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(RESEARCH ARTICLE)

Mycochemical analysis, anti-inflammatory and cytotoxic activities of *Pycnoporus sanguineus* (L.) Murrill, a medicinal mushroom from Gabon

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Abstract

A mycochemical study followed by an evaluation of anti-inflammatory and cytotoxic activities of *Pycnoporus sanguineus*, a species of lignicole mushroom exploited in traditional medicine in Gabon, was carried out on four extracts. Bioactive compounds were extracted by successive macerations with water, hydro-ethanol (50-50), ethanol solvents and sonication was used for dichloromethane-methanol (50-50) extraction. Agilent LC-MS system was used for the molecular profile of the different extracts and fractions. The fractionation of the extracts was made by flash chromatography and the purification using preparative chromatography. Structures of compounds responsible for the pharmacological activities were determinated by NMR. Cytotoxicity essay was carried out on PNT2 cells and the anticancer activities on A549 and PC3 cell lines.

Two molecules have been isolated from this fungus; namely m/z 301.04 $[M-H]^+$ corresponding to cinnabarinic acid and m/z 287.06 $[M-H]^+$ for which the NMR data were insufficient to confirm the molecular identification. Anti-inflammatory activity of the aqueous extract was stronger (IC50 = 197.82 µg/ml) than niflumic acid (IC50 = 809.27 µg/ml) but weaker than diclofenac (IC50 = 15.28 µg/ml). However, the hydro-ethanolic and ethanolic extracts of *P. sanguineus*, have a weaker anti-inflammatory activity than niflumic acid and diclofenac. About cytotoxicity, the crude extracts and fractions tested have anticancer and cytotoxic activities on A549, PC3 and PNT2 lines. However, these activities are more pronounced in the healthy line PNT2 than in the cancerous lines A549 and PC3.

Keywords: Mycochemical Analysis; Anti-Inflammatory Activity; Anticancer and Cytotoxic Activities; *Pycnoporus Sanguineus*; Gabon

1. Introduction

Non-Timber Forest Products (NWFP) taken from the forests of Central Africa have experienced renewed interest in recent years, according to the number of studies and scientific meetings devoted to them [1-8]. Many people depend directly or indirectly on these resources for their food and medicinal uses, and sometimes to improve their family income [9, 10]. The populations of Gabon exploit around sixty species of edible [11, 12] and medicinal [13-15] mushrooms. Among the medicinal species inventoried in northern Gabon, *Pycnoporus sanguineus* used by these populations to treat painful menstruation is one of the most popular fungal species in the area with a med.UVS of 0.66 [14]. More generally, *P. sanguineus* has been used in empirical medicine in Africa [15] and South America to treat many diseases such as skin lesions, arthritis, gout, sore throat, ulcer, dental pain, fever and hemorrhage [16-17]. It has a high

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potential in reducing blood glucose levels and reduces insulin resistance and the risk of diabetes complications by inhibiting alpha-amylase and alpha-glucosidase [18]. According to Smânia et al. [16], *P. sanguineus* has antifungal, antiradical, antimicrobial, antiviral and cytotoxic activities.

The present work aims to evaluate the anti-inflammatory and cytotoxic activities of *P. sanguineus* after an analysis based on a mycochemical screening of this species.

2. Material and methods

2.1. Collection and identification of the studied fungus

The fungus studied was collected from decaying dead wood in a plantation in Malibé 2 at 172m above sea level (geographical coordinates 0° 34′0′′N and 9° 27′0′′), then described, dried and identified. According to the procedure described by Eyi Ndong *et al*. [12].

2.2. Extractions

The extraction techniques used are maceration and sonication. Maceration for 48 hours was used for successive extractions with water, hydro-ethanolic (50-50) and ethanol solution. Sonication for 3 h was used for dichloromethane-methanol (50-50) extractions.

2.3. Chromatographic analyses

Analytical HPLC runs were carried out using an Agilent LC-MS system comprising an

Agilent 1260 Infinity HPLC hyphenated with an Agilent 6530 ESI-Q-TOF-MS operating in positive polarity.

Full scan mass spectra were acquired in the positive-ion mode in a mass range of m/z 100 to 1200 Da, with the capillary temperature at 320°C, source voltage at 3.5 kV and a sheath gas flow rate at 10 L/min. Capillary, fragmentor and skimmer voltages were set at 3500, 175 and 65 V, respectively. MS/MS acquisition used a collision energy of 30, 50 and 70 eV. A Sunfire® preparative C18 column (150 * 30 mm, i.d. 5 µm, Waters) was used for HPLC separations using a Waters Delta Prep (Waters Co., Milford, MA, USA) consisting of a binary pump (Waters 2525) and a ultraviolet (UV)–visible diode array detector (190–600 nm, Waters 2996). Silica 40 g Grace™ cartridge was used for flash chromatography using an Armen Instrument–Spot Liquid Chromatography Flash apparatus.

TLC analyses were carried out on precoated silica gel 60F254 (Merck), and spots were visualized with U.V and by spraying with freshly prepared sulfuric vanillin. Chemicals and solvents were purchased from Sigma-Aldrich.

2.4. Structural elucidation

2.4.1. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were recorded at 20°C using deuterated solvents in a 400 MHz NMR spectrometer. The chemical shifts of ^{1}H protons (δH) and ^{13}C carbons (δC) in ppm were determined by comparison with tetramethylsilane (TMS), used as a reference molecule [19]. The data obtained was processed with the NMR notebook.

2.5. Anti-inflammatory activity

2.5.1. Protein Denaturation Inhibition Method

The in vitro anti-inflammatory activity of the fungi was carried out according to the protein denaturation inhibition method described by Chandra *et al*. [20] with slight modifications. For this, the reaction medium was composed of 0.1 ml of albumin, 1.4 ml of PBS at pH 6.4 and 1 ml of extract with concentrations ranging from 1000 µg/ml to 62.5 µg/ml. In the control, the extract was replaced with an equal volume of distilled water.

Once prepared, the reaction mixture was incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, the absorbances were measured at 660 nm. Sodium diclofenac and nifluric acid were used as reference molecules. The percentage inhibition was calculated using the following formula:

$$\%=100-(DO\ extract\ /\ DOcontrol)*100$$

2.6. Cytotoxicity essay and anticancer activity

The cytotoxicity essay and the anticancer activity consisted in measuring the viability of the PNT2, A549 and PC3 cells in culture when they were placed in the presence of mushroom extracts. They were made using the MTT (3-(4,5-diMethylThiazol-2-yl)-2,5-diphenyl Tetrazolium bromide) staining method.

This test gives an indication of mitochondrial functioning. It consists of measuring the mitochondrial succinate dehydrogenase activity in living cells. This enzyme, by cutting the tetrazolium ring, transforms MTT, which is yellow in color, into blue formazan crystals [21-22].

Cells from 4 to 5 days of culture were seeded in 96-well plates at a concentration of 5000 cells per well. After 24 h, they were incubated with mushroom extracts (100 µg/ml) or purified molecules (10 mM) dissolved in DMSO for 48 h after which, 20 µl of MTT (5 g/l of MTT in 0.1 M PBS) were added and the cells were again incubated for 1 h 30 min. In the control, the extract was replaced by DMSO.

The mixture (extract-culture medium-MTT) was then eliminated and 200 µl of DMSO were introduced into each well in order to dissolve the formazan crystals formed. The plates protected from light rays by aluminum foil were subjected to shaking at 750 rpm for 5 min and the relative cell viability was determined by measuring the absorbance at 570 nm on a microplate reader (Molecular Devices, Menlo Park, CA, USA). Viability is based on the measurement of the number of living cells that are metabolically active and capable of reducing the tretrazolium ring contained in the tretrazolium salt by their mitochondrial succinate dehydrogenase to formed purple formazan crystals. The intensity of this staining is proportional to the number of living cells during the test. It is quantified by spectrophotometry at 570 nm.

3. Results and discussion

3.1. Studied Mushroom

The species studied was identified as *P. sanguineus* (Fig. 1). This saprotrophic species grows on dead wood, in open forests, or in plantations.

Figure 1 Fruit bodies of *P. sanguineus*. Photograph by Hugues Eyi Ndong

3.2. Main chemical species present in the dichloromethane-methanol extract of Pycnoporus sanguineus.

Chromatographic analysis of dichloromethane-methanol extract of *P. sanguineus* for a 5-100 gradient (water + 0.1% formic acid-methanol) on a normal phase sunfire column is represented by figure 2.

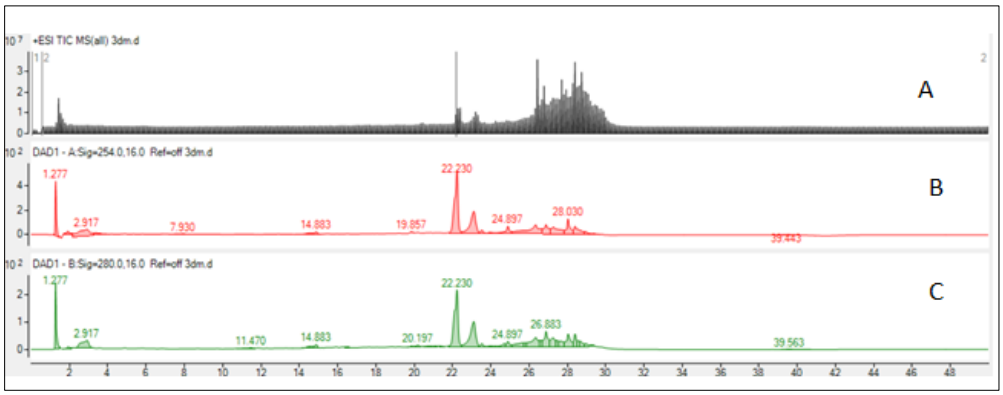

Figure 2 MS2 chromatogram (A), PDA chromatograms (254 nm [B]; 280 nm [C]) of dichloromethane-methanol extract of Pycnoporus sanguineus

This chromatogram (Fig. 1) made it possible to identify the majority ions present in the analyzed extract (Table 1).

Table 1 Main ions present in the dichloromethane-methanol extract of *P. sanguineus*

m/z	Molecular formula (adduct)	Score (%)	Retention time (min)
287,06	$C_{14}H_{10}N_2O_5$ (H+)	96,41	22,28
301,04	$C_{14}H_8N_2O_6$ (H+)	92,15	22,39
285,04	$C_{14}H_8N_2O_5$ (H+)	98,61	23,32
230,24	$C_{14}H_{31}NO$ (H+)	99,91	26,43
249,14	$C_{13}H18N_3O_2$ (H+)	98,74	26,66
318,30	$C_{18}H_{39}NO_3$ (H+)	99,71	26,77
330,30	$C_{17}H_{37}N_4O_2$ (H+)	98,79	27,70
297,24	$C_{16}H_{30}N_3O_2$ (H+)	85,2	28,39
328,31	$C_{20}H_{41}NO_2$ (H+)	83,07	28,737
358,33	$C_{19}H_{41}N_4O_2$ (H+)	98,67	29,083

Two molecules have been isolated from this fungus. These are m/z 301.04 [M-H]+ (Figure 3) corresponding to cinnabarinic acid and m/z 287.06 [M-H]+ (Figure 4) whose NMR data did not allow to elucidate the structure of the molecule, both molecules being of red-orange color which precipitate in the presence of acid.

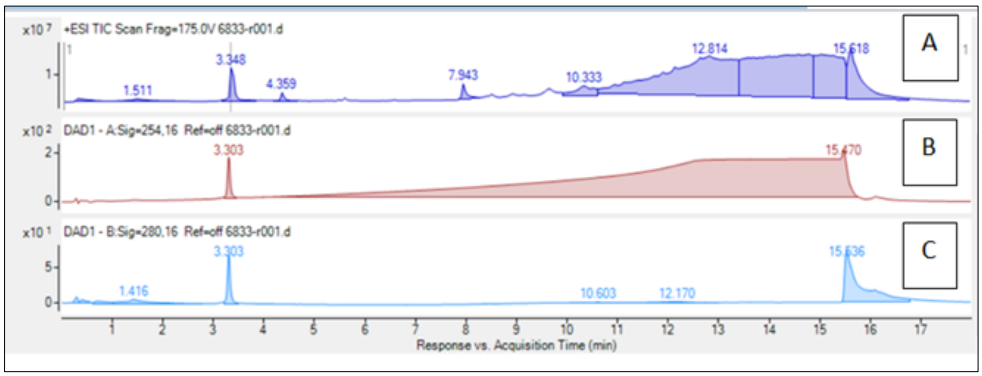

Figure 3 MS^2 chromatogram (A), PDA chromatograms (254 nm [B]; 280 nm [C]) of m/z 301.04 $[M-H]^+$ molecule

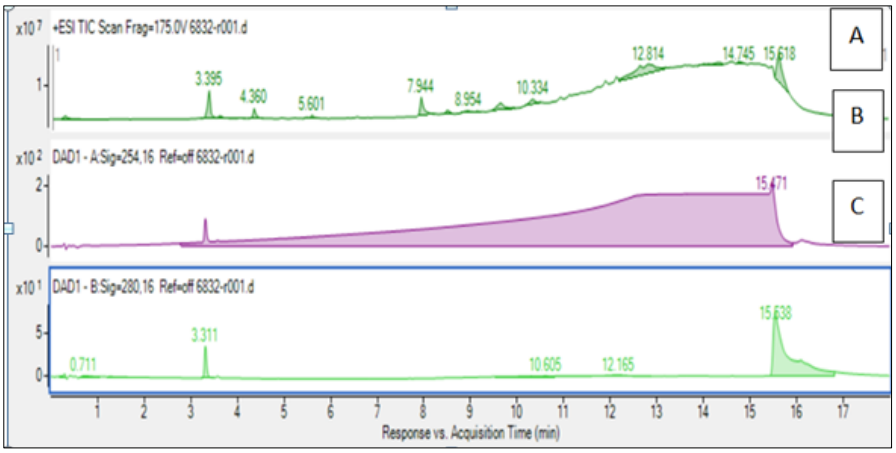

Figure 4 MS^2 chromatogram (A), PDA chromatograms (254 nm [B]; 280 nm [C]) of m/z 287.06 $[M-H]^+$ molecule

In the panel of Pycnoporus sanguineus molecules described in the literature, we find cinnabarinic acid (2-amino-3-oxo-3H-phenoxaine-1,9-dicarboxylic acid) and cinnabarine (2-amino-9-hydroxymethyl- 3-oxo-3H-phenoxazine-1-carboxylic acid), two molecules derived from the 2-amino-phenoxazinone skeleton.

However, these two masses corresponding to the m/z ratios 301.04 and 287.06 do not fragment in the same way at the same collision energies. Moreover, the NMR data from the molecule at m/z equal to 287.06 did not make it possible to elucidate the structure of the molecule. However, the proton NMR spectrum shows a methyl singlet signal at 2.02, a methyl doublet signal 2.30 ppm (1.2 Hz), and a singlet signal from an isolated methylene at 1.29 ppm. To these signals are added the signals of the aromatic protons at 7.46; 7.55 and 7.87 ppm. The NMR data of the cinnabarinic acid here isolated were similar to those of Dias and Urban [23].

3.3. Anti-inflammatory activity

The IC50 values (µg/ml) of the anti-inflammatory activity of the tested extracts, based on the albumin denaturation method are recorded in Table 2.

Table 2 IC50 values (µg/ml) linked to the anti-inflammatory activity of the different extracts of *P. sanguineus*

IC_{50} (µg/ml)			
Mushrooms	**EAQ**	**EHE**	**EE**
P.S	197,82	1359,62	916,61
Niflumic acid	809,27		
Diclofénac	15,28		

All the *P. sanguineus* extracts studied show anti-inflammatory activity. The anti-inflammatory activity of the aqueous extract is stronger (IC50 = 197.82 µg/ml) than that of niflumic acid (IC50 = 809.27 µg/ml) but weaker than that of diclofenac (IC50 = 15 .28 µg/ml). The hydro-ethanolic and ethanolic extracts of *P. sanguineus*, on the other hand, have a weaker anti-inflammatory activity than those of niflumic acid and diclofenac (IC50 = respectively 1359.62 µg/ml and 916.61 µg/ml). This result confirms that of Li *et al*. [24], who found that polysaccharides from the aqueous extract of *P. sanguineus* improved colitis in mice by decreasing the inflammatory disease activity index. Similarly, the study of the anti-inflammatory activity of the ethanolic extract of *P. sanguineus* conducted by Chen *et al*. [25] in an experimental model of colitis induced by sodium dextran sulphate (DSS) showed that this extract induced a decrease in the disease activity index. In addition, Lambertellin isolated from *P. sanguineus* MUCL 51321 exerted an anti-inflammatory effect on lipopolysaccharide-stimulated RAW 264.7 macrophage cells [26]. The activities of the extracts tested are linked to the metabolite composition of these fungi. Indeed, the fatty acids contained in the mushroom are able to support the anti-inflammatory process [27].

In addition, a dosage of the phenolic compounds of *P. sanguineus* (Orango *et al*. [15] revealed their abundance in the treated sample. These phenolic compounds have proven anti-inflammatory properties *in vitro* and *in vivo*.

3.4. Cytotoxicity test

The data collected at the end of these tests are recorded in Table 3.

Table 3 Results of the cell viability test in the presence of crude extracts of P. sanguineus and associated fractions

Cell viability (%)			
Cell lines	A549	PC3	PNT2
Doxorubicin	55±6	70±20	50±4
P.SEDM raw extract	47±24	37±17	19±1
P.S fraction	97±5	90±6	92±3
Doxorubicin	31±4	54±20	40±7
Cinnabarinic acid	89±1	92±9	90±0

The crude dichloromethane-methanol extract of *P. sanguineus* (100 µg/ml) has greater cytotoxic activity than the fraction studied and doxorubicin on the three cell lines tested. However, this cytotoxicity is more felt on the PNT2 healthy cell line. This is in agreement with the work of Piet *et al*. [28] who indicated that an extract of *P. sanguineus* at 200 µg/ml significantly decreased the viability of HT-29 and SW948 cells but also possessed pro-apoptotic properties towards these cancer cell lines; it also had a moderate effect on normal CCD841 CoTr cells. Cinnabarinic acid for its part had weak activities on the three lines. These results are however contradictory with those of Hayashi *et al*. [29] who showed that cinnabarinic acid has a strong induced apoptotic activity resulting from the production of ROS with a loss of mitochondrial membrane potential and a reduction in the activation of capsases.

In the study by Ahmad *et al*. [30], the aqueous extract of the mycelium of *P. sanguineus*, hot and cold, inhibited the growth of cells of the A549 line after 72 hours of incubation. On the other hand, the dichloromethane extract of *P. sanguineus* studied by Usha *et al*. [31] showed low cytotoxicity (IC50>1000 µg/ml) against human breast carcinomas (MCF7).

4. Conclusion

The present study made it possible to isolate two molecules of the studied fungus; m/z 301.04 $[M-H]^+$ corresponding to cinnabarinic acid and m/z 287.06 $[M-H]^+$ whose NMR data did not make it possible to elucidate the structure of the molecule.

Regarding anti-inflammatory activity, all the *P. sanguineus* extracts studied show anti-inflammatory activity; the aqueous extract having a stronger anti-inflammatory activity than that of niflumic acid but weaker than that of diclofenac unlike the other extracts.

As for the cytotoxicity and anticancer activity, the crude dichloromethane-methanol extract of *P. sanguineus* has greater cytotoxic activity than the fraction studied and doxorubicin on the three cell lines tested. Unfortunately, the observed cytotoxicity was not selective for cancer cells; healthy cells (PNT2) were more sensitive than cancer cells. Nevertheless, these data show that *P. sanguineus* could strengthen the scientific database for the search for new bioactive molecules against cancers but also against other diseases linked to oxidative stress.

Compliance with ethical standards

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Author contributions

The data collection and identification of the fungus by Eyi Ndong and Orango Bourdette was supervised by Bourobou Bourobou. The mycochemical analysis by Orango Bourdette was supervised by Obame Engonga, finally all the authors read, accepted and approved the final manuscript.

Disclosure of conflict of interest

The authors declare that they have no potential conflict of interest regarding the publication of this article. In addition, ethical issues have been attested by the authors.

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