

Anti-inflammatory and antimicrobial potential of three natural polyketides isolated from Endophytic fungus *Phomopsis sp*CAM212 against to dysenteric causing pathogens.

ABSTRACT:

Aims: (Infectious)(the)The present work aimed to evaluate the anti-amoebic, antibacterial, and anti-inflammatory potential of three natural polyketides from *Phomopsis sp.* CAM212.

Study design: Clinical isolates of *E. histolytica*, *E. coli* ATCC25922 strain, primary peritoneal mouse macrophages and three polyketides were used.

Places and duration of study: Laboratory of Pharmacology and Toxicology, Laboratory of Medical Microbiology, Faculty of Science, University of Yaounde 1 between May and December 2022.

Methodology: During this work, we evaluated the ability of three natural polyketides from *Phomopsis sp* to inhibit the growth of germs responsible for amoebic and bacillary dysentery. First, the anti-amoebic activity was carried out on clinical isolates of *E. histolytica* in polyxenic culture. Subsequently, we evaluated the antibacterial potential on a strain of *E. coli* ATCC25922. Finally, the anti-inflammatory potentials were evaluated on a primary culture of SC activated macrophages through inhibition of nitric oxide (NO) production, activation of phosphatase alkaline (ALP) and inhibition of 5-lipoxygenase (5-LOX).

Results: It emerges from this work that among compounds, phomopsinin B, presented the highest anti-amoebic potential (84.4 % inhibition after 72h) and the highest antibacterial potential (MIC=12.5µg/mL and MBC/MIC=2). Phomopsini A and phomopsini A acetate showed moderate anti amoebic and antibacterial potentials. However, all these activities remain lower than that of metronidazole and ciprofloxacin (90% of amoebic inhibition after 72h; MIC=0.72µg/mL and MBC/MIC=4). Subsequently, all tested compounds were nontoxic on primary macrophages. Phomopsinin B exhibited a great anti-inflammatory potential through the inhibition of NO production (IC₅₀=1.72±0.91µg/mL); inhibition of 5-LOX activity (IC₅₀=36.97±7.12µg/mL) and activation of ALP activity (IC₅₀=0.13±0.01µg/mL) as compared to Baicalin the standard. The anti-inflammatory potential of phomopsinin A and phomopsinin A acetate were lower compared to baicalin.

Conclusion: Ultimately, among compounds tested, phomopsinin B exhibited the best anti-amoebic, antibacterial and anti-inflammatory potential similar to the respective standards within the limits of the tests carried out.

Keywords: polyketides, anti-amoebic, antibacterial, anti-inflammatory, dysentery.

1. INTRODUCTION

Dysenteries are endemo-epidemic diseases of a microbial origin characterized by an ulcerous inflammation of the large intestine. They are manifested by frequent evacuations of bloody mucus accompanied by violent colic [1]. These pathologies are frequently linked to numerous enteric germs, including the bacterium *Escherichia coli* (*E. coli*) responsible for bacillary dysentery and the protozoan *Entamoeba histolytica* (*E. histolytica*) causing amoebic dysentery [2]. *E. coli* is a type of fecal coliform of the Enterobacteriaceae family divided into various pathotypes that cause different manifestations of the disease (traveler's diarrhea; infantile gastroenteritis, etc.). *E. histolytica* is a unicellular protozoan that successively takes two forms during its evolutionary cycle: the mobile vegetative form and the resistant cystic form that allows (its) its dissemination. Epidemics caused by these germs result in significant morbidity and mortality, which are often underestimated. Indeed, nearly 4 million deaths are deplored in the world, including more than 525,000 children each year as a result of infectious dysentery [3]. Amoebiasis affects about 50 million people worldwide with a mortality rate approaching 100,000 deaths per year [4]. In the Republic of China, *E. coli* bacillary dysentery was in the top 10 of 39 reported infectious diseases from 2004 to 2014 revealing a high frequency of bacillary dysentery in children under 5 years of age [5]. In Cameroon, according to recent studies conducted on parasitic infections;

prevalences would be 33% in Yaounde, 27.8% in Douala, 59.5% in Dschang and 28.7% for HIV co-infected individuals[6,7].

Pathogens responsible for dysentery are transmitted between humans via the fecal-oral route and create lesions in the intestinal wall that causes inflammation of the digestive tract regardless of the etiological agent. Intestinal inflammation is therefore a defensive response of the immune system at the level of the intestinal wall due to the stimulation of the organism by microbial toxins or pathogenic agents [8]. Its essential role is the elimination of the pathogen and the repair of the injured tissue [9]. To this end, inflammatory reactions are accompanied by the production of numerous enzymes such as alkaline phosphatase involved in the repression of the NF- κ B signaling pathway of cellular inflammation and lipoxygenases that synthesize pro-inflammatory mediators such as leukotrienes and prostaglandins from arachidonic acid [10].

Furthermore, activated macrophages secrete various inflammatory mediators such as chemokines and cytokines (IL-1, IL-6, TNF- α , NO), all in an effort to eliminate antigen [11]. However, failure in antigen removal and repair of injured tissue causes overproduction of these mediators and in a persistent manner leading to progression into chronic inflammation that can be fatal to the organism [12]. Thus, the inhibition of these inflammatory mediators and enzymes (appears) appear to be an important target for the prevention of chronic inflammatory diseases. The conventional therapeutic armamentarium used for the treatment of dysentery mainly includes imidazole molecules for amebiasis and fluoroquinolone antibiotics for *E. coli* bacillary dysentery. These drugs exhibit carcinogenic, teratogenic and mutagenic effects over time [13]. Also, this therapeutic treatment does not take care of the inflammatory response disturbances that are common during the physiopathological process of this disease. Added to this are the economic costs associated with microbial resistance to *E. coli* frequently leading to therapeutic failures that result in complications and even death.

Due to the recurrence of toxicity and undesirable side effects of these synthetic molecules, it is imperative to direct the search for new therapeutic agents towards endophytic fungi which today constitute a potential resource of natural compounds [14]. These are secondary metabolites from plants for the most part (80% of the secondary metabolite), bacteria, fungi and many marine organisms (sponges, tunicates, corals and snails). They produce various bioactive molecules grouped into several structural categories such as alkaloids, flavonoids, polyketides and terpenoids [15, 16]. Many compounds from endophytic fungi have already shown interesting pharmacological activities like those isolated from *Phomopsis* species.

Very few research works have investigated their anti-dysenteric potentials on pathogenic strains of *E. coli* and *E. histolytica*. Thus, in (our) continuous search for therapeutic alternatives based on natural antimicrobials and in the perspective of making available to local populations a wide range of biological, natural, effective and low cost products, we proposed to study the antimicrobial and anti-inflammatory potential of some natural polyketides from *Phomopsis* sp CAM212 on two types of dysenteries: amoebic and bacillary dysenteries.

2. MATERIAL AND METHODS

2.1. Ethical statement

The present study was approved by the Joint Institutional Review Board for Animal and Human Bioethics (Ethical Clearance No BTC-JIRB2022056). All procedures followed the Cameroon National Veterinary Laboratory guidelines.

2.2 Biological material

The biological animal material used consisted of macrophages prepared from mice, clinical isolates of *E. histolytica* maintained on polyxenic culture at the Laboratory of Pharmacology and Toxicology of the Faculty of Sciences, University of Yaounde 1 and the enteropathogenic strain of *E. coli* ATCC25922 maintained at the Laboratory for Phytochemistry and Medicinal Plant Study.

2.3 Preparation of polyketides natural compounds.

Polyketide natural compounds isolated from *Phomopsis* sp CAM 212 strains following the previously described (protocole) protocol [17], were obtained from the Department of bioorganic chemistry, Leibniz Institute of Plant Biochemistry, Halle (Saale) Germany. The structures of tested compounds are shown in Figure1.

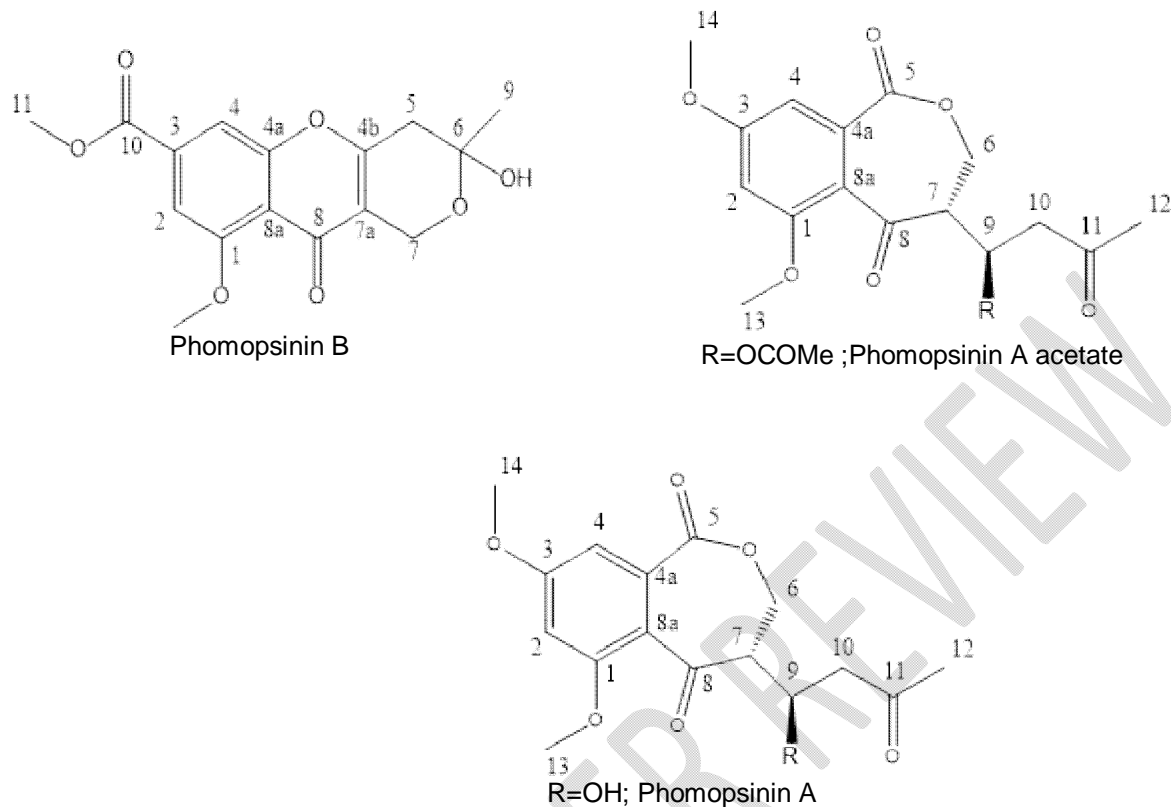


Figure 1: Structures of natural polyketides isolated from the fungus *Phomopsis sp* CAM212

2.4 Antiamoebic testing

2.4.1 Polyxenic culture of *Entamoeba histolytica*

A de Boeck and Drbohlav two-phase medium that involves a solid phase (Ringer's solution + egg) and a liquid phase (nutrient-containing lock solution) was used for polyxenic culture of *E. histolytica*. Prior to inoculation, complete media were pre-incubated at 37°C for 30 min and 10 µL of polyxenic culture maintained at the Laboratory of Pharmacology and Toxicology, University of Yaounde 1 containing clinical isolates of *E. histolytica* trophozoites were added to each tube. The tubes were incubated at 37°C and the growth of *E. histolytica* trophozoites was checked every 48 and 72 h. Then, the tubes were removed from the incubator and shaken to detach the parasites from the solid phase and left for 5 min, and the supernatant was decanted to remove the culture medium. The pellet containing the parasites was placed in a tube containing new pre-incubated medium and incubated as previously described [18].

2.4.2 Assessment of amoebic viability

The Trypan blue counting method was used. For this purpose, tests were performed using clinical isolates of *E. histolytica* in polyxenic culture counted using the Malassez cell, harvested in log phase at a concentration of 1.67×10^7 cells/mL and inoculated into 2.5 mL of new culture medium in the presence of six compounds from *Phomopsis sp*. All compounds were tested at the concentration of 25 mg/mL during the course of the tests. Metronidazole used as the reference anti-amoebic compound was also tested at the concentration of 25 mg/mL. During these tests a control tube, a standard and test tubes were used. The control contained parasites incubated with sterile distilled water; the standard contained parasites incubated with metronidazole; and the test tubes contained parasites incubated with the different compounds. Before each incubation, each tube containing the new culture medium, previously introduced 30 min in the incubator, received a pinch of rice starch. The experiment was performed in triplicate for each compound and all tubes were placed in the incubator at 37.5°C. Tubes removed from the incubator were immediately placed on ice. In a 1.5mL eppendorf tube; 25µL of parasite suspension was introduced and 225µL of 0.4% Trypan blue solution prepared in 0.9% NaCl was added. The whole mixture was

homogenized by vortex. Then, 20 μ L of the mixture was introduced into the Malassez cell which was then covered with a glass slide [19]. Viable amoeba were counted under a light microscope at 40X magnification and the amoebic concentrations in the culture medium were calculated using the following formula: $N = n \times Nr \times Vr \times Fd$

In which: N= concentration of viable amoebae (amoebae/mL); n= number of live amoebae counted in the Malassez cell; Fd= dilution factor; Nr = Number of rectangles (100); Vr = Volume of a rectangle (1000 mm³)

The percentages of inhibition were calculated using the following formula:

$$\% \text{ d'inhibition} = \frac{Nt - Nc}{Nc} \times 100$$

In which :

Nc = Number of living amoeba in the control tube; Nt = Number of living amoeba in the test tube.

After determining the percentages of inhibition, the percentages of viability were calculated according to the following formula: Percentage of viability = (100 - % inhibition)

2.5 Antibacterial testing

2.5.1 Formulation of Luria Bertani culture medium

One liter of liquid culture medium was prepared by adding 10 g of peptone, 5 g of yeast extract and 10 g of NaCl was added inside a volumetric flask containing 900 mL of sterile distilled water. The volume was completed to one liter using sterile distilled water then, followed by homogenization with a magnetic stirrer until the components of the medium were completely dissolved. The prepared medium was autoclaved for 15 min at 121°C for sterilization. For the preparation of LB agar culture medium, 15 g of Agar was added to the components of the liquid medium and prepared as previously described. The culture media were poured into petri dishes while hot; the dishes were then sealed with film paper. Finally; the media were stored in the refrigerator at +4°C until use [20]

2.5.2 Evaluation of bacterial activity

The determination of the inhibition parameters of the compounds was done according to the micro-dilution method on liquid media according to a previously described protocol M7-A7 [21]. 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT) was used for growth revelation of bacterial cells exposed to decreasing concentrations of compounds after incubation. A 96-well microplate, allows to study the activity in triplicate of two antimicrobial stock solutions on a bacterial strain. In each well of a sterile microplate placed horizontally, 100 μ L of Luria-Bertani Broth (LBB) culture medium was introduced. Subsequently, 100 μ L of an antimicrobial stock solution (compound or ciprofloxacin) was introduced into the first four wells (1A, 1B, 1C, 1D) of column 1. Thus there's three first test wells and one first negative control well. In lines 1 to 12, successive dilutions following a geometric progression of reason 2 were performed (from the wells of the first columns (1A, 1B, 1C and 1D) to the 11th well by taking after homogenization 100 μ L of the previous well to put in the next well; thus varying the concentration range from 25 to 0.0244 μ g/mL for the compounds and ciprofloxacin because they were tested at the same concentration (100 μ g/mL). Finally, 100 μ L of *E. coli* bacterial inoculum was added to each well except for those in lines D and E (used as negative controls) which were instead supplemented with 100 μ L of LBB culture medium. The final volume of each well was 200 μ L and the tests were performed in triplicate. The microplate wells containing only the antimicrobial and culture medium was used as a negative control and the wells in the 12th column containing only the culture medium and inoculum were used as positive controls for bacterial growth. The microplate was covered and sealed with film and incubated for 24 hours at 37.5°C. After incubation, microbial growth was demonstrated by adding 3 drops of 0.02% INT solution to two (02) of the three (03) test wells, the test wells of the non-INT labeled line were used for the determination of the MBC. MIC was defined as the lowest concentration of antimicrobial for which there was no bacterial growth visible to the naked eye (CLSI, 2015). For BMC determination, 50 μ L of the unlabeled line test wells with a concentration greater than or equal to their MICs, were spiked into 150 μ L of sterile Luria-Bertani broth contained in the microplate wells. The plate was incubated for 24 h at 37, 5°C. After incubation 3 drops of 0.02% INT were added to the wells and left for 15 min. The experiment was performed in duplicate. MBC was considered the lowest concentration of compound or ciprofloxacin for which no visible germ growth was observed. The MBC/MIC ratio was used to determine the bacteriological profile of the tested compounds. As previously described when MBC/MIC < 4, the substance is considered to be bactericidal; and when 4 \leq MBC/MIC \leq 16 the substance is bacteriostatic [21]. MBC/MIC > 16: tolerant substance.

2.6 Determination of the in vitro anti-inflammatory potentials of natural polyketides on primary culture of peritoneal macrophages

2.6.1 Primary macrophages cells culturing

Macrophages were isolated and maintained in culture as described [22]. Mice were elicited by intraperitoneal injection of 0.5 mL of a 2% starch solution (inflammatory agent). Four days later, the animals were sacrificed by cervical dislocation. Then the primary peritoneal macrophages obtained by the previously described method were suspended in 2 mL of DMEM culture medium, and 25 μ L (2.3×10^7 cells/mL) of the suspension were used for the Trypan blue viability assay. Counted cells were dispensed into 96-well microplates at a concentration of 10^4 cells/mL. In the test and positive control wells, 150 μ L of cells were introduced with 50 μ L of *Saccharomyces cerevisiae* (250 μ g/mL). In the blank wells, 150 μ L of cells were introduced with 50 μ L of DMEM. The microplate was incubated for 1h at 37°C (5% CO₂), then 50 μ L of compounds at different concentrations (0.1, 1, 10 and 100 μ g/mL) were added to the test wells and 50 μ L of DMEM was added to the positive control wells and finally 50 μ L of baicalin to the standard. The microplate was again incubated for 3h at 37°C (5% CO₂). The supernatants were used for nitric oxide assays while the pellets were used for alkaline phosphatase, 5-lipoxygenase and MTT cytotoxicity assays.

2.6.2 MTT Cell cytotoxicity

The cell pellet from the different incubations was taken up in 100 μ L of MTT solution (0.5 mg/mL in PBS) and the mixture was incubated at 37°C for 1h 30 min, then the supernatant was removed and 100 μ L of acidified isopropanol was added to each well to dissolve the formazan crystals formed. Finally, the absorbance of the blue-violet solution was read at 550 nm against the acidified isopropanol solution [23]. The percentages of cell viability were calculated using the following formula:

$$\% \text{ of Viability} = \frac{\text{Sample OD}}{\text{Control OD}} \times 100$$

2.6.3 Evaluation of the effect on nitric oxide production

The supernatants obtained during the previous incubations were used for the realization of this test. Indeed, 100 μ L of supernatant were mixed with 100 μ L of Griess reagent (1% sulfanylamide, 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% v/v phosphoric acid). The mixture was incubated (5% CO₂) for 10 min and the absorbance was measured at 550 nm [24]. The amount of nitrite was measured against the calibration curve of the sodium nitrate standard. The percentage of inhibition of nitric oxide production was calculated according to the formula:

$$\% \text{ inhibition} = \frac{(\text{OD}_{\text{control}} - \text{OD}_{\text{assay}})}{\text{OD}_{\text{control}}} \times 100$$

2.6.4 Evaluation of the effect on the alkaline phosphatase activity

The cell pellets obtained after the incubation of macrophages were used. The pellets obtained were solubilized by adding 25 μ L of Triton X-100, followed by the addition of 50 μ L of p-nitrophenylphosphate (10 mM) and 50 μ L of glycine buffer (0.1 M, pH 9.0). All solutions were incubated (5% CO₂) for 30 min at 37°C. The reaction was stopped by adding 100 μ L of NaOH buffer (0.2 M, pH 12) [25]. The absorbance was measured at 405 nm and the percentage change in lysosomal enzyme activity was calculated taking into account the control tubes according to the formula below:

$$\% \text{ de variation de l'activité de l'enzyme lysosomale} : \frac{(\text{DO}_{\text{assay}} - \text{DO}_{\text{control}})}{\text{DO}_{\text{control}}} \times 100$$

2.6.5 Evaluation of the effect on 5-lipoxygenase activity

The activity of 5-lipoxygenase was performed in sterile test tubes [10]. After isolating mouse macrophages and recovering in DMEM culture medium, 950 μ L of macrophage cells were introduced into each tube (100000 cells per tube). Then we added 300 μ L of *Saccharomyces cerevisiae* suspension (250 μ g/mL) to each tube, except for the negative control where the culture medium was added. This was followed by a first incubation of one hour at 37°C (5% CO₂). Then 50 μ L of compounds at concentrations of 0, 1, 1, 10 and 100 μ g/mL were introduced in the test tubes; 50 μ L of ascorbic acid, acetylsalicylic acid and baicalin for the standard tubes and 50 μ L of DMEM culture medium for the control tubes; a second incubation of 3 hours at 37°C (5% CO₂) followed. After that we centrifuged each tube at 2000rpm for 10 minutes at 4°C and removed the supernatant. The pellet containing the cells was recovered in 50 μ L of

Triton X-100 and then the tubes were vortexed within 2 minutes. Finally we added 1000µL of linoleic acid (125µM) and incubated for 30 minutes. All tests were performed in triplicate and the optical density of the supernatant was read at 234nm. The percentage of inhibition of the activity of this enzyme was calculated using the following formula:

$$\% \text{ of inhibition: } \frac{(OD_{\text{positive control}} - OD_{\text{assay}})}{OD_{\text{positive control}}} \times 100$$

2.7 Statistical analysis

Statistical analyses of the values obtained were performed using Graphpad Prism 9.0.0 software. The results were expressed as mean \pm standard deviation and the different values were compared using the analysis of variances test "one-way ANOVA" with the Turkey multiple comparison test and the differences were considered significant for a p-value < 0.05 .

3. RESULTS

3.1 Anti-amoebic potential

Clinical isolates of *E. histolytica* maintained on de Boeck and Drbohlav diphasic medium were incubated with different polyketides. The variation of trophozoite number with concentration and incubation time observed by light microscopy showed a significant decrease in percentage of viable parasites in the tested tubes compared to the control tubes after 24h, 48 and 72h post-treatment (Table 1). Among compounds, phomopsinin B, presented the highest anti-amoebic potential (84.4 % of amoebic inhibition after 72h). Phomopsini A and phomopsini A acetate showed moderate anti amoebic potentials (respectively 64 and 73% of amoebic inhibition after 72h). However, all these activities remained lower than that of metronidazole (90% of amoebic inhibition after 72h).

Table 1: antiamoebic effect of natural polyketides against clinical isolates of *E. histolytica*.

Compounds	Amoebicidal activities (% of viable parasites)		
	24 hours	48 hours	72 hours
Control	100 \pm 0,00	100 \pm 0,00	100 \pm 0,00
phomopsinin A	40,04 \pm 2,29	38,38 \pm 1,02	35,24 \pm 0,78
Phomopsinin A acétate	29,74 \pm 0,82	29,19 \pm 1,65	26,14 \pm 0,86
Phomopsinin B	37,78 \pm 1,50	24,94 \pm 12,77	15,54 \pm 3,66*
Metronidazole (standard)	17,08 \pm 5,18	13,15 \pm 6,42	10,19 \pm 3,85

*= Value significantly non different from the standard

3.2 Anti-bacterial potential

Determination of inhibition parameters of polyketide natural compounds was done by liquid microdilution technique described by CLSI (2015). Following this logic, the inhibition parameters (MIC, BMC) of compounds from Phomopsis species on the enteropathogenic *E. coli* strain ATCC25922 represented in (Table 2) were determined. Ciprofloxacin which was the reference drug taken as standard showed the best antibacterial potential against enteropathogenic *E. coli* strain (MIC=0.72µg/mL and MBC/MIC=4). The highest antibacterial potential was observed with phomopsinin B (MIC=12.5µg/mL and MBC/MIC=2) among natural tested compounds. No significant difference was observed between the antibacterial potential of phomopsinin A and that of phomopsinin A acetate (MIC=25µg/mL and MBC/MIC=1).

Table 2: Antibacterial potential of natural polyketides against *E. coli* strain ATCC25922.

Compounds	Bactericidal activities		
	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
phomopsinin A	25	25	1
Phomopsinin A acétate	25	25	1
Phomopsinin B	12.5	25	2
Ciprofloxacin (standard)	0.718	3.12	4

MIC= minimal inhibitory concentration; MBC= minimal bactericidal concentration

3.3 Anti-inflammatory activity

3.3.1 Effect on macrophage viability

Evaluation of the cytotoxicity of natural compounds from *Phomopsis* sp and plants on primary macrophages was done in MTT at different concentrations of the compounds (Figure 2). The results revealed that the viability of macrophages in the presence of the different compounds showed no significant difference except in the presence of phomopsinin B compounds at the concentration of 100 µg/mL although the percentage of viability did not go below 80%. Then the concentration of 100 µg/mL was chosen as the highest testing concentration for the anti-inflammatory assays.

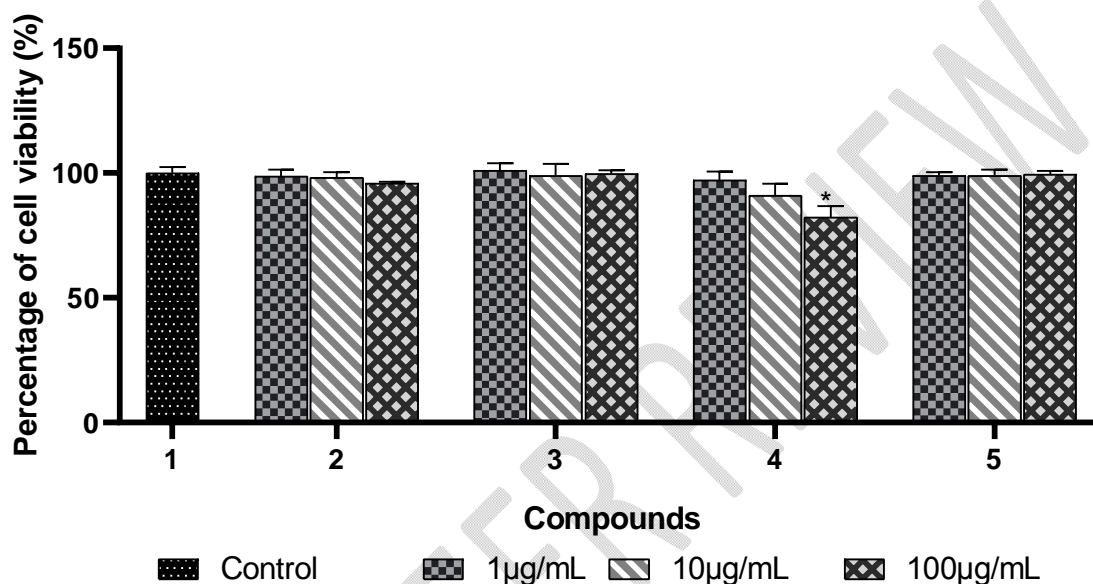


Figure 2: Effect of polyketides on the viability of primary mouse macrophages

1= Control; 2= Phomopsinin A; 3= Phomopsinin A acetate; 4= Phomopsinin B 5= Baicalin

*= value significantly different from the control ($p < 0.05$).

3.3.2 Inhibitory effect on nitric oxide production

Natural polyketides from *Phomopsis* sp effectively inhibit nitrite oxide production and the inhibitory effect was concentration dependent (Figure 3). Determination of IC_{50} (Table 3) demonstrated that phomopsinin B showed the best inhibitory potential on the synthesis of this mediator ($IC_{50} = 1.72 \pm 0.91 \mu\text{g/mL}$). Phomopsinin A ($8.05 \pm 2.19 \mu\text{g/mL}$) exhibited a moderate inhibitory potential and phomopsinin A acetate showed the lowest inhibitory potential ($41.61 \pm 7.28 \mu\text{g/mL}$). The inhibitory potential of phomopsinin B was significantly higher as compared to that of baicalin ($4.26 \pm 0.93 \mu\text{g/mL}$; $p < 0.05$) the reference drug taken as standard.

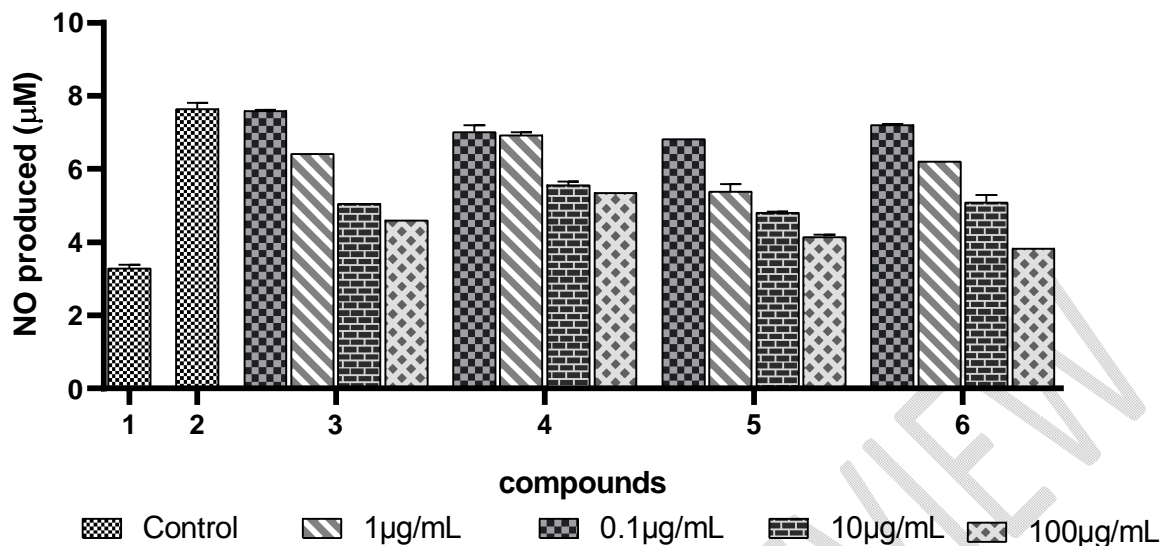


Figure 3: Inhibitory effect of natural polyketides on NO production. 1= Cells; 2= Cells + *Saccharomyces cerevisiae* 3= Phomopsinin A; 4= Phomopsinin A acetate; 5= Phomopsinin B 6= Baicalin

3.3.3 Stimulating effect on (alkalin)alkaline phosphatase (alp) activity

Phomopsis sp polyketides effectively boost the activity of this lysosomal enzyme (Figure 4) in a concentration-dependent manner. The determination of IC_{50} (Table 3) showed that no significant difference was observed between the modulatory effect of Phomopsinin A ($IC_{50}=0.63\pm 0.08\mu\text{g/mL}$), phomopsinin B ($IC_{50}=0.13\pm 0.01\mu\text{g/mL}$) and baicalin ($IC_{50}=0.92\pm 0.09\mu\text{g/mL}$) ($p<0.05$). However, phomopsinin A acetate ($IC_{50}=2.03\pm 0.28\mu\text{g/mL}$) exhibited a good modulatory activity on ALP, this remained lower compared to baicalin.

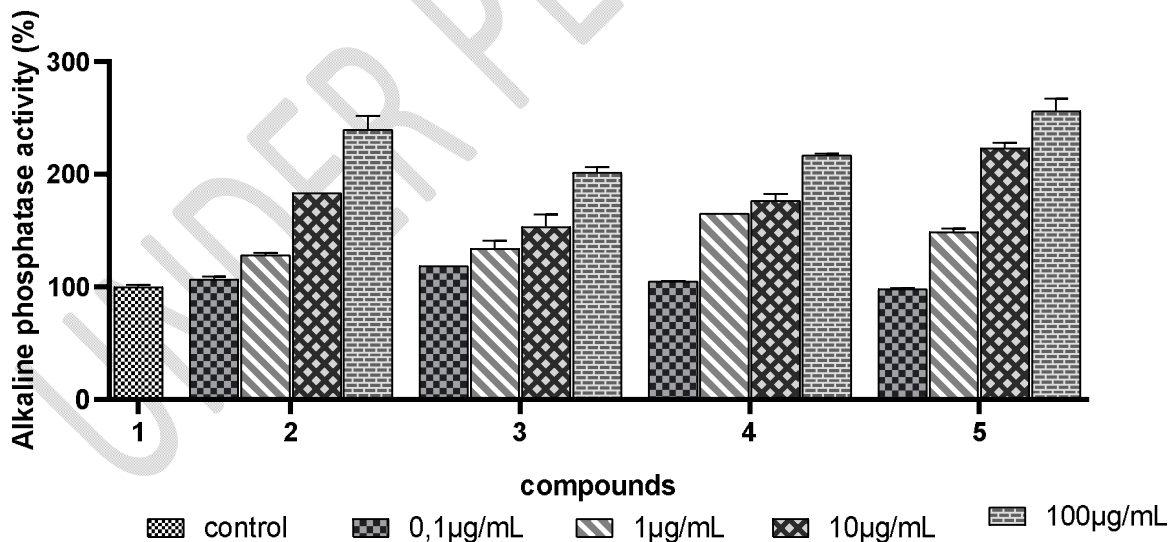


Figure 4: Effect of natural polyketides on the alkaline phosphatase activity 1= Cells + *Saccharomyces cerevisiae*; 2= Phomopsinin A; 3= Phomopsinin A acetate; 4= Phomopsinin B 5= Baicalin

3.3.4 Inhibitory effect on 5-lipoxygenase activity

Compounds from *Phomopsis* sp effectively inhibit 5-lipoxygenase activity and the inhibitory effect was concentration dependent (Figure 5). Determination of IC_{50} (Table 3) showed that the phomopsinin A ($IC_{50}=47.47\pm6.45\mu\text{g/mL}$) phomopsinin B ($IC_{50}=36.97\pm3.12\mu\text{g/mL}$) presented the moderate inhibitory potentials on the activity of this enzyme. Those inhibitory potentials were significantly lower compared to that of baicalin ($IC_{50}=10.77\pm2.16\mu\text{g/mL}$) ($p<0.05$). no inhibitory effect was observed with Phomopsini A acetate.

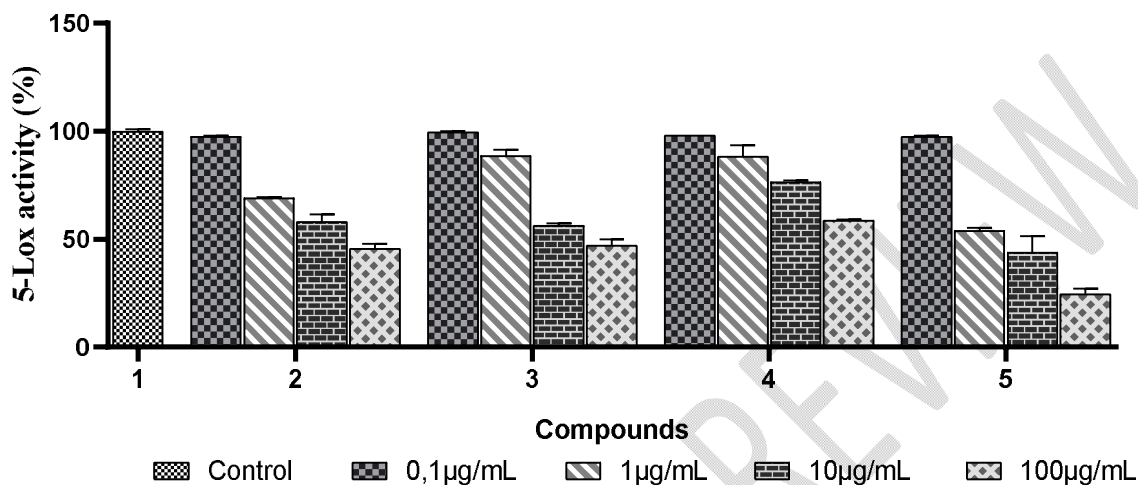


Figure 5: Inhibitory effect of natural polyketides on 5-lipoxygenase activity.

1 = Cells + *Saccharomyces cerevisiae*; 2 = Phomopsinin A; 3 = Phomopsinin A acetate; 4= Phomopsinin B 5 = Baicalin.

Table 3: efficacy of natural polyketides on the modulation of pro-inflammatory mediators

Compounds	IC_{50} ($\mu\text{g/mL}$)		
	Inhibition of NO	Inhibition of 5-LOX	Activation of ALP
Phomopsinin A	8,05±2,19**	47,47±6,45	0,63±0,08**
Phomopsinin A acetate	41,61±7,28	>100	2,03±0,28
Phomopsinin B	1,72±0,91*	36,97±3,12	0,13±0,01**
Baicalin	4,26±0,93	10,77±2,16	0,92±0,09

*= value significantly lower than the standard; **=Value significantly non different from the standard

4. DISCUSSION

Endophytic fungi are becoming more and more interesting to explore in the discovery of new therapeutic alternatives due to their great capacities to produce this wide range of biologically active compounds [26]. Indeed, following the recurrence of toxicity and undesirable side effects of synthetic molecules used in the treatment of dysenteric diseases, we evaluated in this work the effect of three natural polyketides isolated from *Phomopsis* sp CAM212 on a polyxenic culture of *E. histolytica*. The polyxenic culture medium is a commonly used in vitro model for culturing clinical isolates of *E. histolytica* and performing anti-amoebic tests [27, 28]. Indeed, the parasites grow in this environment in the presence of certain species of bacteria and yeasts that they use as a protein source; rice starch being the main energy source. During our study we noted an effectiveness of the anti-amoebic activity of natural polyketides

through the inhibition of the growth of *E. histolytica* which can be due to the destabilization of the constituents of the cell membrane and the DNA helix leading to the inhibition of the protein synthesis, which proves to be fatal for the parasite. From the results obtained, it appears that the compound phomopsinin B presented the highest anti-amoebic potential (84.4% inhibition after 72h) not significantly different from that of metronidazole (90% inhibition after 72h). These results are similar to those previously obtained [8] which demonstrated an inhibitory activity of *Sidarthombifolia* extracts on *E. histolytica* polyxenic culture. In addition, many studies have demonstrated that endophytic fungi are the origin of several antiparasitic molecules. One example is the endophytic fungus *Aspergillus terreus*-F7, associated with *Hyptissuaveolens* (L.) against *Schistosoma mansoni*, *Leishmania amazonensis* and *Trypanosoma cruzi*. The results showed that the three molecules, terrein, butyrolactone I and V from this endophyte killed at concentrations of 1297.3, 235.6 and 454.1 μM , after 24, 48 and 72 hours, 100% of *Schistosoma mansoni* worms. They also had moderate leishmanicidal activity with IC50s ranging from 78.6, 26.0 and 23.7 to μM respectively. Against *Trypanosoma cruzi*, only butyrolactone I and V were active and killed 100% of the cells at concentrations of 94.2 and 181.6 μM respectively [29]. Finally, we could observe an increase in activity in some compounds with the acetate moiety. This is the case for the compound phomopsinin A acetate having a percentage of parasite inhibition of 73.86% after 72h of incubation compared to the compound phomopsinin A without the acetate moiety and having a percentage of parasite inhibition of 64, 76%. The increase in efficacy observed during the tests suggests that the compounds' constituents may act synergistically on single or multiple targets associated with the physiological process leading to the destruction of *E. histolytica* trophozoites. These results are similar to those obtained by [30] who showed that the use of the compound phomopsinin A acetate obtained after acetylation of the compound phomopsinin A significantly increased the activity of the acetylated compound in the nitric oxide assay in LPS-stimulated RAW 264.7 macrophages. *E. coli* bacillary dysentery due to its various pathotypes remains a global public health concern. In the present work, *E. coli* strain ATCC25922 was grown on Luria-Bertani culture medium. This nutrient culture medium used for bacterial culture of pathogenic *E. coli* strains was used in par [20] in an interaction study between an EPEC and *E. histolytica*. It consists of peptones as sources of peptides, vitamins and trace elements from yeast extracts and sodium ions for transport and osmotic balance provided by Sodium Chloride. This culture medium was therefore adopted in our study for the culture of *E. coli* ATCC25922. This is a virulent enteropathogenic strain usually used in antibacterial tests [31]. The results obtained after our analyses revealed that out of six compounds tested, 05 compounds had bactericidal inhibitory activity of *E. coli* ATCC25922 with MICs ranging from 12.5 $\mu\text{g/mL}$ to 25 $\mu\text{g/mL}$ compared to ciprofloxacin (MIC 0.7812 $\mu\text{g/mL}$). The observed low sensitivity to bioactive compounds of this *E. coli* strain could be attributed to the different constituents of the bacterial cell wall [32]. Indeed, the cell wall of Gram-negative bacteria is complex. It consists of a periplasmic space and a thin layer of peptidoglycan adjacent to the cytoplasmic membrane, it is also surrounded by an additional outer membrane composed of phospholipids and lipopolysaccharides [33], which could make the cell wall impermeable to bioactive compounds. This would therefore tend to expel compounds from cells by acting as a selective barrier [34]. These results are similar to those obtained by [35] who tested two isocoumarins from *Xylaria* species on the same *E. coli* strain and obtained MICs ranging from 12.5 to 25 $\mu\text{g/mL}$. Another study on the same *E. coli* strain ATCC25922 showed inhibitory activity with a MIC of 100 $\mu\text{g/mL}$ using three alkaloids from *Fusarium proliferatum* [36]. On the other hand, lower results than ours were observed when studying antibacterial activities on several *Vibrio cholerae* and shigella strains using compounds from Phomopsis CAM240 that revealed MICs ranging from 512 to 218 $\mu\text{g/mL}$ [37].

Macrophages play an important role in the body's defense system by orchestrating the inflammatory response through the release of several pro- and anti-inflammatory mediators (NO, TNF α , IL-1, IL-6, IL-10, TGF β). These can act through the production or inhibition of reactive oxygen species and the activation of enzymatic pathways (Lipoxygenases, cyclooxygenases). All of this is done in order to eliminate a pathogen [38]. Excessive release of these mediators is a risk factor for chronic inflammation, implicated in the pathogenesis of many human diseases [39]. Therefore, in this work we evaluated the effect of our compounds on the production of some of these elements. Cytotoxicity was performed using MTT which is a tetrazolium salt that mainly targets the effectiveness of the respiratory process through the activity of mitochondrial succinate dehydrogenase. It is only from the concentration of 100 $\mu\text{g/mL}$ that we started to observe an effective cytotoxicity especially for the compound Phomopsinin B which was derived from *Phomopsis*. This therefore showed a slight decrease in mitochondrial succinate dehydrogenase activity from this concentration. This concentration was therefore set as the maximum working concentration for

the following. We found a significant ($p < 0.05$) decrease in the amount of NO produced by our macrophages compared with the control and in a concentration-dependent manner. Nitric oxide being a pro-inflammatory mediator synthesized by NO synthase from arginine and involved in vasodilation of blood vessels during the inflammatory response [40]. Inhibition of its synthesis is therefore a sign of anti-inflammatory activity; thus, the compound phomopsinin B showed the best inhibitory activity ($IC_{50} = 1.72 \pm 0.91 \mu\text{g compound/mL}$). These results are similar to those of [17] who had observed a significant decrease in the amount of NO produced by LPS-stimulated RAW 264.7 macrophages in the presence of compounds from *Phomopsis* species. Subsequently, the immunomodulatory activity of these compounds was evaluated through their effect on the activity of alkaline phosphatase. We realized that these compounds have the ability to boost the activity of this lysosomal enzyme compared to normal with the compound phomopsinin B which boosted more effectively this enzyme ($IC_{50} = 0.14 \pm 0.15 \mu\text{g of compounds/mL}$). Alkaline phosphatase is involved in the processes of repression of the nuclear factor NF κ B of cellular inflammation and in the dephosphorylation of bacterial compounds, so its activation would reflect the ability of our compounds to contribute to the protection of the intestinal barrier by modulating the composition of the microbiota through action on the microorganisms. These results are superior to those obtained by [41] who also obtained an increase in the activity of this enzyme ($IC_{50} = 0.274 \mu\text{g/mL}$) in the presence of the hydroethanolic extract of *Codiaeum variegatum*. Finally, we evaluated the effect of our compounds on the activity of 5-lipoxygenase. This enzyme allows the synthesis of leukotriene (LTB $_4$) responsible for the recruitment of immune cells at the site of inflammation. We were able to observe that the compound phomopsinin B inhibited 5-lipoxygenase in a concentration-dependent manner ($IC_{50} = 36.97 \pm 7.12 \mu\text{g/mL}$) and with a higher inhibitory activity than baicalin ($IC_{50} = 4.77 \pm 2.16 \mu\text{g/mL}$). The inhibition of this enzyme by our compounds may reflect their abilities to prevent the influx of immune cells to the site of inflammation. These results are similar to those of [10] who were able to show a significant decrease in 5-lipoxygenase activity in the presence of *Sabasenegalensis* extracts and superior to those obtained by [8] with a decreasing activity of 5-lipoxygenase ($IC_{50} = 73.22 \mu\text{g/mL}$) produced by Wistar mice macrophages with the ethanolic extract of *Sidarhombifolia* (L).

5. CONCLUSION

These findings demonstrated that polyketides natural compounds from *Phomopsis* sp CAM 212 are potent anti-dysenteric and anti-inflammatory agents. However, further in silico and in vivo studies are needed to better elucidate the mode of action of those natural compounds.

REFERENCES

1. Rajaona H (2003). Dysentery. Medicalhepato-gastroenterology. 2nd cycle gastroenterology course, 3rd ed. Antananarivo. 2003: 35.
2. Boi K (2022). Antibioticresistance profile of Escherichia coli and Salmonella sppstrainsisolatedfromenteric infections in childrenaged 0 to 15 years in the community: case of the CSCom of Yirimadjo, Bamako. University of Sciences, Techniques and Technologies of Bamako. Pharmacologicalfaculty. Doctoral thesis.
3. Aubry P, Gaüzère B (2019). Infectiousdiarrhea, News 2019. Center René Labusquière. Institute of Tropical Medicine, University of Bordeaux. 33076 Bordeaux (France). 8 p.
4. Houpt E, Hung C, Petri W (2016). Entamoeba histolytica (Amebiasis). InfectiousDiseases and Antimicrobial Agents. 3(24):24-28.
5. Chang Z, Zhang J, Ran L, Sun J, Liu F, Luo L, Zeng L, Wang L, Li Z, Yu H, Liao Q (2016). The changingepidemiology of bacillarydysentery and characteristics of antimicrobialresistance of Shigella isolated in China from 2004–2014. BioMedCentralInfectious Diseases.16:685.
6. Nkenfou C, Nana C, Payne V (2013). Intestinal parasitic infections in HIV infected and non-infected patients in a low HIV prevalenceregion, West-Cameroon. PLoS One. 8(2):e57914.
7. Pechangou NS, Upninder K, Kapil G, Rakesh S, Moundipa FP (2015). Moleculardifferentiation of Entamoeba Spp. isolatedfromCameroonianhumanimmunodeficiency virus (HIV) infected and uninfected patient. Academy Journal of Parasitology and Vector Biology.7(7): 139-150
8. Pechangou NS, Ndiandoukoue NR, Mfotie NE, Enang II EB, Njyou FN, Moundipa FP (2023). Comparative study of potent anti-amoebic and ant-inflammatoryactivities of differentextractsfrom Sida rhombifolia (L). InvestigationalMedicinal Chemistry and Pharmacology.6(1): 70.

9. Ashley NT, Weil ZM, Nelson RJ (2012). Inflammation: mechanisms, costs a natural variation. *Annual Review of Ecology, Evolution and Systematics*. 43: 385-406.
10. Yougbaré-Ziebrou MN, Ouédraogo N, Lompo M, Bationo H, Yaro B, Gnoula C, Sawadogo WR, Guissou IP (2015). Anti-inflammatory, analgesic and antioxidant activities of the aqueous extract of the leafy stems of *Saba senegalensis* Pichon (Apocynaceae). *Phytotherapy*. 8p.
11. Noack M, Kolopp-Sarda MN (2018). Cytokines and inflammation: physiology, physiopathology and therapeutic use. *French Journal of Laboratories*. 489(3): 28-37.
12. Lee HN, Surh YJ (2012). Therapeutic potential of resolvins in the prevention and treatment of inflammatory disorders. *Biochemistry Pharmacology*. 84: 1340-1350.
13. Mfotie NE, Weber C, Hernandez-Cuevas NA, Hon CC, Janin Y, Kamini FGM, Paul F, Moundipa PF, Guillen N (2014). Bioassay-Guided fractionation of extracts from *Codiaeum variegatum* against *Entamoeba histolytica* discovers compounds that modify expression of ceramide biosynthesis related genes. *PLOS Neglected Tropical Diseases*. 8(1): e2607.
14. Sadrati N (2021). Isolation, identification and culture of endophytic fungi isolated from Algerian medicinal plants for the production of biologically active secondary metabolites. Doctoral thesis in Microbiology. Ferhat Abbas Sétif University 1 Faculty of Nature and Life Sciences.
15. Zheng YK, Qiao XG, Miao CP, Liu K, Chen YW, Xu LH, Zhao LX (2016). Diversity, distribution and biotechnological potential of endophytic fungi. *Annual Microbiology*. 66: 529-5
16. Rana KL, Kour D, Kaur T, Devi R, Negi C, Yadav AN, Yadav N, Singh K, Saxena AK (2020). Endophytic fungi from medicinal plants: biodiversity and biotechnological applications. In: *Microbial Endophytes, Functional Biology and Applications*. Elsevier Masson. pp:273-305.
17. Jouda JB, Mfotie NE, Mbazoa CD, Yuan ZZ, Meli LA, Wandjia J, Shionoe Y, Wangb F (2018). Lambertellin from *Pycnoporus sanguineus* MUCL 51321 and its anti-inflammatory effect via modulation of MAPK and NF- κ B signaling pathways. *Bioorganic Chemistry*. 80: 216-222.
18. Parija S, Rao R (1995). Stool culture as a diagnostic aid in the detection of *Entamoeba histolytica* in the faecal specimens. *Indian Journal Pathology Microbiology*. 38: 359-363.
19. Bansal S (1987). Carbohydrate metabolism in the rat peritoneal macrophages. *Journal of Biological Sciences*. 12: 415-420.
20. Luz A, Fernandez-Lopez, Gil-Becerril K, Galindo-Gomez S, Teresa-Garcia, Ximenez C, Leon-Coria A, Moreau F, Chadee K, Tsutsumi V (2019). *Entamoeba histolytica* interaction with enteropathogenic *Escherichia coli* increases parasite virulence and inflammation in amebiasis. *Infectology Immunology*. 87(00): 279-19.
21. Rakholiya KD, Kaneria MJ, Chanda SV (2015). In vitro assessment of novel antimicrobial from methanol extracts of matured seed kernel and leaf of *Mangifera indica* L. (Kesar Mango) for inhibition of *Pseudomonas* spp. and their synergistic potential. *Amrongs Journal Drug Discovery*. 5:13-23.
22. Titus RG, Gueiros-Filho FJ, De Frietas LR, Berveley SM (1995). Development of a safe live *Leishmania* vaccine line by gene replacement. *Proceeding of the National Academy of Sciences*. 92 :10267-10271
23. Cedillo-Rivera R, Ramirez A, Muñoz O (1992). A rapid colorimetric assay with the tetrazolium salt MTT and phenazine methosulfate (PMS) for viability of *Entamoeba histolytica*. *Archives Medical Research*. 23: 59-61
24. Sun J, Zhang X, Broderick M, Fein H (2003). Measurement of nitric oxide production in biological systems by using Griess reaction assay. 3(1): 276-284.
25. Suzuki Y, Orellana MA, Schreiber RD, Remington JS (1988). Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science*. 240 (4851) :516-524.
26. Atiphasaworn P, Monggoot S, Gentekaki E, Brooks S, Pripdeevech P (2017). Antibacterial and anti-oxidant constituents of extracts of endophytic fungi isolated from *Ocimum basilicum* var. *thrysifloraleaves*. *Current Microbiology*. 74 : 1185-93.
27. Moundipa F, Kamini G, Bilong BC, Bruchhaus I (2005). In vitro amoebicidal activity of some medicinal plants of the Bamun region (Cameroon). *African journal of traditional complementary and alternative medicines*. 2(2): 113-121.
28. Pechangou NS, Moundipa P, Sehgal R (2014). In vitro susceptibilities of the clinical isolate of *Entamoeba histolytica* to *Euphorbia hirta* (Euphorbiaceae) aqueous extract and fractions. *African Journal of Microbiology Research*. 8(36), pp. 3354-3361.

29. Da Silva IP, Brissow E, Kellner Filho LC, Senabio J, de Siqueira KA, Vandresen Filho S, Damasceno JL, Mendes SA, Tavares DC, Magalhães LG (2017). Bioactive compounds of *Aspergillus terreus*-F7, an endophytic fungus from *Hyptissuaveolens* (L.) Poit. *World Journal MicrobiologyBiotechnology*. 33: 62.
30. Jouda JB, Mfotie NE, Tanemossu FSA, Yuan ZZ, Qiang Z, Mbazoa CD, Wolfgang B, Guo-lin Z, Wandji J, Fei W (2020). Natural polyketides isolated from the endophytic fungus *Phomopsis* sp. CAM 212 with a semisynthetic derivative downregulating the ERK/I κ B α signaling pathways. *Planta Medicine*.
31. Minogue TD, Daligault HA, Davenport KW, Bishop-Lilly KA, Broomall SM, Bruce DC, Chain PS, Chertkov O, Coyne SR, Freitas T, Frey KG, Gibbons HS, Jaissle J, Redden CL, Rosenzweig CN, Xu Y, Johnson SL (2014). Complete genome assembly of *Escherichia coli* ATCC 25922, a serotype O6 reference strain. *Genome Announcements*. 2(5): e00969-14.
32. Stefanovic O, Radojevic I, Vasic S, Comic LJ (2012). Antibacterial activity of naturally occurring compounds from selected plants. In: *Antimicrobial Agents*. Bobbarala V, (eds). 1st edition. Rijeka: InTech. pp: 1-24.
33. Praptiwi, Fathoni A, Ilyas M (2020). Diversity of endophytic fungi from *Vernonia amygdalina*, their phenolic and flavonoid contents and bioactivities. *Biodiversitas*. 21: 436–441.
34. Sebola TE, Uche-Okerefor NC, Tapfuma KI, Mekuto L, Green E, Mavumengwana V (2019). Evaluating antibacterial and anticancer activity of crude extracts of bacterial endophytes from *Crinum macowanii* Baker bulbs. *Microbiology open*. 8:1–10.
35. Zheng N, Yao F, Liang X, Liu Q, Xu W, Liang Y, Liu X, Li J, Yang R (2018). A new phthalide from the endophytic fungus *Xylaria* sp. GDG-102. *National Product Research*. 32: 755-760.
36. Jiang CX, Li J, Zhang JM, Jin XJ, Yu B, Fang JG, Wu QX (2019). Isolation, identification, and activity evaluation of chemical constituents from soil fungus *Fusarium avenaceum* SF-1502 and endophytic fungus *Fusarium proliferatum* AF-04. *J. Agriculture Food Chemistry*. 67: 1839–1846.
37. Jouda JB, Tamokou JDD, Mbazoa CD, Douala-Meli C, Sarkar P, Bag PK, Wandji J (2016). Antibacterial and cytotoxic cytochalasins from the endophytic fungus *Phomopsis* sp. harbored in *Garcinia kola* (heckel) nut. *BMC Complementary Alternative Medicine*. 16(462).
38. Ondua M, Njoya EM, Abdalla MA, Lyndy J, Gaw M (2018). Anti-inflammatory and antioxidant properties of leaf extracts of eleven South African medicinal plants used traditionally to treat inflammation. *Journal of Ethnopharmacology*.
39. Hunter P (2012). The inflammation theory of disease. The growing realisation that chronic inflammation is crucial in many diseases opens new avenues for treatment. *European Molecular Biology Organisation Rep*. 13: 968-970.
40. Nairz M, Schleicher U, Schroll A, Sonweber T, Theurl I, Ludwiczec S, Talasz H, Brandacher G, Moser PL, Muckentaler MU, Fang FC, Bogdan C, Weiss G (2013). Nitric oxide-mediated regulation of ferroportin-1 controls macrophage iron homeostasis and immune function of salmonella infection. *Journal of Experimental Medicine*. 210(5) : 855-873.
41. Pechangou NS, Enang II EB, Nghobha SV, Mfotie NE, Njyou FN, Moundipa FP (2022). Crude Extracts of *Codiaeum Variegatum* stem exhibit potent antioxidant and anti-inflammatory activities in vitro. *Journal of Exploratory Research in Pharmacology*. 000(000).