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Comparative Study of the *in vitro* Antibacterial Activity of Extracts of Two *Penicillium oxalicum* Strains on the Growth of Multi-resistant Bacteria

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Abstract In order to discover new antibiotics, microorganisms, in this case fungi, are explored. However, the composition of secondary metabolites and their biological activity can be influenced by their habitat. Thus, the objective of this study was to compare the antibacterial activity of two *Penicillium oxalicum* strains, one from the rhizosphere of the *Solanum lycopersicum* crop (Pos) and the other from the leaves of *Solanum lycopersicum* (Poe). The antibacterial activity was performed on six (6) clinical multidrug resistant strains and two (2) reference strains. The agar diffusion and Muller-Hinton liquid methods were used for susceptibility testing and determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), respectively. For the susceptibility test, the Pos extract was active on all strains tested, whereas the Poe extract had a low activity on *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* 931/18. The MIC and MBC of the Pos extract ranged from 0.156 to 5 mg/mL. The lowest MIC and MBC values were observed with *K. pneumoniae* 815/18 while those of Poe extract ranged from 2.5 to 5 mg/mL. And the lowest MIC and MBC value was observed with *S. aureus* ATCC 25923. The Pos extract gave the best antibacterial activity showing that, fungi from the rhizosphere would therefore be the best candidates for antibiotic research.

Keywords: *Penicillium oxalicum, habitat, antibacterial activity, acetate extract, multi-resistant bacteria*

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1. Introduction

Since the advent of antibiotics, there has been a marked improvement in the quality and duration of life [1]. Unfortunately, the overuse of antibiotics in both animals and humans has resulted in the adaptation of bacteria to these substances [2]. Several bacteria can be resistant to one or more molecules, hence the notion of multidrug resistant bacteria (MDR). Among the latter, methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum ß-lactamase-producing Enterobacteriaceae (EBLSE) are the most worrying, given their pathogenic power [3]. In Côte d'Ivoire, the rate of MRSA was 39% in hospitalised patients in 2011 [4] and the frequency of EBLSE, from 5.3% in 2005, rose to 24.6% in 2012 [5,6]. This alarming and worrying situation constitute a real public health problem. To cope with this, the search for new molecules is imperative [7] as the most recent antibiotic molecules are derived from the synthesis of existing molecules [8]. Microorganisms remain the ideal biological material for obtaining new compounds. Among these, fungi are an interesting group. Indeed, they are responsible for the production of 22% of active antibacterial molecules on an industrial scale [9]. However, the selection of fungi that produce antibiotic molecules can be long and tedious. One of the selection criteria is the habitat. Indeed, several authors have reported that the yield, the composition of secondary metabolites and especially the biological activity can be influenced by the habitat of the fungus [10,11,12,13]. The genus *Penicillium*, a ubiquitous fungus known for its production of antibacterial compounds, provides a model for understanding the influence of habitat on the ability to produce antibacterial compounds [14,15,16,17]. The objective of the present study was therefore to compare the antibacterial activity of extracts of two strains of *Penicillium oxalicum*. One strain isolated from the rhizosphere of the *Solanum lycopersicum* crop and another isolated from the leaves of *Solanum lycopersicum* of the same crop on the growth of multi-resistant bacteria.

2. Materials and Methods

2.1. Fungal Material

Two strains of *Penicillium oxalicum were* studied. The first strain, coded Pos, was isolated from the rhizosphere of the *Solanum lycopersicum* crop as described previously by [18]. The second strain, coded Poe, was isolated from the leaves of *Solanum lycopersicum* at the same site where the first strain was isolated and provided by the Laboratory of Bacteriology and Virology of the UFR of Medical Sciences (Felix Houphouët-Boigny University).

2.2. Bacterial Material

The strains to be tested came from the Bacteriology and Virology Unit of the Institut Pasteur of Côte d'Ivoire (IPCI). These were two (2) reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923) and six (6) multi-resistant strains presented in Table 1.

2.3. Preparation of Fungal Extracts

For the extraction of metabolites, each *Penicillium oxalicum* strain was pre-cultured on PDA medium and incubated at 28°C for 7 days. From the fungal cultures obtained, a suspension of spores was made in sterile distilled water. One millilitre (1mL) of suspension was transferred to rice medium. In total, the fungal suspension was spread on ten plates of rice medium and incubated at 28°C for 21 days for each of the fungi. At the end of the incubation period, the cultures were harvested and then macerated for 24 h under agitation at room temperature (approximately 28°C) in 500 mL of ethyl acetate. The macerates obtained were filtered three times on cotton wool and once on wattman paper. The recovered filtrates were concentrated using a rotary evaporator at 45°C. The extracts obtained were respectively the crude extract of Pos and Poe**.**

2.4. Sterility Test of Pos and Poe Extracts

A mixture of 0.1 g of the test extract in 10 mL of thioglycholate broth was incubated at 37°C for 24 h. The mixture was then plated onto a Petri dish containing the regular agar and incubated at 37°C for 24 h. The substance was declared sterile if no colonies were visible in the agar plate [19].

2.5. Evaluation of the Antibacterial Activity of the Extracts

The susceptibility of the strains to the fungal extracts was tested by the agar diffusion technique. By swabbing, Mueller Hinton media was inoculated. Using a sterile cookie cutter, wells of approximately 6 mm diameter were formed in the agar. Each well received 50 µL of the test substance at concentrations of 5; 2.5; 1.25 and 0.625 mg/mL. For Amoxicillin/clavulanic acid (AMC, 20/10 µg) and ampicillin (AMP, 10 µg) which served as positive controls, each disc received 20 µL of the antibiotic. The negative control was 10% dimethylsulfoxide (DMSO). After 15 min of diffusion at laboratory temperature, the Petri dishes were incubated at 37 °C for 18-24 h. Subsequently, the action of the extracts was assessed by measuring the diameter (in mm) of the zone of inhibition around each cup with a ruler [20]. Interpretation was done according to Ponce *et al* [21]. Based on the inhibition diameter, the strain is classified as:

Resistant: diameter less than 8 mm;
Sensitive: diameter between 9 and 14 mm;
Very sensitive: diameter between 15 and 19 mm,
Extremely sensitive: diameter greater than 20 mm.

2.6. Preparation of the Inoculum

The bacterial inoculum was prepared from young colonies less than 24 h in Mueller Hinton Broth (MHB). Using a platinum loop, one to two isolated colonies of the bacterial culture were picked and homogenised in 10 mL of the broth. The homogenate was then incubated at 37°C for 3-5 h to obtain a pre-culture. A volume of 0.1 mL or 1 mL was taken for Enterobacteriaceae and Staphylococci respectively and diluted in a tube containing 10 mL of sterile MHB. This bacterial suspension obtained is estimated to be about 10^6 cells/mL and was the 10^0 dilution or pure *inoculum* [22].

Table 1. Phenotypic profile of bacterial strains

Bacterial strains	Code	Origins	Antibacterial profiles
E. coli ATCC	25922	Sr	AMXS, AMCS, TICS, TCCS, PIPS, CFS, FOXS, CTXS, CAZS, ATMS, GMS, TMS, KS, ANS, NETS, TES, CSS, SXTS, NAS, PEFS, CIPS
E. coli	937/18	Pus	ESBL, fluoroquinolone resistant
E. coli	942/18	Pus	ESBL, fluoroquinolone resistant
S. aureus ATCC	25923	Sr	PS, AMS, AMXS, AMCS, TICS, TCCS, PIPS, CFS, OXS, CTXS, CAZS, ATMS, GMS, TMS, KS, SPS, LS, TES, CIPS
S. aureus	931/18	blood	Kana R, Multi-resistant
S. aureus	934/18	blood	Kana R, Multi-resistant
S. aureus	1872	blood	MRSA, Multi-resistant,
K. pneumoniae	815/18	urine	ESBL

ATCC: American Type Culture Collection, Sr: Reference strain, R: Resistant, S: Susceptible, TZP: Piperacillin, TIC: Ticarcillin, TCC: Ticarcillin + Clavulanic acid, ATM: Aztreonam, CAZ: Ceftazidine, CF: Cefsulodine, SS: Sulfonamide, IPM: Imipenem, GM: Gentamycin, TM: Tobramycin, AN: Amikacin, NET: Netilmicin, CIP: Ciprofloxacin, CS : Colistin, FOS: Fosfomycin, C: Chloramphenicol, RA: Rifampicin, TE: Tetracycline, AMX: Amoxicillin, AMC: Amoxicillin + clavulanic acid, PIP: Piperacillin, CF: Cephalothin, TM: Tobramycin, NET: Netilmicin, NA: Nalidixic acid, PEF: Pefloxacin, Kana : Kanamycin , CTX: Cefotaxime, OX: Oxacillin.

2.7. Inoculum Count

To perform the count, the pure bacterial *inoculum* (10^0) was diluted to 10^{-4}. Four dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were obtained. The pure inoculum and the four successive dilutions were plated with a calibrated loop of 2 µl per 5 cm long streak on Mueller Hinton agar and incubated at 37 °C for 24 h. This preparation constituted plate A.

2.8. Determination of the Minimum Inhibitory Concentration (MIC)

A series of eleven (11**)** haemolysis tubes was prepared. This series consisted of **9** experimental tubes numbered from T_1 to T_9 and 2 control tubes including the growth control tube (T_C) and the sterility control tube (T_S). Each experimental tube received 1 mL of the bacterial inoculum. Then, 1 mL of fungal extract of well-known concentration according to the prepared concentration range was added to these same tubes. Thus, tube 1 received a concentration of 10 mg/mL; tube 2, a concentration of 5 mg/mL; and so, on up to tube 9. The control tube (T_C) received 2 mL BMH inoculum and the sterility tube (T_S) received 1 mL fungal extract and 1 mL sterilised BMH (without bacterial suspension). These tubes were then incubated at 37°C for 18-24 hours. The results were read with the naked eye in daylight. The clarity of the medium implies the antibacterial effect of the extract or compound tested, while the presence of cloudiness indicates its ineffectiveness (sign of bacterial growth) [23,24].

2.9. Determination of the Minimum Bactericidal Concentration (MBC)

For its determination, the contents of the tubes in which no cloudiness was observed were taken and plated on Muller-Hinton agar on 5 cm strips using a 2 µl calibrated loop. This Petri dish is called (B). This plate is also incubated at 37°C for 24 hours. The number of germs obtained on the streak of the 10^{-4} dilution of dish A is compared to that of each streak of dish B. The concentration of the test tube with fewer or equal numbers of germs on its streak than the 10^{-4} dilution will be the MBC. Thus, the extract concentration of the first test tube for which the number of germs on its streak is less than or equal to that of the 10^{-4} dilution will correspond to the MBC [25].

2.10. Statistical Analysis

Statistical analysis was done using ANOVA-one way followed by the Tukey test for multiple comparison between the control and the tests, and between the tests. Values of $P \leq 0.05$ were considered statically significant. All results were analysed using GraphPad Prism 5.0 statistical analysis software.

3. Results

3.1. Sensitivity Tests

Sterility tests showed that the Pos and Poe extracts presented no signs of contamination after three readings at 24-hour incubation intervals.

3.2. Diameters of the Inhibition Zones

Table 2 shows the different diameters of the inhibition zones of the *Penicillium oxalicum* strain extracts. The results showed that the crude Pos extract inhibited the growth of all the bacterial strains tested. On the other hand, the crude Poe extract proved to be weakly active on two of the bacteria tested. Indeed, the largest zones of inhibition of the Pos extract were 21.00±1.0 mm, 19.67±0.57 mm and 18.33±0.57 mm respectively on *K. pneumoniae*, *S. aureus* ATCC and *S. aureus* 1870. However, this extract was not very active on *E. coli* 937/18 (8.00±0.0 mm). As for the Poe extract, on *S. aureus* ATCC the zone of inhibition was 10.00±0.0 mm and 8.00±0.0 mm on *S. aureus* 931/18.

3.3. Determination of Antibacterial Parameters (MIC and MBC)

For the POs extract, the antibacterial parameters ranged from 0.156 to 5 mg/mL. The lowest values were obtained with the *K. pneumoniae* strain. For this germ, the MIC was equal to the MBC (0.156 mg/mL). For the POe extract, the antibacterial parameters ranged from 2.5 to 5 mg/mL. The lowest values were observed with the *S. aureus* ATCC strain. For this bacterium, the MIC was equal to the MBC (2.5 mg/mL). The effect of the POs and POe extracts on the bacterial strains was evaluated by the MBC/MIC ratio. This ratio is less than or equal to 2 for all strains tested. Based on this ratio, both extracts had bactericidal effects. The results of the antibacterial parameters of the POs and POe extracts are shown in Table 3.

Table 2. Diameter of inhibition zones (mm) produced by Pos and Poe extracts against bacteria tested

Substances tested	Concentrations	Bacterial strains							
		S. aureus ATCC	*S. aureus* 931/18	*S. aureus* 934/18	*S. aureus* 1870	*E. coli* ATCC	*E. coli* 937/18	*E. coli* 942/18	*K. pneumoniae* 815/18
Ampicillin	10 µg	19	-	-	-	22	-	-	-
Amoxicillin /clavulanic acid	20/10 µg	35	15	19	16	28	-	13	16
POs extract	5 mg/mL	19,67±0,57	18,00±0,0	16,00±0,0	18,33±0,57	15,00±1,0	08,00±0,03	14,00±0,0	21,00±1,0
POe extract	5 mg/mL	10,00±0,0	08,00±1,0	-	-	-	-	-	-

(-): no zone of inhibition.

Table 3. Antibacterial parameters of POs and POe extracts

Extracts	Parameters antibacterial	Gram-negative bacteria				Gram positive bacteria			
		E. coli ATCC 25922	*E. coli* 937/18	*E. coli* 942/18	*K. pneumoniae* 815/18	*S. aureus* ATCC 25923	*S. aureus* 931/18	*S. aureus* 934/18	*S. aureus* 1870
POs	MIC (mg/mL)	0,625	5	0,625	0,156	0,312	0,312	0,625	0,312
	MBC (mg/mL)	0,625	5	0,625	0,156	0,312	0,312	0,625	0,312
	MBC/MIC	1	1	1	1	1	1	1	1
POe	MIC (mg/mL)	ND	ND	ND	ND	2,5	5	ND	ND
	MBC (mg/mL)	ND	ND	ND	ND	2,5	5	ND	ND
	MBC/MIC	ND	ND	ND	ND	1	1	ND	ND

ND: not determined; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

4. Discussion

The work consisted of studying the antibacterial activity of extracts of two strains of *Penicillium oxalicum*, one from the rhizosphere of the tomato crop and the other from the tomato leaves of this crop, on the *in vitro* growth of multi-resistant bacteria. The results of the antibacterial activity showed that the POs extract inhibited the growth of almost all the bacteria tested, except for the *E. coli 937/18* strain, for which the inhibition diameter was less than 10 mm. POe extract inhibited the growth of only two bacteria (*S. aureus* ATCC and *S. aureus* 931/18). The analysis of the MICs obtained was consistent with the diameters of the inhibition zones. Indeed, the POs extract that induced the largest diameter of inhibition presented the smallest. The greater efficiency observed with the POs extract can be explained by the habitat of the fungus. Indeed, the latter comes from the soil where interactions are strong due to the high density of the microbial population [26]. In addition, farmers in Côte d'Ivoire use several types of fertilisers for soil fertilisation: organic fertiliser and poultry manure [27,28]. The use of poultry manure on soil, in addition to having a beneficial effect on the soil, constitutes a danger because it contributes to the increase of multi-resistant bacteria populations in the soil [29,30]. *Penicillium oxalicum* living in this rhizosphere where the parasitic pressure is dense would have produced molecules called specialised metabolites which are a means of communication and/or a weapon in this competition for space and nutrients. Indeed, work by Gallo *et al*., [31] and Schroeckh *et al*., [32] has shown that fungi produce metabolites either for chemical defence or for competition for substrates. For the biosynthesis of secondary metabolites, fungi use different genes called clusters [33,34]. For example, according to the studies of Gneho *et al*., [18], the extract of POs contains phenolic compounds and quinones. The antimicrobial activity of these secondary metabolites has been demonstrated by several authors [35]. Concerning the mechanism of action of phenolic compounds, they act by mechanisms other than those of the usual antibiotics. This involves the deprivation of microbial cells of metal ions or by non-specific interactions, including the establishment of hydrogen bridges with cell wall proteins [36]. For quinones, they could act as antibiotics either by interfering with bacterial respiratory enzymes or by inhibiting the synthesis of essential cellular components [37]. On the other hand, the ineffectiveness observed on E. coli 937/18 would be due to the production of several types of enzymes (beta-lactamases) by the latter [38]. As for the POe extract, the low activity observed can be explained by the fact that *Penicillium oxalicum* from the leaves of *Solanum lycopersicum* comes from a low-interaction environment. Indeed, host plants protect endophytic fungi against different types of biotic and abiotic stress [39,40]. On the other hand, the low phenolic compound content and the absence of quinones in the POe extract would explain this low activity observed. In the present study, POe extract was active on one of the two kanamycin-resistant strains of *Staphylococcus aureus*. This would be explained by the fact that the resistance of Staphylococci to kanamycin is expressed by several phenotypes (K, KT and KTG) depending on the enzyme that catalyses the reaction [41]. Also, sensitivity and resistance are dependent on the type of phenotype [42].

5. Conclusion

The crude extract of *Penicillium oxalicum* from the rhizosphere of tomato showed the best antibacterial activity. The results showed that this strain produced antibacterial compounds probably due to a strong interaction between *Penicillium oxalicum* and other rhizosphere microorganisms. Soil-borne fungi, in this case those from the rhizosphere, may offer better prospects for exploring new avenues of research into new antibiotic compounds in the fight against antibiotic resistance.

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