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Evaluation of the antifungal and antibacterial activities of crude extracts of three species of *Rigidoporus* (Basidiomycota, Polyporaceae) from Cameroon

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ABSTRACT

This study aimed at assessing the antimicrobial activities of hexane and chloroform crude extracts of three species of *Rigidoporus* including *R. microporus*, *R. ulmarius* and *R. vinctus* on eleven strains of bacteria of which five gram-positive (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Mycobacterium smegmatis*) and six gram-negative (*Enterobacter cloacae*, *Proteus vulgaris*, *Klebsiella oxytoca*, *Klebsiella aerogenes*, *Proteus mirabilis*, *Escherichia coli*), as well three species of human pathogenic fungi including *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus ochraceus*. The assessment was done by determining the Minimal Inhibition Concentration (MIC). Results recorded globally show a strong activity of crude extracts on pathogenic fungi with MIC values ranging from 0.39 to 6.25 mg/mL. This activity was rather weak on all strains of bacteria tested with MIC values ranging from 6.25 to 12.5 mg/mL. More precisely, a strong activity (MIC = 0.39 mg/mL) of extracts of *R. microporus* and *R. vinctus* was recorded on *Aspergillus fumigatus*, a weak activity (MIC = 6.25 mg/mL) of *R. ulmarius* on the same *Aspergillus* species, and rather a strong activity (MIC = 0.39 mg/mL) of extracts of *R. microporus* and *R. ulmarius* on *Aspergillus ochraceus*. These results show that pathogenic fungi are generally much more sensitive to crude extracts of *Rigidoporus* than bacteria. Based on these preliminary and rather interesting results, it clearly appears that carpophores of these three species of *Rigidoporus* could constitute a source of new natural compounds that could be used to manufacture new pharmaceutical products potentially more efficient against some bacterial and fungal infections.

Keywords: *Rigidoporus*, Wood-rotting macrofungi, Crude extracts, Antimicrobial activity, Minimal Inhibitory Concentration (MIC), Cameroon.

INTRODUCTION

Besides higher plants that have been used for millenaries and nowadays still largely used worldwide by peoples of diverse origins in the traditional pharmacopoeia to cure multiple human pathologies, numerous mushrooms species are also known to be used in traditional medicine in some parts of the world and are now reported to also contain compounds with proven efficacy on numerous human diseases. In addition to their activities against fungal and bacterial disease [1-10], which is also the aim of this study, numerous other reports have been published by various researchers on other potential or effective healing properties of mushrooms including their proven cardioprotective [11-13] effect, action in regulating blood pressure [11-14] and action as liver-[12-15] as well as kidneyprotector [12, 13, 16]. Elsewhere, other mushrooms species have rather revealed properties as hypoglycemic [12, 13], anti-inflammatories [12, 13], antioxydants [12, 14], antitumoral [12-14], antiinfectious and anti-HIV [12-14] agents. Numerous antibiotics and antifungal products have been produced by pharmaceutical industries over the past decades to combat infectious diseases. Although these products have most often proven their efficacy, microbes have continued to develop resistance, therefore leading researchers to continue investigating other potential sources of new and more efficient compounds both physiologically beneficial and safe for the human organism [17].

Research works on medicinal properties of natural substances have so far focused essentially on green plants and very little on mushrooms. Over an estimated potential of more than 145 000 mushrooms species of which just about 10% (≈ 14 500 species) described and only about 2500 edible or with medicinal properties, very few have so far been investigated for their genuine pharmaceutical potential. Field works in the tropics have shown that mushrooms including Agaricales and Polyporales are numerous and diversified in the tropical rain forests of Africa, but very few have been reported in published scientific papers [18-21] and are mostly cited for their uses in African traditional pharmacopoeia [22-24].

The largest part of the mycoflora of tropical Africa remains uninvestigated and therefore stands as a very rich potential where new compounds could be identified and used to combat disease resistance to

conventional pharmaceutical products. This research work falls in line with the above mentioned goals by studying the antimicrobial activities of crude extracts of three species of *Rigidoporus* including *Rigidoporus microporus*, *R. ulmarius* and *R. vinctus*. *Rigidoporus* is a genus belonging to the family of Polyporaceae within the Polyporales of the higher Basidiomycetes [25]. Species of this genus offer an interesting model for this study since many other Polyporaceae were reported by several authors as used in the African traditional pharmacopoeia [22-24] to cure several diseases and very few in scientific published papers [4, 18, 19, 20]. Therefore, this study aimed at assessing comparatively the antifungal and antibacterial properties of hexane and chloroform crude extracts of the above mentioned three species of *Rigidoporus* of which two are investigated here for the first time ever. The experiment was carried out by determining and comparing the Minimal Inhibitory Concentrations (MIC) of their crude extracts and thereby determine whether these species could be considered as a new source of potentially more efficient antifungal and antibacterial compounds.

MATERIALS AND METHODS

Mushrooms collection, identification and extraction

Mushrooms samples were made of carpophores of the three species of *Rigidoporus* broken into pieces. These carpophores were mostly collected on tree stumps in Yaoundé, Cameroon and outskirts, and determined according to Ryvarden and Johansen [25]. The three species are saprophytic macrofungi belonging to the Polyporaceae and causing white rot on numerous forests and commercial trees including rubber on which *Rigidoporus microporus* is particularly very destructive. Once carpophores were cut or broken into pieces, they were dried and conserved in the Mycological Herbarium of the Faculty of Science of the University of Yaoundé 1 under the numbers HUY1-DM 1023 for *Rigidoporus microporus*, HUY1-DM 551 for *R. ulmarius* and HUY1-DM 537 for *R. vinctus*. They were first cleaned of impurities and cut or broken into small pieces using a knife or a hammer before being powdered separately in an electric blender. The powder obtained was thereafter conserved in plastic bags labelled differently for the three species.

Test Microorganisms

The microorganisms tested here were the same used by Metsebing *et al*. [26]. They included eleven strains of bacteria and three strains of human pathogenic or toxic fungi, the list of which is given in the above mentioned paper [26] and in the tables below.

Extraction of crude natural substances

Crude extracts of mushrooms were obtained according to the method described by Boonsong and Klaypradit [27] with slight modifications. Dried mushroom samples were milled to powder and extracted using hexane and chloroform. 400 mL of hexane or chloroform were added to 5 g of powder, stirred for 24 hours and filtered using Whatman No. 4 filter paper. The filtrate was evaporated at 40° C using a rotary-evaporator. 0.05 g of each of both extracts were mixed to give 0.1 g hexane: chloroform (1:1) extract that was used for antimicrobial assay.

Preparation of bacterial and fungal suspensions

The test bacterial and fungal suspensions were prepared to the concentration of $1x10^5$ bacteria/mL according to McFarland [28] and $1x10^5$ spores/mL respectively. Bacteria were grown in nutrient broth at 37°C for 12–16 hrs and the fungi strains on RPMI 1640 medium at 30°C for 24 h. These spores were thereafter dipped into the Ringer's solution, the role of which was to stabilize them by temporally inhibiting their germination. The spore suspension was prepared 24 hrs before the tests and kept at 4°C-8°C in the refrigerator.

Determination of the Minimal Inhibitory Concentration (MIC)

The MIC was determined by microdilution according to Eloff [29] with slight modifications. Nutrient Broth culture medium was used for bacteria and RPMI 1640 medium for pathogenic fungi. Briefly, 100 µL aliquots of culture suspension were dispensed into each of the 96 microwells test-plate (Fig. 1), then 100 µL of 25 mg/mL extract solution dissolved in DMSO (25 mg/mL or 100 mg in 4 mL DMSO) were added to line B, column 2 to 11 (Fig. 1). Serial dilutions were successively realized from line B to G by mixing suspension of line B and pipetting 100 µL in wells of line C which were also mixed and 100 µL pipetted in wells of line D and so on and so forth until wells of line F were pipetted into those of line G. 100 µL of mixture was thereafter pipetted out of each well of line G. In this process, the concentration of the mixture in the wells of each line from B to G (Fig. 1) is that of the preceding line divided by two and by so doing, six test concentrations (12.5 mg/mL, 6.25 mg/mL, 3.13 mg/mL, 1.56 mg/mL, 0.78 mg/mL, 0.39 mg/mL) were realized according to Maragesi *et al.* [30]. Wells of line A (A1 to A12) and H (H1 to H12) and columns 1 to 12 (1A to 1H and 12A to 12H) were set as positive and negative controls respectively. The positive control was inoculated with bacterial or fungal suspension only, while only DMSO was added to the negative control wells. Aliquots (100 µL) of each bacterial or fungal suspension were inoculated into the wells to obtain a final volume of 200 µL in each well of the plate. The plates were sealed and incubated at 37°C for 24 hrs for bacteria and 72-84 hrs for fungi at the same temperature. In order to visualize the concentrations showing bactericidal or fungicidal effect in the plates, 10 µL of 2-(4-iodophény)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) 2mg/mL was added to each of the 96 wells and incubated at 37°C for 1 to 5 hrs for bacterial test and 1 to 2 (sometimes until 7) days for fungal test still at 37°C [31-33].

After incubation, the plates were visualized for MIC determination by spotting the concentration line were colour change occurred. In fact, wells with bacteria and fungi still alive turned pink (Fig. 1C) due to reaction of their metabolism products with INT-dye, whereas in wells where the crude extracts concentrations inhibited their growth, the original colour of the crude extract remained unchanged.

RESULTS

The antimicrobial activity of the three species of *Rigidoporus* were assessed by their Minimal Inhibitory Concentration (MIC) values shown in tables 1 and 2 and Figs. 2 and 3. The MIC values recorded clearly show that crude extracts of the 3 species of *Rigidoporus* develop a much higher inhibition activity on pathogenic fungi than bacteria. Table 1 and Fig. 2 compare the MIC values of 3 species of *Rigidoporus* on 11 strains of bacteria and according to the values recorded, *Rigidoporus microporus* shows a generally weak inhibition activity on almost all the 11 strains of bacteria with a MIC of 12.5 mg/mL, except for *Proteus vulgaris* (PV) with a value of 6.25 mg/mL. Concerning *Rigidoporus ulmarius*, it shows the same pattern with a similar MIC value of 12.5 mg/mL for most of the 11 strains of bacteria, except for *Staphylococcus aureus* (SA) and *Enterobacter cloacae* (ECL) recording each a MIC value of 6,25 mg/mL. As concerning *Rigidoporus vinctus*, it equally shows a weak inhibition activity on bacteria, but with MIC values more balanced between 6.25 mg/mL and 12.5 mg/mL among the 11 strains of bacteria.

Table 2 and Fig. 3 compare MIC values of crude extracts of the three species of *Rigidoporus* on three species of human pathogenic or toxic fungi including *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus ochraceus*. With a MIC value of 0.39 mg/mL, some species of *Rigidoporus* show a strong inhibition activity on some pathogenic fungi. According to MIC values in Table 2 and Fig. 3, they include *Rigidoporus microporus* on *Aspergillus fumigatus* and *Aspergillus ochraceus; Rigidoporus ulmarius* on *Aspergillus ochraceus* and *Rigidoporus vinctus* on *Aspergillus fumigatus*. However, the three species of *Rigidoporus* show a weak inhibition activity on *Candida albicans* with a MIC value of 3.13 mg/mL. A weak inhibition activity is equally recorded for *R. ulmarius* on *Aspergillus fumigatus* and *R. vinctus* on *Aspergillus ochraceus* with MIC values of 6.25 mg/mL and 3.13 mg/mL respectively.

Table 1: Minimal inhibition concentration (MIC) of crude extracts of three species of *Rigidoporus* against human pathogenic bacteria

	Minimal Inhibition Concentration (MIC, mg/mL)										
	Gram-positive (+) bacteria					Gram-negative (-) bacteria					
	BS	**EF**	**SE**	**SA**	**MS**	**ECL**	**PV**	**KO**	**KA**	**PM**	**EC**
R. microporus	12,5	12,5	12,5	12,5	12,5	12,5	6,25	12,5	12,5	12,5	12,5
R. ulmarius	12,5	12,5	12,5	6,25	12,5	6,25	12,5	12,5	12,5	12,5	12,5
R. vinctus	12,5	12,5	12,5	12,5	6,25	12,5	6,25	6,25	12,5	6,25	12,5

BS: Bacillus subtilis, EF: Enterococcus faecalis, SA: Staphylococcus aureus, SE: Staphylococcus epidermidis; MS: Mycobacterium smegmatis; ECL: Enterobacter cloacae, EC: Escherichia coli, PV: Proteus vulgaris, PM: Proteus mirabilis, KO: Klebsiella oxytoca, KA: Klebsiella aerogenes.

Table 2: Minimal inhibition concentration (MIC) of crude extracts of three species of *Rigidoporus* against human pathogenic fungi

	Minimal Inhibition Concentration (MIC, mg/mL)		
	Candida albicans	***Aspergillus fumigatus***	***Aspergillus ochraceus***
R. microporus	3,13	0,39	0,39
R. ulmarius	3,13	6,25	0,39
R. vinctus	3,13	0,39	3,13

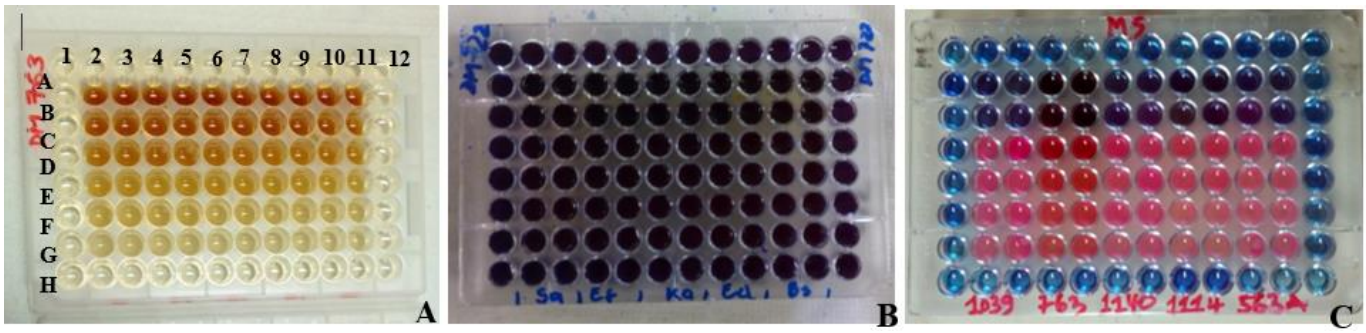
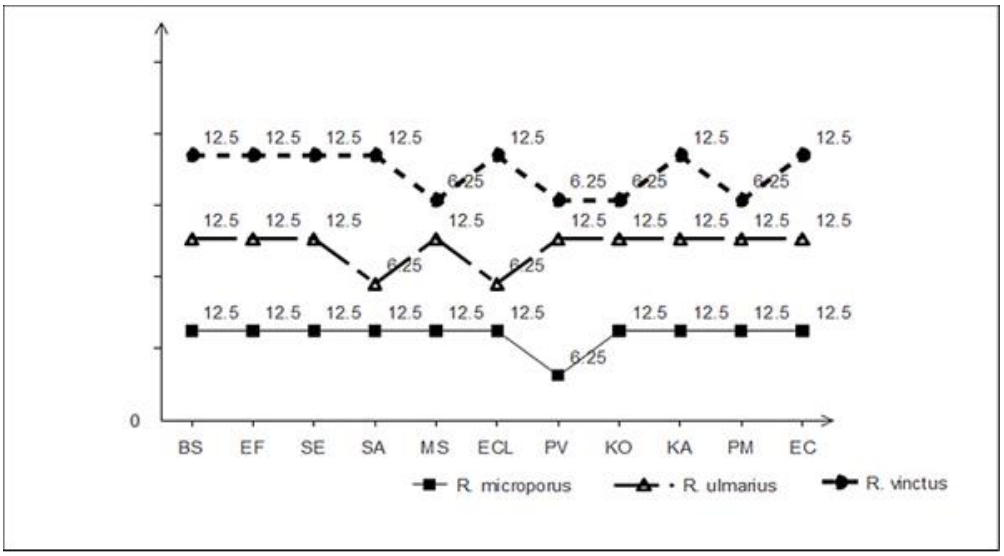


Figure 1: Antibacterial and antifungal tests on the 96 microwells plate. **A.** Plate with wells containing crude extracts at various concentrations and suspensions of bacteria or fungi. **B**. Plate after pipetting drops of the INT-dye in the wells. **C.** Plate ready for reading the MIC values after reaction of the INT-dye with microorganisms suspensions in the wells. **Plate A/B/C.** Positive control wells: A1 to A12 and H1 to H12; Negative control wells: 1A to 1H and 12A to 12H



BS: Bacillus subtilis, EF: Enterococcus faecalis, SA: Staphylococcus aureus, SE: Staphylococcus epidermidis; MS: Mycobacterium smegmatis; ECL: Enterobacter cloacae, EC: Escherichia coli, PV: Proteus vulgaris, PM: Proteus mirabilis, KO: Klebsiella oxytoca, KA: Klebsiella aerogenes.

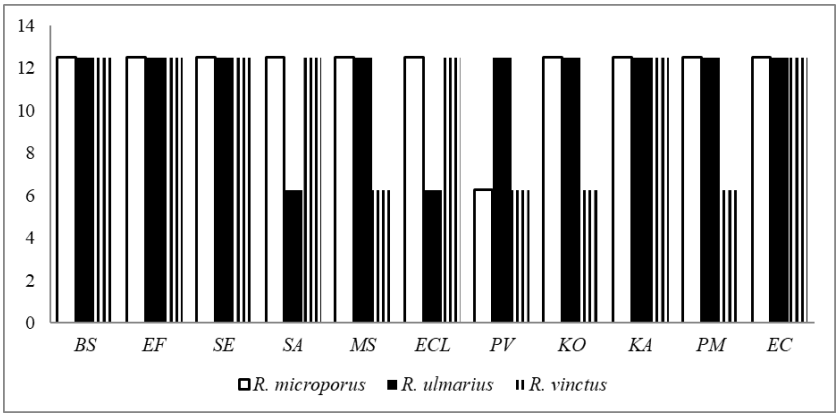


Figure 2: Histograms (below) and curves (above) comparing the MIC of crude extracts of carpophores of 3 species of *Rigidoporus* on 11 strains of human pathogenic bacteria

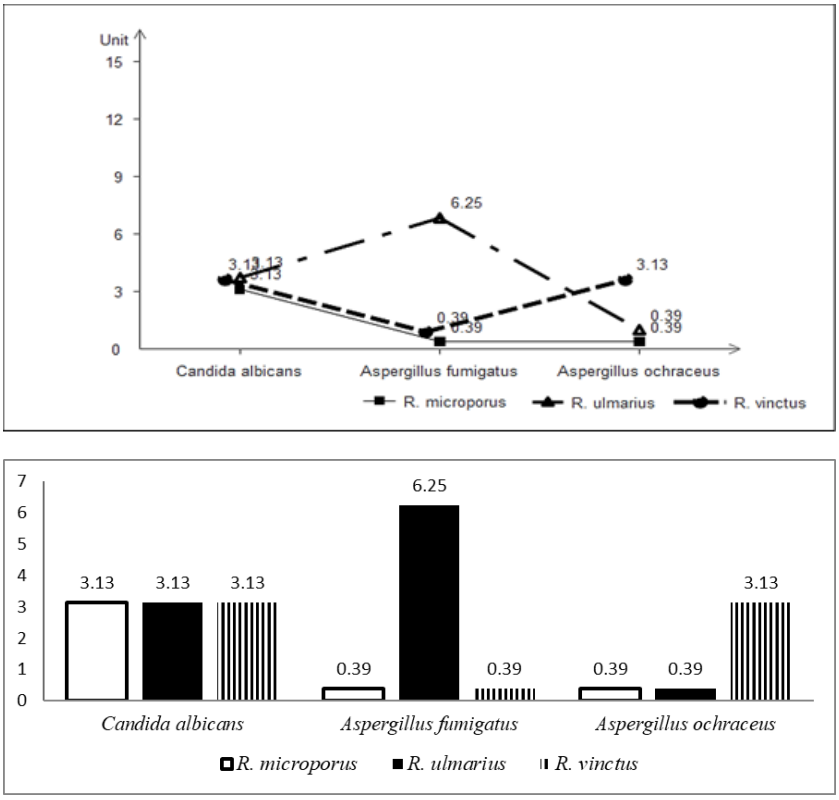


Figure 3: Histograms (below) and curves (above) comparing the MIC of crude extracts of 3 species of *Rigidoporus* on 3 strains of human pathogenic fungi

DISCUSSION

Algiannis *et al.* [34] set up reference values to assess the efficacy of antimicrobial activity of crude extracts. According to the scale designed by these authors, MIC values lower than 0.5 mg/mL are strong inhibitors, those with values between 0.6-1.5 mg/mL are moderate inhibitors and MIC values higher than 1.6 mg/mL are weak inhibitors. Referred to the above mentioned scale, MIC values recorded in this study lead to one key remark that crude extracts of the three species of *Rigidoporus* are strong fungal inhibitors of the three species (*Candida albicans*, *Aspergillus fumigatus*, *Aspergillus ochraceus*) of human pathogenic fungi and rather weak inhibitors of the eleven strains of bacteria.

Among the three species of *Rigidopus* tested in this study, only *R. microporus* has been once tested before by Falade *et al.* [35] for its antimicrobial potency. Although these authors [35] tested mostly different bacteria and microfungi strains, their results generally corroborate those of our study, considering that they equally demonstrate at various level of efficacy the antimicrobial activity of *R. microporus* on the tested microorganisms. Concerning *R. ulmarius* and *R. vinctus*, they are tested in this study for the first time ever to assess their antimicrobial potential. In a rather different and also important scientific domain, it is worth remarking that *R. microporus* is also well known as a white rot macrofungus causing devastating effects in rubber plantations and other forests trees. Elsewhere it is also worth mentioning that among the three species of *Rigidoporus* tested, *R. ulmarius* is much more than the two others (*R. microporus* and *R. vinctus* are typically tropical polypores) reported also in the European continent where, according to Ryvarden and Melo [36], a collection of this species measuring about 1.5 m in diameter was reported in the Royal Botanic Garden, Kew, UK, and based on this extraordinarily conspicuous size was assumed to be the largest polypore ever recorded worldwide.

In a recent study, Metsebing *et al.* [26] used also MIC values to assess for the first time in a comparative manner the antifungal and antibacterial activities of crude extracts of carpophore of *Pleurotus*

tuber-regium on the one hand and its self-prefabricated sclerotium on the other hand. The authors showed that much more than its carpophore, the sclerotium of *Pleurotus tuber-regium*, equally as the three species of *Rigidoporus* tested in this study, stands as a strong fungal inhibitor on the same three human pathogenic fungi (Table 2) and rather also a weak inhibitor on the same eleven strains (Table 1) of human pathogenic bacteria.

In another study of antibacterial and antifungal activities of wild mushrooms using as well MIC values for assessment, Chelela *et al*.. [21], included besides crude extracts of mushrooms, Gentamycin and Fluconazole as positive control for antibacterial and antifungal tests respectively. The MIC values recorded by the authors ranged from 0.0015 to 0.0061 mg/mL for Gentamycin according to the pathogenic bacteria and from 0.003 to 0.006 mg/mL according to the pathogenic fungi. Referred to the scale of Algiannis *et al.* [34], these synthetic antibacterial and antifungal agents could be ranked as very strong inhibitors equally as crude extracts of the three species of *Rigidoporus* on the three species of pathogenic fungi tested (Table 2 and Fig. 3), but different from the tests results of these crude extracts on the eleven strains of bacteria (Table 1 and Fig. 2) on which they reacted rather as weak inhibitors. However, the MIC values of the extracts of the three species of *Rigidoporus* could eventually be improved by testing different solvents and varying the concentrations of the extracts.

Although few works have so far been published on antimicrobial activity of mushrooms of tropical Africa in general and Polyporales in particular, we can remark that MIC values recorded in the present study are more or less close to those obtained in similar works carried out with crude extracts of edible and/or toxic mushrooms [9, 10, 20, 21].

CONCLUSION

Considering on the one hand that two (*Rigidoporus ulmarius* and *R. vinctus*) of the three species of *Rigidoporus* are here investigated for the first time as far as antifungal and antibacterial activities are concerned, and on the other hand that the three species showed interesting results particularly for their strong antifungal activities, these results confirm that hexane and chloroform crude extracts of carpophores of the three species of *Rigidoporus* contain among others compounds, molecules with antimicrobial properties against some human pathogenic fungi and bacteria. Our results therefore naturally stand as an outstanding contribution in the search of tropical species of mushrooms in general and Polyporales in particular showing antifungal and antibacterial properties. In future prospect, considering that these promising results were obtained only with crude extracts of the three species, more refined investigations could be planned in order to accurately identify and separate compounds of the crude extracts that are active as antifungal and antibacterial molecules against the strains of bacteria and fungi tested, the ultimate goal being to manufacture new and more efficient pharmaceutical products to combat microbes resistance as well as current or new infectious diseases caused by the above mentioned strains of human pathogenic fungi and bacteria.

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