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Isolation, identification of endophytic fungi extracts from *Codiaeum variegatum* and assessment of their anti-amoebic and anti-inflammatory potential

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ABSTRACT

Intestinal amoebiasis (IA) remains a global public health concern. It is often confused with an inflammatory bowel disease, and is caused by *Entamoeba histolytica*. Although it is more widespread in tropical countries, IA is increasingly becoming a subject of concern in so-called developed countries. The limits of conventional therapy and the concern to promote phytotherapy in a sustainable and ecological manner inspired this work, during which the anti-amoebic and anti-inflammatory potential of endophyte fungi extracts isolated from Codiaeum variegatum were evaluated. C. variegatum leaves and bark was harvested at the University of Yaounde 1. Those two fragments undergo surface decontamination then were placed in petri dishes containing PDA (Potato Agar Dextrose) supplemented with amoxicillin. The cultures obtained were then subcultured in SDA (Sabouraud Dextrose Agar) and CZA (CZapek dextrose Agar) media. The resulting growth was observed using an optical microscope with an x40 objective for grouping on the basis of macroscopic and microscopic characteristics. Screening of extracts of these fungi by the Trypan blue counting method on clinical isolates of *E. histolytica*, made it possible to retain 4 extracts (6, 8, 14, 18) of fungi with anti-amoebic activity. Subsequently, the anti-inflammatory potential of these extracts was evaluated on a primary culture of macrophages activated by *Saccharomyces cerevisiae* through the inhibition of nitric oxide (NO) production, NADPH oxidase activity, and activation of alkaline phosphatase (ALP). From that culture, 28 endophytic fungi were isolated, 13 belonging to 7 genus (*Absidia* sp, *Alternaria* sp, *Aspergillus* sp, *Trametes* sp, *Trichoderma* sp, *Fusarium* sp and *Geotrichum* sp). Anti-amoebic tests showed that extract 14 was the most active with an inhibitory concentration 50 (IC_{50}) of 289.87±18.57; 207.73±22.36 and 87.79±12.51µg/mL respectively after 24, 48 and 72 hours of incubation. Chemical screening of the extracts revealed the majority presence of flavonoids in the 4 extracts (6, 8, 14, and 18). The anti-inflammatory tests of these 4 extracts revealed better anti-inflammatory activity in extract 6 (IC_{50}= 95.94 ± 21.07 µg/mL for NO; IC_{50}= 11.78±0.27 µg/mL for NADPH oxidase). Extract 14 had the best effect on the inhibition of NO production (IC_{50} = 8.56±2.13 µg/mL) and on the inhibition of the growth of trophozoites. Endophytic fungi extract from *C. variegatum* have demonstrated promising anti-amoebic and anti-inflammatory potential and therefore deserve special attention for the development of natural and ecological alternative treatment.

Keywords: Endophytic fungi, *Codiaeum variegatum*, Anti-amoebic, Anti-inflammatory, Plant extract.

INTRODUCTION

Intestinal amoebiasis is a parasitic disease of the intestine, caused by a unicellular and microaerophilic eukaryote, with mainly enteric tropism, called *Entamoeba histolytica*. It is a ubiquitous microorganism with a predominance in urban and rural environments of tropical and subtropical countries. Globally, approximately 500 million people are infected, but 10% develop symptoms associated with amoebiasis each year. In Africa, the prevalence is more than 40% of the population; and in Cameroon it varies between 14 and 35% depending on the city. Symptoms usually appears 2 week after ingestion of food or water contaminated with *E. histolytica's* cysts. In the large intestine, the cysts turn into trophozoïtes and feed on gut microbiota [1]. Their multiplication create lesions that leads to inflammation that will contribute to tissue damage manifested by bloody, often mucus-like diarrhea, abdominal pain, weight loss and fever. Other pathologies are encounter in patient infected by *E. histolytica* such as liver abscess, purulent pericarditis, pneumonia and cerebral amoebiasis; the most common case of extra intestinal amoebiasis is liver abscess [2,3]. *E. histolytica* has developed mechanism to escape the host immune response and to be more invasive by producing mammalian like cytokine called MIF (macrophages migration inhibitory factor) which makes intestinal inflammation last through the constant production of inflammatory mediators and enzymes such as nitric oxide synthase (NOS) and NADPH oxidase and the

reduction in the activity of alkaline phosphatase (ALP) essential in stopping intestinal inflammation [4]. The treatment prescribed for *E. histolytica* infections is most often metronidazole (MTZ). It has, despite his efficacy, showed side effects such as nausea, vomiting, headache, metallic or bitter taste, ataxia, anorexia and rash; and has been proved to induced resistance when *E. histolytica* is exposed to high level of MTZ [5–7]. To provide alternatives to MTZ clinical uses, medicinal plants as *Codiaeum variegatum* have been the a target for the search of new drug, due to their less side effect [8]. However, not only the simple use of plant is not sufficient to meet the need at a large scale, but also their potential caused them to be endangered. In other to preserve the biodiversity, a number of conventions and organizations have been established and has lead scientist to another source of bioactive compounds such as endophytes [9,10].

Endophytes are symbiotic microorganisms that live inside plants without causing any apparent damage. They are involved in the adaptation of plants to biotic and abiotic factors. In return the plant provides the endophytes with shelter and nutrients. Endophytes, the most common of which are fungi, can be isolated from any part of the plant [11]. These are microorganisms rarely encountered due to their existence in the plant, and therefore producers of new secondary metabolites, unknown to the majority of scientists and therefore unexploited; but also, molecules identical or similar to the secondary metabolites of their host plant; having multiple pharmacological properties including antimicrobial, anticancer, antioxidant and anti-inflammatory. According to other studies, some molecules contained in plant extracts come from those endophytes [12]. They therefore represent a potential means of producing biological medicines on a large scale with the consensus of the scientific community and the various members of biodiversity protection organizations; given that, from an economic and ecological point of view, the intensification of the fermentation process by microorganisms is easier and allows meeting industrial demand. This is due to the fact that they can be isolated and cultivated in an appropriate medium. It is following the same way that the present study evaluated the anti-amoebic and anti-inflammatory potential of extract obtained from endophytic fungi isolated from *C. variegatum.*

MATERIALS AND METHODS

Biological material

C. variegatum leaves and bark harvested at the garden of the University of Yaounde 1 were used for the isolation of endophytic fungi; in the month of October 2022. The other biological material used consisted of macrophages isolated 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 Yaoundé 1.

Isolation and culture of endophytic fungi

The plant tissues were washed thoroughly distilled water and surface sterilized through sequential dip in 70% ethanol (1 min), in 2.5% sodium hypochlorite (NaOCl) (4 min) and in 70% ethanol (30s), followed by a final rince in 3 containers of sterile distilled water**.** They were air dried under a microbiological hood. Some milliliter of the final rinse water was inoculated on potato dextrose agar (PDA) medium to check the effectiveness of the sterilization procedures. Segments of surface sterilized plant tissue measuring about 7mm in diameter were cut and inoculated at 28 °C±2°C onto PDA medium supplemented with amoxicillin (15µg/ml). The plates were checked daily for any fungal growth; single isolates grown out from the tissues were re-inoculated on fresh PDA medium and then stored at 4°C for further study [13].

Identification of endophytic fungi

Endophytic fungi isolates were identified according to their cultural and morphological characteristics on PDA, Sabouraud Dextrose Agar (SDA), and Czapek Dox Agar (CDA) media at 28 ±2°C and microscopic features already described [14,15]**.** The macroscopic characteristics observed were color and surface colonies (granular, such as flour, mounting, slippery), texture, growth area, the lines of radial and concentric, reverse color, and exudate drops. the microscopic examinations of the hyphae, fruiting bodies and spores were carried out with methylene blue reagent [16,17].

Extraction of secondary metabolites of endophytic fungi

Fermentation

The isolates presenting macroscopic and microscopic features searched were chosen for the liquid phase fermentation. Some mycelia agar cylinder from endophytic fungi culture were introduced into two 500ml flask containing 300ml of potato dextrose broth (PDB) for one endophyte. The flasks were incubated at room temperature for 1 week [18].

Extraction of endophytic fungal culture

After incubation period, biomass and culture media were filtered and 300ml of ethyl acetate was added. The acetic fraction was evaporated under reduced pressure by Heidolph brand rotary evaporator.

Anti-amoebic potential assessment

Polyxenic culture of Entamoeba histolytica

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. Then 10 µl of polyxenic culture maintained at the Laboratory of Pharmacology and Toxicology, University of Yaounde 1 containing clinical isolates of *E. histolytica* trophozoites was added to each tube. The tubes were incubated at 37°C and the growth of *E. histolytica* trophozoites was checked every 24, 48 and 72 h. Then, the tubes were removed from the incubator and shaken to detach the parasites from the solid phase, left for 5 min, and the supernatant was decanted and a small amount was introduced in the tube cap. That amount of supernatant containing the parasites was placed in a tube containing new pre-incubated medium and incubated as previously described [19]**.**

Assessment of anti-amoebic potential

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 counting cell, taken in the log phase at a concentration of 1.67×10^7 cells/m and inoculated into 2.5 ml of new culture medium in the presence of 26 endophytic fungi extracts. All extracts were screened at the concentration of 1000µg/ml. Metronidazole (MTZ) used as the reference anti-amoebic compound was also tested at the concentration of 10 mg/mL. During these tests a control tube, a standard and test tubes were used. The control contained parasites incubated with DMSO; the standard contained parasites incubated with MTZ; and the test tubes contained parasites incubated with the different extracts. After the screening, extract with the ability to reduce parasite growth were chosen and then retested at the concentration of 1000µg/ml, 500µg/ml, 250µg/ml, 100µg/ml and 10µg/ml. Before incubation, each tube containing the new culture medium received a pinch of rice starch. The experiment was performed in triplicate for each extract 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, 20µL of the mixture was introduced into the Malassez cell which was then covered with a glass slide. Viable amoeba was counted under a light microscope at 40X

magnification and the amoebic concentrations in the culture medium were calculated using the following formula:

$$N = n \times Nr \times Vr \times Df$$

In which: N= concentration of viable amoebae (amoebae/mL); n= number of live amoebae counted in the Malassez cell; Df= dilution factor; Nr = Number of rectangles (100); Vr = Volume of a rectangle (1000 mm^3) [20].

Determination of the anti-inflammatory potential

Macrophages cells culture

Macrophages were isolated and maintained in culture as described [21]. Their production was elicited by intraperitoneal injection of 0.5 mL of a 2% starch solution in PBS (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 25µL of the suspension was used for the Trypan blue viability cell count. Counted cells were dispensed into 96-well microplates at a concentration of 10^4 cells/well. In the test, standard and positive control wells, 150 µl of cells were introduced with 50µl of *Saccharomyces cerevisiae* (250 µg/ml). In the blank wells, 150µl of cells were introduced with 50µl of DMEM. The microplate was incubated for 1h at 37°C (5% CO_2), then 50µl of extracts at different concentrations (0.1, 1, 10 and 100 µg/ml) was added to the test wells and 50µl of DMEM was added to the positive control wells and finally 50µl of baicalin to the standard. The microplate was again incubated for 3h at 37°C (5% CO_2). The supernatants were used for nitric oxide assays while the pellets were used for alkaline phosphatase, NADPH oxidase and MTT cytotoxicity assays.

Assessment of the cell cytotoxicity using MTT

The cell pellet from the different incubations (without *Saccharomycces cerevisiae*) was added to 100 µl MTT solution (0.5 mg/mL in PBS) and the mixture was incubated at 37°C for 1h 30 min. The supernatant was removed and 100 µl of acidified isopropanol was added to each well to dissolve the formazan crystals formed. At the end, absorbance of the blue-violet solution was read at 550 nm against the acidified isopropanol solution [22]. The percentages of cell viability were calculated using the following formula:

$$\% \text{ viability } = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times 100$$

Determination of the effect on nitric oxide synthesis

The supernatants obtained during the previous incubations were used for the realization of this test. Indeed, 100 µl of supernatant were mixed with 100 µl of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% v/v phosphoric acid). The mixture was incubated (5% CO_2) for 10 min and the absorbance was measured at 550nm [23]. The quantity of nitric oxide produced was calculated using a sodium nitrate calibration curve. The percentage of inhibition of nitric oxide production was calculated according to the formula:

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

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 25µl of SDS, followed by the addition of 50µl of p-nitro phenyl phosphate (10mM) and 50µl of glycine buffer (0.1M, pH 9.0). All solutions were incubated (5% $C0_2$) for 30min at 37°C. The reaction was stopped by adding 100µl of NaOH buffer (0.2M, pH 12) [24]. The absorbance was measured at 405nm and the percentage change in alkaline phosphatase activity was calculated taking into account the control tubes according to the formula below:

$$\% \text{ change in alkaline phosphatase activity } = \frac{(OD\ sample - OD\ control)}{OD\ control} \times 100$$

Determination of the effect on NADPH oxidase

Micro-plates were used for this experiment and the protocol used was the one previously described [25]. Here 150µl of cells (10^4cells/well) was incubated with 50µl of endophytic fungi extracts at 37°C, 5% CO_2 during 3h. After that, 50µl of *Saccharomyces cerevisiae* and 50µl of nitro blue tetrazolium (NBT) was added to the medium then incubated at 37 °C, 5% CO_2 during 1h. The supernatant was eliminated, the pellets was solubilized inside 20µl methanol then left at room temperature during 10min. After that 50µl of KOH 2M and 50µl of DMSO was added to the medium. The absorbance was read at 570nm.

$$\% \text{ NBT } = \frac{(DO\ essai - DO\ témoin)}{DO\ témoin} \times 100$$

Statistical analysis

Statistical analyses of the values obtained were performed using Graphpad Prism 8.0.1 software. The results were expressed as mean ± standard deviation. IC_{50} and EC_{50} were calculated using a logarithmic dose response curve. The different values (IC_{50} and EC_{50}) were compared using the analysis of variances test "one-way ANOVA" with the Turkey's multiple comparison test and the differences were considered significant for a p-value $p<0.05$

RESULTS

Isolation and identification of endophytic fungi

A total of 28 endophytic fungi were isolated from *C. variegatum* and grouped into 26 fungi isolates. It was found that 13 fungi isolates were identified, belonging to seven different genus: *Absidia, Alternaria, Aspergillus, Geotrcichum, Trichoderma* and *Trametes*. Those genera belong to Deuteromycota division, more precisely to the Hyphomycetes class. They were divided into hyaline hyphomycetes (Moniliaceae order) with *Absidia* spp, *Alternaria* spp, *Aspergillus* spp, *Trichoderma* spp and *Trametes* spp; and dark hyphomycetes (Dematiaceae order) with the only genus *Geotrichum spp*. being identified. However, the remaining 13 endophytic fungi could not be identified due to their issues related infertility.

Fungi 1 and 17 presented colonies whose aerial mycelium was white in color and the substrate mycelium was greyish in the PDA medium; in the SDA the colonies were white and velvety; in the CDA, the colonies presented a milky texture with a gray color. Microscopy revealed non-septate hyphae branching into sporangiophores presenting a circular swelling: these different elements refer to the genus *Absidia*. A species of this genus *Aspergillus*, originating from the leaf was identified in this study. (Figure 1) The mycelial colonies observed were powdery black on the reverse side of the Petri dish and white on the reverse side in the PDA media; in the SDA medium, the mycelial colonies were light yellow on the inside and on the back of the Petri dish; in the CDA medium, the mycelial colonies were powdery black on the back of the Petri dish and white on the back. Microscopy of this isolate showed a septate thallus and a long unbranched conidiophore, ending in a swelling bearing phialides inserted on the end of the conidiophores via metulae; what is called aspergillus head and characteristic of the genus *Aspergillus*. Fungi 10, 18 and 20 exhibited various colors and textures (Figure 1). They were white, cottony in the PDA, woolly in the CDA, orange-pink and glabrous in the SDA for fungus 10. In isolate 18, the colonies are white and woolly in the CDA, cottony in the PDA and woolly white in the SDA on the obverse and on the reverse of the petri dish. In isolate 20, the colonies are woolly, white and pink at the tips in SDA and woolly and white in PDA and SDA. Microscopy showed septate hyphae, a cluster or chain arrangement of microconidia, fusiform

macroconidia, curved and quite pointed at the ends: which is characteristic of the Fusarium genus. Colonies produced by fungus 25 were white, fluffy in PDA and SDA media; white in CDA environment. The colonies of fungus 15 were woolly white in color on the obverse and reverse of the petri dish in PDA and SDA medium; and transparent in the CDA environment. Microscopy shows the presence of septate and hyaline hyphae dissociating into arthroconidia with flat ends; which refers to the genus *Geotrichum*. Fungi 12 presented grayish cottony mycelial colonies on the obverse and reverse of the petri dish, in PDA, SDA and CDA media. Microscopic observation allowed us to observe dark and septate hyphae, elongated spores presenting horizontal partitions; which refers to the genus *Alternaria*. The colonies of fungus 28 were velvety and white on the obverse and reverse of the petri dish; woolly white in SDA and cottony white in CDA. Macroscopic observation shows us a highly branched non-septate hyaline hypha. These different characteristics pointed towards the genus *Tramestes*. Fungi 8, 13 and 16 showed green to yellowish green colors and a flaky texture in PDA medium. In the CDA medium, the colonies were granular and green in color on the obverse and reverse of the petri dish; and cottony white-yellow in the SDA for fungus 8, yellow for fungus 13, and white for fungus 16. Microscopy showed septate hyphae and a branched conidiophore producing lateral branches bearing short phialides directly inserted at the end of conidiophores and grouped in groups of 3; from which conidia or round spores are produced: which is characteristic of the genus *Trichoderma*. Unfortunately, fungi isolate such as 2, 5, 6, 23 and 26 were unidentified as they did not produce sporing structures on PDA, SDA, and CDA media but presented dirty white cotton growth that darkened with age differently in each isolate. Microscopy presented non-septate and dark hyphae in those fungi. Fungi 11, 14, 21, 22, 24 and 27 presented various texture and color. Their hyphae were non-septate and dark.

Anti-amoebic potential of endophytic fungi extracts

The anti-amoebic potential of extracts of endophytic fungi isolated from *C. variegatum* was revealed a dose dependent inhibitory activity at those different point of time as represented on figure 1. The activity was realized at different concentrations of the extracts. That permits us to determine the IC_{50} for each extract as presented on the Table 1 above. It is a key parameter that was useful for the comparison of the different extract in order to bring out the more active extract. Results in the table 1 shows in fact that the 04 extracts selected during the screening present an anti-amoebic potential, but not significant compared to that of MTZ. Despite the fact that no extract induced total mortality (100%) in the performed assay, we did not observe any stationary phase in amoebic growth except for the extract 18 which has better activity than all the other extracts between 24 and 48 hours, but from 72 hours we see that there is again growth of the amoebae reflected by an increase in the IC50 at 72 hours. Extract 6 only begins to be active after 48 hours, and extract 14 presented better activity compared to extracts 6,8 and 18; but its activity lower than that of MTZ.

Anti-inflammatory activity of endophytic fungi extracts:

Effect of endophytic fungi extracts on macrophage viability

No apparent cytotoxicity was observed in presence of the different extract from 0.1 to 1000µg/ml for all the extracts (Fig.1).

Inhibitory effect of endophytic fungi extracts on nitric oxide production

The capacity to inhibit NO synthesis by activated macrophages of the different extracts was assessed at different concentration from 0.1 to 1000µg/ml. The results obtained are presented on the figure 2 above. We observed that the inhibitory activity was following a concentration dependent aspect. Using a logarithmic dose response curve, we were able to find out the IC_{50} of eachextract as presented on table 2. Extract 14 presented the more effective inhibitory activity (8.56 ± 2.13µg/ml) without a significant difference with that of the standard (1.40 ± 0.22µg/ml).

Inhibitory effect of endophytic fungi extracts on NADPH oxidase activity

The inhibitory potential of those extracts against the activity of this key enzyme involved in the production of reactive oxygen species was done at different extract concentration as previously described. That revealed for all the extract except the extract 18 a dose dependent activity as represented on the figure 2. The IC_{50} obtained from that permit us to compare the different extracts one to another as presented on table 2. Extract 6 was the more active (11.78±0.27µg/ml).

Activating effect of endophytic fungi extracts on alkaline phosphatase activity

Alkaline phosphatase activity has been increased by the different extracts in a dose dependent manner as represented on the figure 2. Amongst the different tested extracts, the extract 8 was the only one presenting an effective boosting potential on alkaline phosphatase activity within the tested range of concentrations (EC_{50}=328.03±16.04) as shown on the table 2. That extract was able to increase the alkaline phosphatase activity up to 180 % of its normal state. With no significant difference the effect of baicalin (EC_{50}=41.87±1.89).

Table 1: Summary of the genera of endophytic fungi obtained according to the parts used

Genera	Plant part	
	Leave (fungi's code)	Bark (fungi's code)
***Absidia* sp**	1 ; 17	/
***Alternaria* sp**	/	12
***Aspergillus* sp**	3	/
***Fusarium* sp**	18	10 ; 20
***Geotrichum* sp**	/	15 ; 25
***Trametes* sp**	/	28
***Trichoderma* sp**	8	13 ; 16 ; 19
Not identified	7	6

Table 2: Morphology and microscopic characters of endophytic fungi isolated from *Codiaeum variegatum* whose extracts have been used to assess the anti-amoebic and anti-inflammatory activity

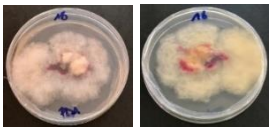
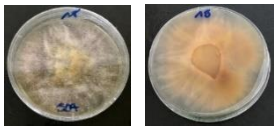
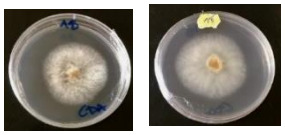
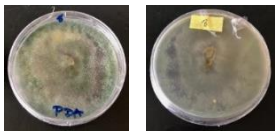
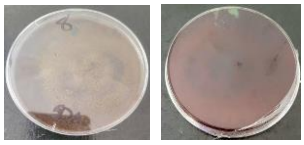
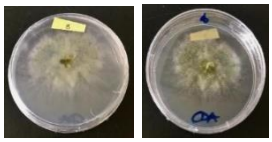
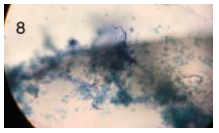
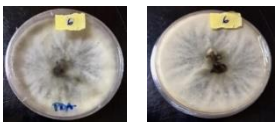
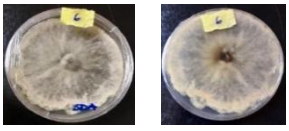
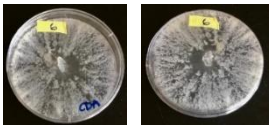
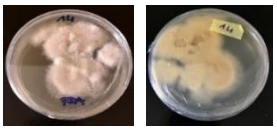
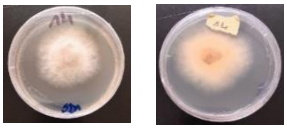
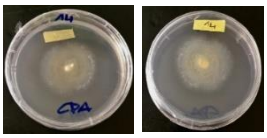
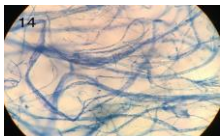
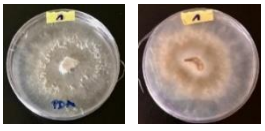
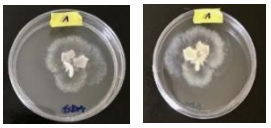
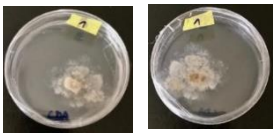
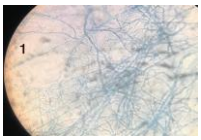
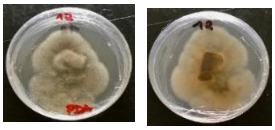
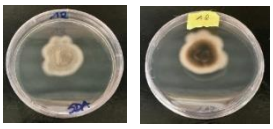
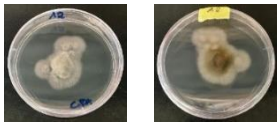
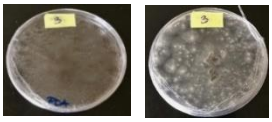
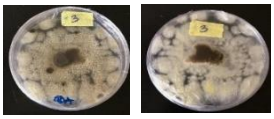
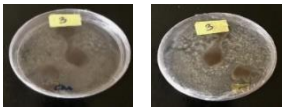
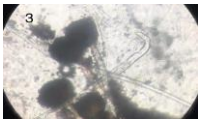
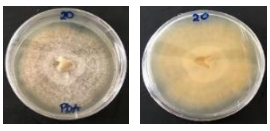
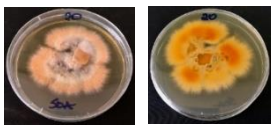
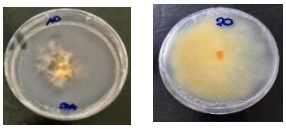
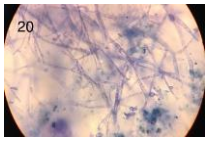
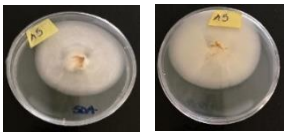
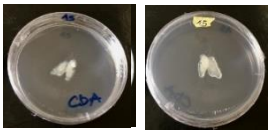
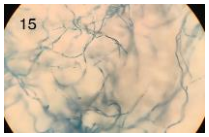
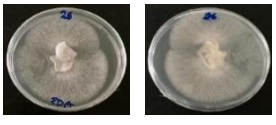
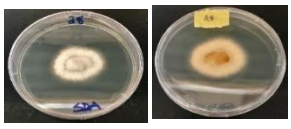
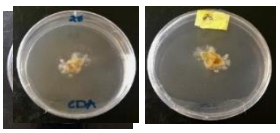
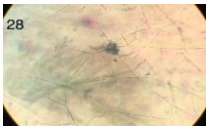
Isolates' codes	PDA medium	SDA medium	CZA medium	Microscopy (methylene blue)
18 *Fusarium* sp 2				
8 *Trichoderma* sp 1				
6 Not identified				
14 Not identified				

Table 3: A few images of endophytic fungi isolated from *Codiaeum variegatum*

Isolates' codes	PDA medium	SDA medium	CZA medium	Microscopy (methylene blue)
1 *Absidia* sp 1				
12 *Alternaria* sp				
3 *Aspergillus* sp				
20 *Fusarium* sp 3				
15 *Geotrichum* sp 1				
28 *Trametes* sp				

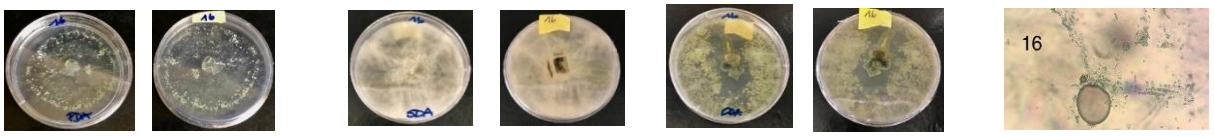
16 *Trichoderma* sp 3		

Table 4: IC_{50} (µg/ml) of anti-amoebic activity of endophytic fungi extracts

Time	Extract 6 (not identified)	Extract 8 (*Trichoderma* sp)	Extract 14 (not identified)	Extract 18 (*Fusarium* sp)	Metronidazole
24h	>1000[a]	594.03±25.28[a]	289.87±18.57[a]	299.27±30,27[a]	2,25±0,42
48h	317±27.06[a]	226.47±31.65[a]	207.73±22.36[a]	78.03±7.52[a]	1.03±0.13
72h	168.27±16.27[a]	173.77±5.08[a]	87.79±12.51[a]	106.02±6.15[a]	0.77±0.10

Values are expressed as means ± standard deviation of 3 tests (triplicate). [a]: significant difference compared to Metronidazole for p<0.05

Table 5: IC_{50}s of the effect of endophytic fungi extracts on NO production

Extracts	NO(IC_{50})	NADPH oxidase (IC_{50})	PAL (EC_{50})
Extrait 6 (not identified)	95.94 ± 21.07 [a]	11,78±0,27	>1000
Extrait 8 (*Trichoderma* sp)	239.9 ± 68.81 [a]	>1000[a]	328.03±16.03
Extrait 14 **(not identified)**	8.56 ± 2.13	>1000[a]	>1000
Extrait 18 (*Fusarium* sp)	69.03 ± 28.99	>1000[a]	>1000
Baicalin	1.40 ± 0.22	0.07±0.002	41.87±1.89

Values are expressed as means ± standard deviation of 3 tests (triplicate). [a]: significant difference compared to Baicalin for p<0.05

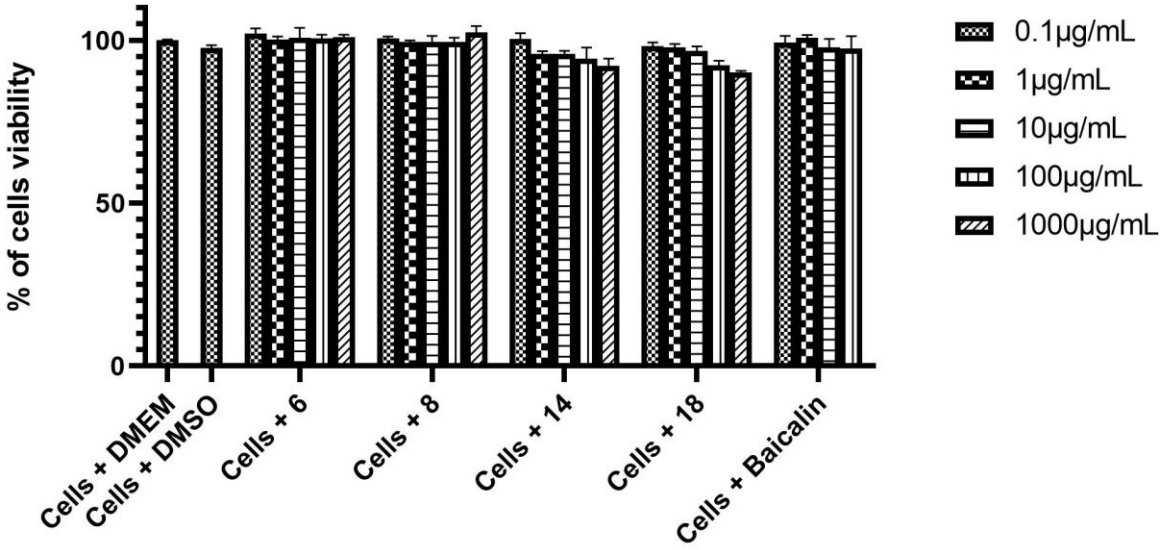


Figure 1: Effect of endophytic fungi extracts on macrophage viability

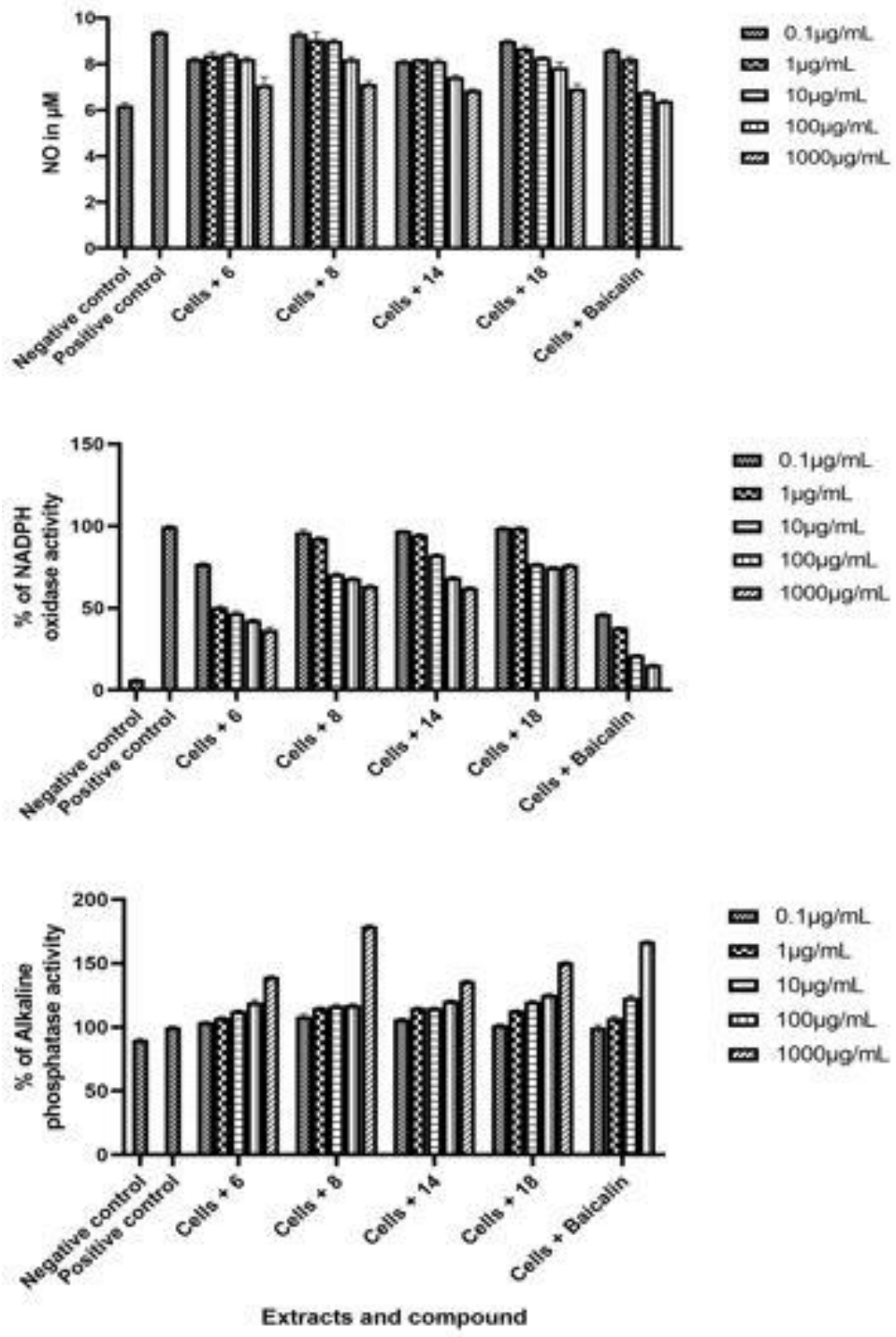


Figure 2: Anti-inflammatory effect of endophytic fungi extracts

DISCUSSION

Endophytic fungi are microorganisms that live inside plants without causing any apparent damage. Their ability to produce secondary metabolites identical or similar to those of their host plant has been demonstrated by numerous studies. Their use not only for the screening of therapeutic molecules, but also for their production, therefore appears to be an ecological and bioconservative alternative respecting the rules established in relation to the conservation of biological diversity [10]. Following the isolation of endophytic fungi from the leaves and bark of *C. variegatum*, 28 fungi were obtained. These were then grouped into 26 based on their morphological characteristics in PDA, SDA and CZA media. The microscopic characterization of these fungi allowed us to obtain 7 genera, namely: *Absidia* sp, *Alternaria* sp, *Aspergillus* sp, *Trametes* sp, *Trichoderma* sp, *Fusarium* sp and *Geotrichum* sp. Fungi of the genus *Trichoderma* sp and *Fusarium* sp constituted the greatest number of species among the isolates obtained during this study. Former studies demonstrated that fungi of the genus *Trichoderma* sp are universal soil resident. Therefore, their hyphae can easily penetrate the plant and be found in its different tissues [26,27]. *Fusarium* sp, is omnipresent molds and widely distributed in nature thanks to its airborne spores that it produces in large numbers. In our finding the frequency of *Fusarium* sp may reflect its presence among endophytes isolated from plants [28].

Among endophytic fungi obtained, the different species belonging to the genus *Absidia* sp and the different species belonging to the genus *Geotrichum* sp were isolated respectively from leaves and bark

exclusively. This result could be due to the fact that endophyte colonization is specific to plant tissues due to the selection pressure that exists in these different locations [29,30]. The fungi of the genus *Fusarium* sp, *Geotrichum* sp and *Alternaria* sp obtained during our study were also previously isolated from an ethnomedicinal plant *Phyllanthus reticulatus* another *Euphorbiaceae* [31] in the same way as *C. variegatum*. This could confirm the hypothesis that fungi of the same genus are likely to colonize plants of the same family [32].

After screening the endophytic fungi extracts, 04 extracts from fungi 6, 8, 14 and 18 were selected on the basis of their anti-amoebic potential at 1000µg/ml. Subsequently, the 04 selected extracts were tested at different concentrations on a polyxenic culture of *E. histolytica*, in order to determine the IC_{50}. The photographs of the trophozoites after the different incubations reveal the anti-amoebic potential of these 04 extracts. These photographs combined with the IC_{50} obtained present extract 14 as having the best activity compared to the other extracts. However, the significant contrast observed between the IC_{50}s of this extract (extract 14) and those of MTZ could reveal the presence in this extract of molecules whose action is antagonistic to those of the compounds responsible for the activity of the extract; which could have reduced its ability to inhibit the growth of trophozoites of *E. histolytica* relative to MTZ [33]. In addition, numerous studies, including those of Mfotie et al., in 2014, made it possible to observe the same contrast between the aqueous extract of *C. variegatum* and MTZ [8].

Comparison of the results obtained by Mfotie et al., in 2014 with the aqueous extract of *C. variegatum* (IC_{50}=204.33µg/ml and 102µg/ml) and those obtained with the extract of the endophytic fungus 14 isolated from *C. variegatum* (IC_{50}=207.73µg/ml and 89.79µg/ml), shows us that there is no significant difference between the aqueous extract and the extract 14 to 48 hours. However, there is a significant difference in favor of the 14 extracts to 72 hours because its IC_{50} is lower than that of the aqueous extract. This could be explained by the ability of endophytes to produce new compounds of their own, and which can thus make their extracts more effective than those of their host plants [34].

In addition, the result obtained with extract 14 is lower than those obtained with the different fractions of the aqueous extract of *C. variegatum* during the work carried out by Mfotie et al in 2014. Indeed, the bio-guided fractionation of this extract would have contributed to the concentration of the active compounds of the extract, thus creating a synergy between these different compounds, resulting in an increase in the activity of the extract. The IC_{50}s ranged from 204.33µg/ml and 102µg/ml for the aqueous extract; at 126.5µg/ml and 53.00µg/ml for the methanolic fraction; then at 18.87µg/ml and 15.62µg/ml for the sub-fraction of the methanolic fraction and finally at 5.71µg/ml and 2.25µg/ml for the crystalline fraction of the sub-fraction of the methanolic fraction, respectively after 48 and 72 hours of incubation.

The chemical screening carried out revealed the presence of flavonoids in the 04 extracts having demonstrated anti-amoebic potential, with a more pronounced content in extract 14. Indeed, many flavonoids have demonstrated amoebicidal activity, and their presence in our extracts from endophytic fungi could justify their ability to inhibit the growth of *E. histolytica* trophozoïtes [35]. In addition, the work of Quintanilla-Licea et *al*., in 2020, was able to isolate flavonoids active on *E. histolytica* such as pinocembrin, naringenin, sakuranetin and cirsimaritin from the plant *Lippia graveolens*, of which extract demonstrated an anti-amoebic activity [36].

Any infection being accompanied by inflammation; the presence of *E. histolytica* in the intestine, not only gradually destroys the intestinal wall but causes an inflammatory reaction which contributes to increased tissue damage. The anti-inflammatory potential of extracts from endophytic fungi 6, 8, 14 and 18 was therefore evaluated.

In the presence of our extracts, we observed a significant drop in the quantity of nitric oxide (NO) produced by macrophages in proportion to the concentration. The inhibitions observed show that our extracts could prevent the migration of cells to the inflammatory site, thus limiting the damage caused by inflammatory cells. Extract 14 inhibited NO production more effectively (IC_{50}=8.56±2.13µg/ml). This result joins that of Pechangou et al in 2022 who had obtained a similar inhibition of NO production in the presence of hydroethanolic extracts of *C. variegatum* (IC_{50}=8.80±4.52µg/ml [37]. This could be due to the capacity of endophytic fungi to produce compounds similar to those of their host plants, making their extracts as active as those of their host plants [38].

The determination of the anti-inflammatory potential of these extracts continued by evaluating the activity of alkaline phosphatase (ALP). Extract 8 had the best capacity to increase the activation of alkaline phosphatase, with an EC_{50}=328.03±16.04µg/ml. This extract could limit the synthesis of certain pro-inflammatory mediators via the dephosphorylation of antigenic motifs by this enzyme [39]. These results agree with those obtained by Medoua et al in 2023 with polyketides isolated from the endophytic fungus Phomopsis sp CAM212 [40].

Finally, we evaluated the effect of these extracts on the activity of NADPH oxidase produced by activated macrophages. The greatest inhibition of NADPH oxidase activity was observed in the presence of extract 6 (IC_{50}=11.78±0.27µg/ml). This result suggests that this extract may be able to modulate phagocytosis through the reduction of the synthesis of reactive oxygen species produced by NADPH oxidase [41]. This result is better than that of Enang II et al., in 2023 with the aqueous extract of *Alchornea cordifolia* (IC_{50}=36.20µg/ml) [42].

CONCLUSION

These findings demonstrated that extracts of endophytic fungi 6, 8, 14 and 18 are potent anti-amoebic and anti-inflammatory agent, thus showing the potential of endophytic fungi to meet the needs of populations while complying with the requirements of international organizations on nature conservation. However, further molecular characterization of active fungi as well as *in silico* and *in vivo* studies are needed to better elucidate the mode of action of those natural compounds.

Author contributions

EAMT, SNP, BEE carried out all experiments reported in the manuscript. SNP, BEE, JPAA and PFM designed the study. All authors read and approved the final manuscript.

Ethical statement

All procedures in this study followed the Cameroon National Veterinary Laboratory guidelines and were approved by the Animal Ethical Committee of the Laboratory of Animal Physiology of the Faculty of Sciences, University of Yaoundé I–Cameroon.

Conflict of interest

The authors declared no conflict of interest.

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