_{50\ (P.\ falciparum)}$). So, we observe that IC_{50} values to IMR90 cells of AcOET fraction (103.6 µg/mL) and F3 fraction (127.6 µg/mL) lead selectivity indices to lie between 0.23 and 0.35, depending on the sample and the plasmodial strain (Table 3), which confirms that they are more cytotoxic to the cells than for the parasite. The norditerpenoid dilactone detected in our samples, although previously described with antiplasmodial activity, also had cytotoxic activity against mammalian kidney fibroblasts (Vero cells) (BANDARA HERATH et al., 2010). The same behavior were found by Sadorn et al., (2018) applying *Cytospora eugeniae* extracts that exhibited excellent antimalarial activity and cytotoxic activity against cancerous (MCF-7, KB, NCI-H187) and non-cancerous (Vero, African green monkey kidney fibroblasts) cells. On the other hand, Ateba et al. (2018) pointed out three fungi extracts with great activity against chloroquine-resistant *P. falciparum* and little cytotoxic against the human embryonic kidney HEK293T cells. So, we can again emphasize that the differences found in our results to others studies may be due the differences in the sample compositions, since that the compounds may interact with each other and had different biological behavior, as previously mentioned.

Despite the fact that the goals of the researchs are develop safe new drugs and non-toxic to counter malaria parasites (FIDOCK et al., 2004), *S. sclerotiorum* AcOET fraction and its fraction presented cytotoxic activities more so to the non-cancer and cancer cells than to malaria parasite (Table 2 and 3). The presente study is, however, the first report of the *S. sclerotiorum* antiplasmodial properties against chloroquine resistant strain (W2) and chloroquine sensitive strain (3D7) of *P. falciparum*. Thus, the *S. sclerotiorum* should be more studied, since it is known that this fungus may produce compounds, such as ergosterol, ergosterol peroxide and

these compounds are recognized by their high antimalarial and other pharmacological activities as an important source of leads for drug discovery (DEMBITSKY, 2015).

Table 3- Antiplasmodial activity and cytotoxicity of *Sclerotinia sclerotiorum*, against chloroquine resistant strain (W2) and chloroquine sensitive strain (3D7) after 48 h exposure to AcOET fraction and its fraction (F3):

Samples	Activity against *P. falciparum* (W2 strain) IC_{50} (µg/ ml)	Activity against *P. falciparum* (3D7 strain) IC_{50} (µg/ ml)	Cytotoxicity against IMR90 cell line IC_{50} (µg/ ml)	SI IC_{50} IMR90/IC_{50}W2-3D7		Remark	
				SI W2	SI 3D7	W2	3D7
AcOET	330.4 ± 0.84	449.9 ± 1.93	103.6 ± 1.12	0.31	0.23	weak activity	weak activity
F3	362.3 ± 1.64	465.1 ± 4.22	127.6 ± 4.68	0.35	0.27	weak activity	weak activity
Chloroquine	0.471 ± 5.2	0.0265 ± 2.7	16.24 ± 5.19	34.47	612.83	high activity	high activity

Source: by author

SI: selectivity index = IC_{50} (IMR90)/IC_{50} (W2 or 3D7, respectively), where HCT8 is ileocecal colorectal adenocarcinoma. The inhibition of parasite growth was expressed as IC_{50} (µg/mL): the concentration of the agent that inhibits growth by 50%, is the concentration at which Survival (IC_{50}) = (Fd – Fcn)/(Fcp – Fcn) x 100%, where, Fd is the fluorescence from drug treated infected erythrocytes, Fcp is the fluorescence control positive: fluorescence from non-treated infected erythrocytes, and Fcn is fluorescence control negative: fluorescence from uninfected erythrocytes. The inhibition of cell growth was expressed as GI50 (µg/mL): the concentration of the agent that inhibits growth by 50%, relative to untreated cells, is the concentration at which ([T − T0]/[C − T0]) × 100 = 50, where T and C are the number of treated and control cells, respectively, at time t of treatment and T > T0; T0 is the number of cells at time zero. Antiparasitic activities of AcOETand F3 were classified into four classes according to their IC_{50} values: high activity (IC_{50} ≤5 µg/ml); promising activity (5 µg/ml < IC_{50} ≤15 µg/ml); moderate activity (15 µg/ml < IC_{50} ≤50 µg/ml); weak activity (IC_{50} >50 µg/ml) (JANSEN et al., 2012).

4.4 *In vivo* tests: comet, micronucleus, apoptosis and oxidative stress analyzes

In relation to feed consumption, the results showed that the experimental animals were not affected their diet consumption, when AcOET fraction was added in the diet, which indicates an animal well-being. Taking into account the diet consumption, the group 4 (6mg/100g diet) consumed 25 mg AcOET/ kg b.w. corresponding to 2.1g sclerotia/ kg b.w.; the group 5 (60mg/100g diet) was 240 mg AcOET/ kg b.w. corresponding to 20.9 g sclerotia/ kg b.w. and group 6 (600mg/100g diet) was 2600 mg AcOET/ kg b.w. corresponding to 135.0 g sclerotia/ kg b.w.

The *in vivo* analyses were carried out to explore the mutagenic and genotoxic effects of AcOET fraction by micronucleus assay and comet, respectively. Regards the fungus genotoxic effects, the results demonstrate that the AcOET fraction increased % DNA in comet tail at levels significantly above the negative control by 129.0%, 212.0% and 160% for peripheral blood and 284.0%, 296.0% and 260.0% for liver cells, in the three tested doses 6, 60, and 600 mg/100g diet b.w., respectively. The same results were obtained for tail moment, where there was an increase of 166.0%, 380.0% and 271.0% for peripheral blood and 660.0%, 639.0% and 429.0% for liver cells, in respective treated groups (Fig. 4). Similar damages were observed to micronuclei assay, whose damages were significantly above the negative control by 147.82%-239.13% for erythrocytes and 173.68%-223.68% for colon (Fig. 5). Thus, we highlight that injury levels observed by comet assay and colon micronuclei reached comparable levels of damage effects as same as caused by doxorubicin and N'-dimethylhydrazine, the genotoxics and antioneoplastics agent used as positive control. Taking into account that micronuclei explore the mutagenic effects from chromosomal damage that are displaced to the cytoplasm of the daughter cells (HAYASHI et al., 2000), and the comet assay explore genotoxic effects presents a measure for DNA damage assessed before repair and cell division (MØLLER, 2006), it is possible to conclude that AcOET fraction presented both genotoxic and mutagenic activity. This AcOET fraction presented different behavior compared to aqueous fungi extract (AZEVEDO et al., 2016), which compounds have not caused DNA breakage by comet parameters. Furthermore, Miranda et al. (2008) and a more recently study conduced by Kerche-Silva et al. (2017) pointed out β-D-glucan compound, also found in our sample, did not cause genotoxic and mutagenic effects in Chinese hamster lung fibroblasts (V79), rat hepatoma (HTC) cell lines and in mice.

In this way, the *S. sclerotiroum* compounds could have acted in cromossomal material damage by the following different possibilities: 1) changes in spindle fibers and chromosome rearrangements (SCHMID, 1975), 2) inhibition of the enzyme topoisomerase II, ligases and helicases, formation of adducts with DNA (DESAI et al., 2013), 4) inducing formation of DNA single strand breaks and DNA bases oxidation (LAZZÉ et al., 2003), 5) chromosome segregation (FENECH; FERGUSON, 2001), 6) DNA degradation through generation of reactive oxygen species such as hydroxyl radicals (FERGUSON, 2001), 7) promotes redox state alteration with consequent increase in the free radicals leading to the inactivation or exhaustion of the antioxidant enzymes (LAZZÉ et al., 2003), 8) These free radicals could react with lipids and lead to lipid peroxidation of cell membrane in tissues, causing the breakage of the DNA chain by oxidating the base in DNA and covalent binding between the product of lipid peroxidation and DNA (LAZZÉ et al., 2003), 9) these free radicals could also react with proteins, affect the structures and functions of enzymes, and alter membrane properties. In addition, could attack nucleic acids, especially some spots in purine and pyridine, result in base substitution and breakage of DNA, and eventually induce mutation (REIST; JENNER; HALLIWELL, 1998).

When dealing with citotoxicity and ROS generation, one most be observed pathway triggered is the apoptosis event. So, the apoptotic assay in the intestinal epithelium was performed to further explore these mechanisms. Results from this study showed that positive control (G3) increased 3.01% the frequency of apoptotic cell in enterocytes, while the negative control (G1) identified a rate of 0.62% the frequency of apoptosis (Fig. 5). Furthermore, the tested doses of AcOET fraction increased apoptosis at levels above the negative control by 500.0% and increased apoptotic cells at levels similar the DMH. Indeed, Azevedo et al. (2016) also observed this effect, which was triggered by extrinsic and intrinsic pathway, leading to the activation of Caspase-8, p53, Bax, and the production of ROS, that induce the release of Cytochrome C. Apoptosis is considered a major mechanisms in cancer therapy and plays a crucial role in the cellular progress of proliferation, differentiation, senescence, and death (MA et al., 2014). Thus, in the present study, this increase in the number of apoptotic cells indicates that *S. sclerotiorum* activated mechanisms of cell death. Since that apoptosis is an event of eradication of cells that have suffered DNA damage due to the presence of mutagenic and/or genotoxic compounds (BADAWI et al., 2018), these results agree with damage observed by comet assay. The β-D-glucan, wich was detected exclusively to the AcOET fraction, was described by Queiroz et al. (2015) as exerting an antiproliferative effect in breast cancer MCF-

7 cells, that was associated with apoptosis, necrosis and oxidative stress. Furthermore, another compound detected in our sample, the sclerotiorin, also was found to induce apoptosis in colon cancer (HCT-116) cells through the activation of BAX, and down- regulation of BLC-2, those further activated claved caspase-3 causing apoptosis of cancer cells (GIRIDHARAN et al., 2012).

In the present study the malondialdehyde (MDA) analysis was performed, evaluating one of the most studied consequences of oxidative stress: lipid peroxidation, which is among the processes implicated in oxidative stress-induced celular damage and is associated with cytotoxicity. The treated groups with two higher doses (60 and 600 mg/100g diet b.w.) of AcOET fraction presented a similar increase in oxidative stress when compared to agent doxorubicin at levels significantly above the negative control by 149% and 200%, respectively (Fig. 4). Queiroz et al. (2015) also observed that mechanisms underlying to β-D-glucan antiproliferative effect in breast cancer MCF-7 cells may include the increase of oxidative stress in this cell. It is known that increased basal oxidative stress could promote tumor growth, invasion and metastasis and furthermore, lipid peroxidation may lead to the *in vivo* oxidation of polyunsaturated fatty acids, which are responsible for the maintenance of cell membranes (BALU et al., 2005). So, in this study, we may propose that the two highest tested concentrations of the sample fungus cause lipid peroxidation which is able to induce inflammation, aging, cancer, and cardiovascular/neurodegenerative diseases (GASCHLER; STOCKWELL, 2017).

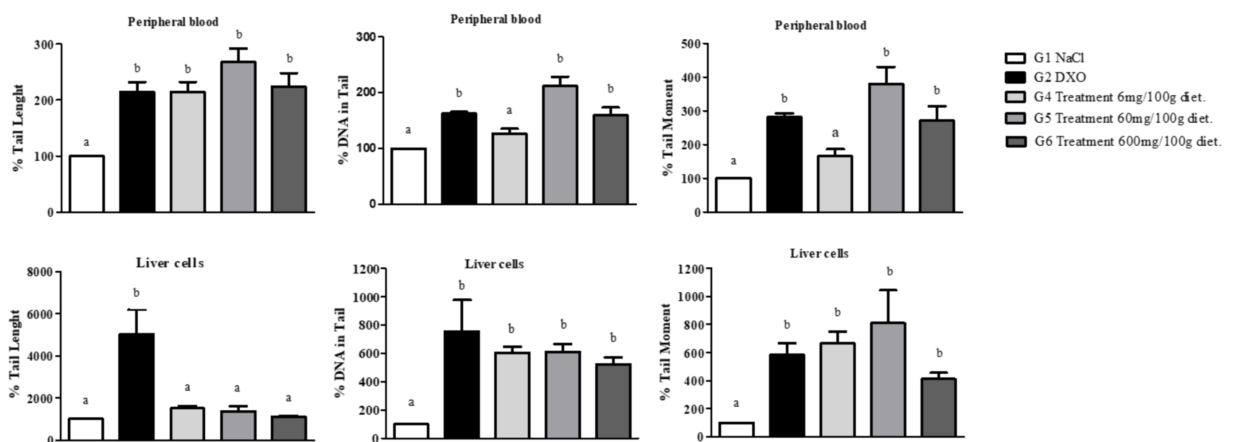

Figure 4- Monitored variables for DNA damage analysis in peripheral blood and liver cells, where G1: 10 mL/Kg b.w. of 0.9% NaCl (negative control); G2: DXO: doxorubicin 30 mg/kg b.w. (positive control); G4: diet added to AcOET 6 mg/100g; G5: diet added to AcOET 60 mg/100g; G6: diet added to AcOET 600 mg/100g. Quantitative data are the mean ± standard deviation. Different letters within same parameter indicate a significant difference ($p < 0.05$, Tukey test).
Source: by author

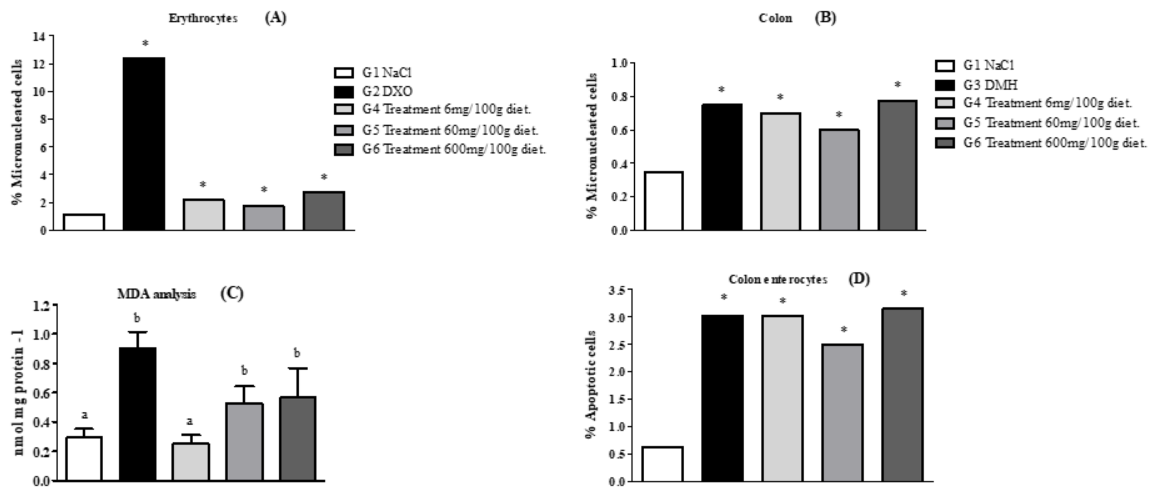

Figure 5- Results of *in vivo* experiments. This picture highlights the mutagenic results from bone marrow and colon micronucleus test, apoptosis and oxidative stress by MDA analysis. Where G1: 10 mL/Kg b.w. of 0.9% NaCl (negative control); G2: DXO: doxorubicin 30 mg/kg b.w. (positive control); G3: DMH: N'-dimethylhydrazine 30 mg/kg b.w. (positive control); G4: diet added to AcOET 6 mg/100g; G5: diet added to AcOET 60 mg/100g; G6: diet added to AcOET 600 mg/100g. (A) Micronucleated cell as percentage of total polychromatic erythrocyte within all animals of each same group (2.000 cells/animal). * means that they are different from negative control (only NaCl) $p \leq 0.05$ ($\chi 2$). (B) Micronucleated cell as percentage of total colon cells within all animals of each same group (1.000 cells/animal). * means that they are different from negative control (only NaCl) $p \leq 0.05$ ($\chi 2$). (C) Lipid peroxidation by malondialdehyde (MDA) analysis in mice´s liver. Quantitative data are the mean ± standard deviation. Different letters within same parameter indicate a significant difference ($p < 0.05$, Tukey test). (D) Frequencies of colon enterocytes apoptosis as percentage of total epithelial cells within all animals of each same group (total of twenty perpendicular well-oriented crypts were examined in each animal). * means that they are different from negative control (only NaCl) $p \leq 0.05$ ($\chi 2$).
Source: by author

5 CONCLUSION

Overall, our findings of *in vitro* and *in vivo* assays indicate that AcOET fraction and its fraction (F3) of sclerotia from *S. sclerotiorum* possess toxic activities, where the GI_{50} in non-cancer cells is lower compared to cancer cells. Furthermore, the AcOET fraction also caused genotoxic DNA-damage observed by the comet test, mutations in polychromatic erythrocytes and colon cells by micronuclei assay, as well as stimulating the process of apoptosis in enterocytes *in vivo*. The oxidative stress assay by ROS generation and lipid peroxidation tests suggests that AcOET caused overproduction of free radicals and may lead these cells to apoptosis and lower GI and IC values to normal cells. However, according with results obtained in anti-malarial activity, we may conclude that the AcOET fraction and F3 fraction from *S. sclerotiorum* presented cytotoxic activities more so to the cells than to malaria parasite. So, the GI_{50} in non-cancer cell value is low, which may be reached by human comsumption of sclerotia contaminated foods. This aspect warrants a more detailed investigation, since this contamination may lead to fungal toxic effects with a risk to human health.

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