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BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS FROM *PENICILLIUM WAKSMANII* ISOLATED FROM MOSQUITOES VECTORS OF TROPICAL DISEASES

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ABSTRACT

Considering that *Penicillium* genus are capable to produce a diversity of secondary metabolites, this work describes the production of bioactive metabolites from *Penicillium waksmanii* Zalessky, using fifteen strains isolated from mosquitoes vectors of tropical diseases. Strains were fermented in malt extract for seven days and extracted with ethyl acetate. All extracts were tested against bacteria and yeast, being active for at least one microorganisms used. Chemical and biological investigation on the ethyl acetate extract of this strain resulted in the isolation of two compounds identified by NRM technique: citrinin and 2-(4-butyl-phenyl)-ethanol.

KEYWORDS: *Penicillium waksmanii*, biological activity, secondary metabolites**,** mosquitoes.

1. INTRODUCTION

Historically, natural products derived from plants have been extensively used in human medicine forming a significant source of drugs in current use, particularly in the anticancer and antihypertensive therapy.[1,2] It was the discovery, and further re-isolation and clinical studies of the penicillin in the early 1940's that launched pharmaceutical industries and research groups into the search of new antibiotics originated from microorganisms, leading to

the isolation of tetracycline, cephalosporin, erythromycin, and vancomycin among other compounds, which are still in use today.[3]

Nonetheless, there has currently been a major concern of the medical community in relation to the emergency of bacterial resistance to the available antibiotics therefore a need for continuing search for novel compounds with antibiotic properties.[4]

Natural products have been the main source of anticancer drugs for the past 30 years comprising 50 % of the drugs used in cancer chemotherapy; because this disease continues to spread with increasing incidence worldwide, anticancer drug discovery continues to be an active area of research.[5]

Fungi are now representing the second most significant and under exploited source of bioactive natural products with a broad range of agricultural and therapeutical applications.[6-8] *Pencilllium* species, among them, have produced a great number of metabolites with a wide array of biological activities including cytotoxic,[9-13] antimicrobial,[14-16] anti-cholinesterasic,[17,18] among other activities.

Despite the evident success of drug discovery through chemical studies undertaken with microfungi isolated mainly from soil samples, there has lately been a great concern about the difficulty of finding new pharmaceutical compounds from microbial sources. Consequently it has been pointed out the importance to study fungal species isolated from unexplored ecological niches for their potential to produce new bioactive natural products.[19] Therefore, we decided to investigate the potential of *Penicillium* spp., a genus that have been frequently isolated from various insects to produce pharmaceutically active secondary metabolites.[20-22] The principal aim of this study was to investigate the *in vitro* antimicrobial activity of the ethyl acetate extracts produced by *Penicillium waksmanii* Zalessky, isolated from adults and larvae mosquitoes vectors of tropical diseases in Brazil.

MATERIALS AND METHODS

General Procedures

Culture medium components, extraction solvents, column grade silica gel and thin layer chromatography silica gel plates were purchased from Merck®. Positive controls and dye reagents were obtained from Sigma-Aldrich®.

Fungal Fermentation

Following published protocols for the isolation of entomopathogenic fungi,[21] fifteen isolates belonging to *Penicillium waksmanii* were obtained from adults and larvae of diseased insects (**Table 1**) collected in Rio de Janeiro, Brazil. Taxonomic characteristics of the isolates were based on cultures grown on Czapek yeast extract agar and the species identification following the method described by Pitt, 2000.[23] All isolates studied were preserved on malt extract agar (MEA) slants and in sterile distilled water in glass screw-top tubes and stored at 4 ºC at the Taxonomy, Biochemistry and Fungal Bioprospecting Laboratory at Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. Fungal isolates were subjected to liquid fermentation for seven days under constant shaking (120 rpm) according to published protocols Rodrigues, 2005.[18] Briefly, Erlenmeyer flasks (250 mL) containing 50 mL of malt extract (20.0 g/L malt extract, 1.0 g/L peptone, 20.0 g/L glucose) were inoculated with three small pieces (ca. 5 mm diameter) of actively growing cultures of all the investigated isolates. The culture broth was filtrated and extracted with ethyl acetate (EtOAc), dried with anhydrous sodium sulfate, filtered and concentrated *in vacuum*. The crude extracts were stored in the freezer for further studies.

Isolation of compounds

All extracts were tested for antimicrobial activity and only one EtOAc extract was selected to follow the investigation. An aliquot of 342,0 mg was taken from crude Pw13 extract and submitted to silica gel Merk® 60H (70-230 mesh) column chromatography and eluted with hexane, hexane-ethyl acetate at increasing proportions, ethyl acetate 100%, ethyl acetate-methanol 50% and methanol 100%. The factions were monitored by thin layer chromatography (TLC), silica gel 60 PF254 (Merck®), n-hexane: ethyl acetate (6:4 v/v) was used as the eluent. The separation led to a number of fractions from where two main fractions were studied by NMR

NMR Analysis

The NMR data were collected using a Varian UNITY-300 NMR spectrometer (1H NMR at 300 MHz and 13C NMR at 75 MHz) and Bruker DRX-400 MHz (1H NMR at 300 MHz and 13C NMR at 100 MHz) spectrometer with 5 mm sample tubes, TMS as internal reference and $CDCl_3$ as solvent. The fractions F6 and F8 from Pw13 extract were available only NMR analysis.

F8 (**c**ompound **1**): yellow crystals, soluble in organic solvents ($CHCl_3$, EtOAC, EtOH) and insoluble in water, IUPAC: (3*R*,4*S*)-8-hydroxy-3,4,5-trimethyl-6-oxo-4,6-dihydro-3*H*-isochromene-7-carboxylic acid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ8.27 (1H,s), 4.79 (3H, q, J 6.8 Hz), 3.00 (3H, q, J 6.4 Hz), 1.36 (1H, d, J 6.8Hz), 1,24 (1H, d, J 7.2Hz), 2,04 (3H, s), 13.13 (1H, s), 15.88 (1H, s). ^{13}C NMR (CDCl3, 100 MHz): δ 162.7 (C1), 81.6 (C3), 34.6 (C4), 138.9 (C4a), 123.1 (C5), 183.8 (C6), 100.3 (C7), 177.2 (C8) 107.4 (C8a), 18.3 (C9), 18.1 (C10), 9.6 (C11), 174.5 (C12);

F6 (compound **2**): brown solid, soluble in chloroform and ethyl acetate, IUPAC: 2 - (4-butyl-phenyl)-ethanol. ^{1}H NMR ($CDCl_3$, 300 MHz):3.81 (2H, t, J 6.7 Hz), 2.79 (2H, t, J 6.7 Hz), 7.08(1H, d, J 8.5Hz), 6.78(1H, d, J 8.5 Hz), 2.33 (2H, t, J 8.1 Hz), 1,73 (s), 1.25 (s), 0.85 (2H, t, J 8.2 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 63.9 (C1), 38.5 (C2), 130.2 (C4/C8), 115.7 (C5,C7), 32.08 (C9), 29.5(C10), 22.8 (C11), 14.1 (C11).

Antimicrobial Assay

The bioautographic TLC agar-overlay method was used as a preliminary test of the fungal extracts and fractions activity. The assays followed the procedure described by Rahalison, 1991,[24] to detect the inhibitory activity against bacteria of medical importance: *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923). Prior to the assay, all bacteria strains were cultured on Müeller-Hinton (MH) medium to ensure purity and viability.

Briefly, crude extracts and fractions were dissolved in AcOEt and MeOH at a concentration of 2 mg/mL. Ten microliters of these solutions were applied on TLC plates (silica gel 60F_{254}, Merck®) as previously described by Corrado and Rodrigues 2004.[25] Plates were covered with molten MH medium containing 100 µl of bacterial inoculum containing 10^6 CFU/mL and incubated for 24 h at 37 ºC. Chloramphenicol was used as positive control. After incubation, the plates were sprayed with a 0,25% aqueous solution of methylthiazolyltetrazolium chloride (MTT), and incubated for another 2 h. White inhibition zones against a purple background on the plates were indicative of antimicrobial activity. The activity was evaluated as diameter (mm) of the inhibition zones.

Determination of minimum inhibitory concentration (MIC)

The bioactivity test with bacteria and yeast were conducted following the CLSI protocols.[26,27] The bacteria species in this study, *Escherichia coli* (ATCC25922),

Staphylococcus aureus (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853), *Bacillus cereus* (ATCC11778), *Bacillus subtilis* (ATCC6633), *Klebsiella pneumoniae* ATCC 700603 e *Enterococcus faecalis* (ATCC 29212), were cultured from frozen freezer stocks and maintained at 37°C on Müller Hinton Agar (MHA). The yeasts: *Candida albicans* (ATCC 10231), *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were activated on Sabouraud dextrose agar (SDA) and incubated for 48h at 35 ° C. All bacteria and yeast used were donated by INCQS/ Fiocruz, Rio de Janeiro, Brazil.

Crude extracts were ressuspended to a concentration of 5.0 mg/mL with DMSO (dimethylsulfoxide) according to Langfield et al. 2004.[28] Test sample (100µL) were added into first well at 96-well microplate and serial dilutions were then performed, using Müller Hinton broth and RPMI-1640 as a diluents to bacteria and yeast. Extracts concentrations ranged from 2500µg/mL to 20µg/mL.

Bacterial cultures (10^7 CFU/ml) were suspended in MH broth and 5µL/well of each bacterium was added, and plates were incubated at 37^o C overnight. Gentamicin and chloramphenicol (64 to 0,5 µg/mL) were used as positive controls. Twenty microliters of p-iodonitrotetrazolium violet (0,2 mg/mL) were added to each well and the plates were incubated for 1 h.[29]

Small portions of the yeasts colonies were transferred to 5.0 mL of sterile saline (0.85 %) solution and the concentration adjusted to a standard value of 1.0 x 10^6CFU/mL. The yeasts standard solutions were diluted in RPMI-1640 medium buffered to pH 7.0 in MOPS obtaining a final solution of 5.0x10^3CFU/mL. Yeast inocula (100 µL) were added to each well and incubated at 35 ° C for 48 h. Ketoconazole and itraconazole were used as controls (16 to 0,125 µg/mL). A resazurin solution (0,01%), fifteen microliters, was added to each well and the plates incubated again under the same conditions for 4h.[30,31] The value of MIC was determined as the lowest sample concentration capable of inhibiting the bacterial growth.

RESULTS

Of fifteen strains activated in this work, only one not grew, despite several attempts, and then the isolated Pw6 was considered dead.

Extracts and fractions of *Penicillium waksmanii* were screened for antimicrobial activity by means of the semi-quantitative bioautographic TLC agar overlay method. All crude extracts

were able to inhibit bacterial growth, but only the most polar fractions showed the highest activity against the bacteria tested. The obtained results indicated that most of the *Penicillium* strain extracts tested showed a variable degree of antibacterial activity ranging from moderate (8 mm diam) to high (16 mm diam) inhibitory activities against both gram-negative and gram-positive bacteria (**Table 1**).

Table 1: Bioautography of *Penicillium waksmanii* crude extracts

Strains Code	Insect Host	Bacteria Inhibition Zone (mm)	
		Sa*	Pa
Pw1	*Anopheles aquasalis*	10	9
Pw2	*Aedes scapularis*	9	9
Pw3	*Culex quinquefasciatus*	10	8
Pw4	*Culex nigripalpus*	11	13
Pw5	*Culex quinquefasciatus*	8	9
Pw6	*Culex quinquefasciatus*	dead	
Pw7	*Aedes scapularis*	12	10
Pw8	*Aedes taeniorhynchus*	10	12
Pw9	*Mansonia titilans*	12	13
Pw10	*Culex quinquefasciatus*	11	10
Pw11	*Culex microculex sp*	15	13
Pw12	*Anopheles aquasalis*	14	15
Pw 13	*Anopheles darlingi*	16	15
Pw14	*Aedes aegypti*	16	14
Pw15	*Aedes fluviatilis*	15	14
Chloramphenicol	-	18	16

*Sa –*Staphylococcus aureus*; Pa –*Pseudomonas aeruginosa* Results are presented by the diameter of inhibition zones (mm)

The MIC values of the tested crude extracts obtained using the microplate assay are shown in **Table 2.** The lowest MIC values recorded against the gram negative bacteria, *E. coli*, *P. aeruginosa* and *K. pneumoniae* were 310 (Pw11 to Pw15), 160 (Pw11 to Pw14) and 625 (Pw9) µg/ml, respectively. In relation to gram positive bacteria, the crude extracts had MIC values of 80 (Pw11 to Pw13), 40 (Pw12 and Pw13) and 160 µg/ml (Pw11 to Pw14) towards *B. cereus*, *B. subtilis* and *S.aureus*, respectively. If we consider the average MIC for each extract investigated our results indicate that the extracts produced by Pw12 and Pw13 demonstrated a higher activity against both, gram negative and gram positive bacteria, with values ranging from 40 to 310 µg/mL.

The fungicidal activities were observed for all the tested extracts. The Pw14 extract showed the best result for *Candida albicans*, with a MIC value of 160µg/mL. In our study, the strains

of *Candida krusei* and *Candida parapsilosis* also demonstrated susceptibility to crude extracts of *Penicillium waksmanii* with MIC values ranging from 310 to 160 µg/mL.

Table 2: Biological Activity from *Penicillium waksmanii* crude extracts

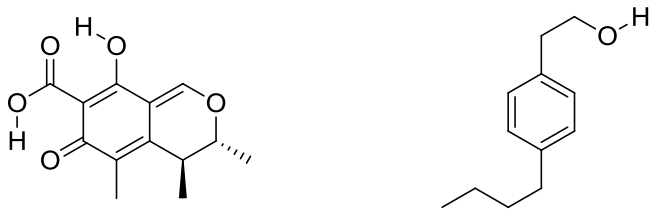
Strains Code	Bacteria MIC** (µg/mL)							Yeast MIC (µg/mL)		
	Ec*	Sa	Pa	Bc	Bs	Kp	Ef	Ca	Ck	Cp
Pw1	>1000	>1000	625	>1000	625	>1000	>1000	625	625	625
Pw2	>1000	>1000	625	>1000	>1000	>1000	>1000	625	625	625
Pw3	>1000	>1000	625	625	>1000	>1000	>1000	625	625	625
Pw4	>1000	625	310	310	160	>1000	>1000	625	625	625
Pw5	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	625	625
Pw6	dead									
Pw7	625	625	625	310	>1000	>1000	>1000	625	625	625
Pw8	625	625	310	310	160	>1000	>1000	625	625	625
Pw9	>1000	>1000	625	625	>1000	625	>1000	310	625	625
Pw10	>1000	>1000	625	>1000	625	>1000	>1000	160	310	625
Pw11	310	160	160	80	80	>1000	160	310	310	625
Pw12	310	160	160	80	40	>1000	160	310	310	160
Pw 13	310	160	160	80	40	>1000	160	310	310	160
Pw14	310	160	160	160	310	>1000	160	160	310	160
Pw15	310	625	310	160	310	>1000	160	310	310	310

*Ec – *Escherichia coli*, Sa – *Staphylococcus aureus*, Pa – *Pseudomonas aeruginosa*, Bc – *Bacillus cereus*, Bs – *Bacillus subtilis*, Kp – *Klebsiella pneumoniae*, Ef – *Enterococcus faecalis*

The NMR spectra showing presence of two compounds **1** (**F8**) and **2** (**F6**) – **Fig.1**, in ethyl acetate extracts from Pw13. Analysis of ^{1}H NMR spectrum for **compound 1** observed methyl signs in δ 1.24, δ 1.36 and one methyl singlet connected to double bond in δ 2.04. It is also noted quartet signals in δ 3.00 and δ 4.79 that values of their coupling constants was methyl groups neighbors. Observed a singlet at δ 8.27 and two singlets typical hydrogen bound to oxygen by making intramolecular hydrogen bonds in δ 15.13 and 15.88 ppm. In the ^{13}C NMR spectrum visualized 11 signs of carbons, three of which are characteristic signs of methyl groups, δ 9.6, δ 18.3 and 18.1. Signals at δ34.6, δ 81.6 and δ162.7 are assigned to carbon atoms with oxygen. Signs of quaternary carbons viewed in δ 107.4, δ138.9, δ 123.1, relating to carbon double bond and signs δ 174.5, δ 177.20, δ 100.3 and 183.8 regarding the *sp2* carbons oxygenates. COSY correlations of δ 1.24 with hydrogen in the δ 3.0 and hydrogen δ 1.36 with a δ4.79 were observed. The experiment has HSQC correlations of signals δ 2.04, δ1.36 and δ 1.24 with the carbons at δ 9.6, δ 18.3 and δ18.1, respectively.

Correlations of δ 3.00 with signs carbon δ34.6 and δ 4.79 with the carbon δ 81.6. In the HMBC spectrum visualized signal corrections carbons δ138.9 with δ1.24, 1.36 and δ2.04, δ81.6 and δ34.6 with hydrogen sings δ 1.24 and δ1.36, and correlations between signs δ 123.1 and 183.8 with δ 2.04. Comparing the data obtained with the literature,[32] observed that the signals of ^{1}H NMR and ^{13}C NMR implied that structure of **compound 1** is similar the citrinin structure, a mycotoxin normally found in species like Penicillium, Aspergillus and Monascus. Early studies with this mycotoxin report it as a potent antibiotic, though, later research showed nephrotoxic activity.[33] The citrinin is commonly produced by *Penicillium citrinum* and until the present time there have been no reports of this toxin in *Penicillium waksmanii* Zalessky isolated from mosquitoes.

Compound 2 is a brown solid, soluble in chloroform and ethyl acetate. The spectra of ^{1}H and ^{13}C NMR showed significant signs, the ^{1}H NMR spectrum showed signs of aromatic hydrogens and methylene. The presence of two doublets in the system of spins AA'XX' in δ6.78 and δ7.08 suggests to di-substituted aromatic ring. Coupling constants of the two triplets in ^{1}H NMR spectrum for signals in δ 3.81 (J 6.7 Hz) and δ2.79 (J 6.7 Hz) indicate that there is coupling between the protons represented by these signals. Still was noticed a strong singlet at δ1.25 relative methylene groups linked to *sp3* carbon and hydrogens attached to methy terminal δ 0.85. The ^{13}C NMR spectrum shows only eight signals which show a signal to the pair C2/C3 (δ 130.27) and one signal to the pair C3/C5 (δ115.69), this similarity is due to the symmetry of these nuclei in system para-disubstituted in aromatic rings. The other two *sp2* carbons of the aromatic ring does not appear in the spectrum, due to the fact that sample is too dilute them being quaternary carbons. There were also signs located one *sp3* bonded carbon to oxygen in the region of δ 63.9 and signals indicating the presence of a carbon chain methylene δ 38.9, δ32.1, δ 29.5 and δ22.8 as well methyl at δ 0.85, which corresponds to one of a methyl group - $(CH_2)_n$ - CH_3. Since there are two signals which appear as triplets CH_2 uniquely coupled to each other (δ 3.81 and 2.79) it is evident that one of the substituents of the aromatic ring is the group -CH_2CH_2OH. By excluding the other group should be a group n-butyl. We suggest that structure of **compound 2** as 2 - (4-butyl-phenyl)-ethanol.



1 **2**

Figure 1: The compounds isolated of *Penicillium waksmanii*

DISCUSSION

Numerous *Penicillium* species have been reported to be potential sources of antimicrobial agents. However, the secondary metabolites produced by those species occurring in ecological niches that have not been thoroughly explored, including insect-associated fungi, are poorly studied.[34] Despite the fact that over 700 species of entomopathogenic fungi are known to date[35] there are relatively few studies on their secondary metabolites,[19] and most current research topics in this area deal with a group of fungi belonging to the Hypocreales (e.g. *Cordyceps*,) including its anamorphs such as *Beauveria*, *Hirsutella*, *Metarhizium* and *Tolypocladium*, because of their potential as insect biocontrol agents.

The strains of *Penicillium waksmanii* studied in this work which displayed the most potentially active extracts regarding the inhibition of bacterial and yeast growth were the ones called Pw12, Pw13 and Pw14 These extracts displayed a stronger degree of inhibitory activity against the tested microorganisms.

Our results showed that the response in terms of sensitivity towards gram-negative and gram-positive bacteria varied among the strains. The differences in bacterial sensitivity have been frequently reported to occur with extracts and/or pure compounds.[36] Similarly Maskey et al., 2003,[37] reported activity against *Staphylococcus aureus* produced by *Penicillium chrysogenum* in agar diffusion test. The different sensitivity between gram-positive and negative bacteria reported for extracts and/or pure compounds have frequently been attributed to cell wall permeability differences found among bacteria. Although both types of bacteria cell walls are composed primarily of peptidoglycan, gram positive bacteria have more layers of peptidoglycan than gram-negative bacteria. Additionally, gram-negative bacteria have an outer phospholipidic membrane and protein channels that regulate the passage of molecules across this membrane.[38] This particular feature disables the access of many antibacterial agents to their cytoplasm. Such remarks lead us to conclude that is well worth pursuing

further investigations with extracts that exhibited stronger inhibition against gram-negative bacterial growth to identify bioactive molecules.

The incidence of fungal infections has been increasing recently with *Candida* species, especially *Candida albicans*, being the major opportunistic pathogen in immunocompromised patients.[39] Although *Candida albicans* is the most prevalent species in candidiasis, [40,41] the number of emerging species resistant as *Candida krusei* and *Candida parapsilosis* to antifungal drugs, has increased. In this work it was found that *Penicillium waksmanii* extracts showed excellent activity against *Candida* species, particularly against *Candida albicans*, thus being an important source for the search of new antifungal compounds.

The NMR studies of individual fractions of Pw13 was essential for structural elucidation of compounds, mainly because the sample concentrations were very low, making it difficult to analysis by other analytical techniques.

CONCLUSIONS

The biological activity against bacteria and yeast shown by different strains of *Penicillium waksmanii* indicate the need for further studies with this species. We observed that ability to microbial inhibition varied depending on the strain, some being more active than others. The strains that showed greater activity against bacteria and yeasts were isolated from those mosquitos *Anopheles* (Pw12 and Pw13) while the isolated strain of the genus *Culex* (Pw5) showed no activity for any of the microorganisms. This difference of bioactivity and the isolation of two compounds indicate that despite of strains belong to the same species, they present different genetic characteristics and consequently differences in the biosynthesis of secondary metabolites. Thus needing to study not only the kind of a fungus, but also their different ecological niches.

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