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ISOLATION AND BIOACTIVITIES OF SECONDARY METABOLITES FROM ENDOPHYTIC FUNGAL STRAINS ISOLATED FROM ROOT SAMPLES OF *Persea americana* (MILL.)

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ABSTRACT

The demand for the synthesis of innovative bioactive agents has been driven by rising rates of antibiotic resistance. *Persea americana* (Mill.) is a plant traditionally implicated for its therapeutic effects. This study aimed to isolate endophytic fungi from the roots of *P. americana*, extract secondary metabolites, and investigate their biological activities. Three (3) endophytic fungi (R12, R22P, R33P) were isolated from the plant root using standard protocols. Using the discontinuous batch method with a combined cooked rice and potato dextrose broth, the fungal isolates were employed in a fermentation system, and secondary metabolites were extracted with ethyl acetate. The antimicrobial activities of the metabolites against *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Serratia marcescens*, *Sarcina lutea*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, R12, R22P, and R33P fungi isolates using the agar-well diffusion method. *In vivo*, acute-inflammatory and toxicological assays were done using standard procedures. Against the bacteria, the antimicrobial effects of metabolites were highest against *B. subtilis* and *E. coli* (IZD = 9 mm), while *S. lutea* had the least across the metabolites (IZD = 0 mm). For fungi, the highest activity was recorded against R33P (IZD = 9 mm), while *C. albicans* have the least (IZD = 4 mm). The secondary metabolite of R12 had the best antibacterial activity, followed by R33P, then R22P, while R22P had the best antifungal activity, followed by R33P, then R12. In the *in vivo* acute inflammatory assay, the fungi metabolites generally showed an increase in inflammation from 0 minutes to 2 hours and a slight decrease from 3 to 4 hours. The toxicity assay carried out shows that the fungi metabolites are less toxic. The study highlights the importance of understanding endophytic fungi and presents room for improvement.

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INTRODUCTION

Globally, antimicrobial resistance is a serious hazard to public health [1]. Antimicrobial resistance is mostly brought about by the inappropriate use of antibiotics in clinical and agricultural settings is one of the main causes of this resistance

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is the exposure of bacteria to sub-inhibitory concentrations of antibiotic medicines [2]. Due to the high toxicity and limited specificity of some currently available molecules, it is essential to look for creative approaches to increase specificity and seek the identification of compounds with higher bioactivity [3]. Throughout history, medicinal plants have been used to treat and prevent disease. Different plant parts, such as leaves, stems, bark, roots, and fruits, have historically been used to cure, prevent, and manage a variety of illnesses [4]. Secondary metabolites are small compounds produced by plants and microorganisms [5]. A variety of secondary metabolites, which are organic sources of substances with biological activity, are produced by medicinal plants. These chemicals exhibit notable pharmacological efficacy and antimicrobial resistance (AMR) [6]. antibiotics via synergistic interaction, thus impeding the establishment of resistance [4]. Many drug leads come from secondary metabolites of plants, fungi, bacteria, and marine organisms. Natural products can be generated by cellular life either as primary metabolites that are involved in normal development, reproduction, and growth, or as secondary metabolites that have no direct part in these processes but typically have an important function [7]. Endophytes found in plants produce a diverse range of useful bioactive metabolites, which have attracted much attention recently from all around the world. Endophytes are microbes or internal plant tissue colonisers that are distinguished by their non-damaging effects on their host plants [8]. The plant used for the research, *Persea americana* Mill., common name “Avocado”, other names are “ube oyibo” (Igbo, Nigeria), “ganyen piya” (Hausa, Nigeria), “ewe pia” (Yoruba, Nigeria). It is a tropical evergreen tree that grows about 7 meters tall. *P. americana* is used for antipyretic and analgesic purposes, as well as for the treatment of diabetes, hypertension, and renal problems. Humans chew the leaves to treat pyorrhea, and its aqueous extract has the potential to cause prolonged hypotension. They demonstrated anti-inflammatory, anti-diabetic, anti-convulsant, and vasorelaxant effects [9]. To address the existing levels of antibiotic resistance, new agents are not being discovered at a rate that can keep up with current processes. The emergence of a "post-antibiotic era" has raised concerns as a result [10]. Endophytes are a novel source for the identification and synthesis of beneficial active compounds. This discovery has made endophytes even more significant because it is simpler and less expensive for the environment to produce potent compounds on an industrial scale in microbes than in plants. Thus, the pharmaceutical sector needs to pay greater attention to endophytes [11]. It is crucial to look for novel treatment medications that are both safe and effective in preventing the illness [12]. The biological activity of endophytic fungi's secondary metabolites is linked to their host, and these fungi are highly diverse when isolated from medicinal plants. properties [13]. One of the least researched but most significant groups of microorganisms is endophytic fungi. They produce a wide range of biologically active secondary metabolites that strengthen their host's defences against abiotic stress, illness, insects, diseases, and mammalian herbivores. For uses in healthcare, pharmacology, and agriculture, they may therefore provide a different source of secondary metabolites [14].

MATERIALS AND METHODS

Sampling and Processing

Healthy *P. americana* Mill. roots were collected from different locations within Nsukka, Enugu state, Nigeria. It was appropriately identified and authenticated (voucher number: PCG/UNN/0079) by Mr Felix Nwafor of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka.

Inoculation, Isolation, and Purification of Endophytic Fungi Isolates

The roots of the plant were thoroughly rinsed with distilled or demineralised water to remove the soil particles. The roots of the plant were then immersed in 70 % ethyl alcohol for 1 to 2 minutes to disinfect the surface. It was transferred into a 2 to 5 % sodium hypochlorite solution and agitated gently for 3 to 5 minutes to kill the surface microbes. It was then rinsed thoroughly with distilled water about 3 to 5 times to remove the residual disinfectants on the root surface.

The sterilised roots were then cut into smaller segments of about 1 to 3 cm under aseptic conditions using a sterile scalpel and forceps. The sterile plant roots were placed on the surface of the potato dextrose agar in a plate prepared according to the manufacturer's specification, which was then properly sealed and incubated for about 1 week with daily checks for endophyte hyphal development on the plates. Inoculum was prepared by transferring a small amount of the fungal mycelium from the plate it was cultured into a freshly prepared potato dextrose agar

plate using an inoculating loop to evenly distribute it on the agar plate under aseptic conditions. The agar plate was incubated at room temperature for about 2 weeks to allow fungal growth.

After incubation, the inoculated plates were examined for the growth of individual fungi colonies. Well-separated and distinct colonies were selected based on morphology, colour, and growth pattern. The fungi colonies were isolated by sub-culturing distinctive colonies in different agar plates using a zigzag pattern of inoculation.

The pure cultures of the fungi isolates were obtained by sub-culturing the distinctive fungi colonies as needed using PDA plates. The pure fungal isolates were stored by cryopreservation.

Discontinuous Batch Fermentation Method

Under aseptic conditions, a small number of pure fungi isolates from the pure stock cultures of fungi isolates were transferred to a fresh PDA plate and allowed to grow until well-developed colonies were formed. The appropriate amount of rice was cooked until it was soft and moist. It was allowed to cool to room temperature. The rice was cooked with the potato dextrose broth (PDB) medium to create a solid substrate suitable for fungi growth and the production of secondary metabolites. It was transferred into sterile containers suitable for fermentation and appropriately labelled. The containers were sealed to prevent contamination and maintain a suitable environment for fungal growth. The mixture was incubated at the appropriate temperature and humidity. 20 ml of PDB was added every week to provide essential nutrients for the fungi isolates growth. The fermentation process was monitored to assess the fungal growth and production of secondary metabolites.

Extraction of Secondary Metabolites of the Endophytic Fungi

After 2 weeks, the container containing the mixture of the cooked rice and the PDA was mixed with the appropriate volume of ethyl acetate and sealed with cotton-filled aluminium foil. The mixture was shaken vigorously for about 5 minutes to ensure thorough mixing of the ethyl acetate and the mixture and for the extraction of the secondary metabolites into the ethyl acetate phase. There were two resulting separate phases, the mixture phase and the ethyl acetate phase, which were allowed to sit for 24 hours. The ethyl-acetate phase was poured into Petri dishes and allowed to dry for 24 h. The 0.5 g of the dried extracts (R12, R22P, R33P) were transferred into small sterile amber vials, and 1 ml of DMSO was added to the dried extracts in the amber vials using a sterile syringe. The amber vials were then stored in the refrigerator.

Antimicrobial Assay

The bacteria include gram-positive bacteria and gram-negative bacteria. The Gram-positive bacteria include *Bacillus subtilis, Staphylococcus aureus*, and *Sarcina lutea*. The Gram-negative bacteria include *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Serratia marcescens.* The fungi microorganism includes *C. albicans*, R12, R22P, and R33P.

Antimicrobial Sensitivity Testing

The secondary metabolite extracts (R12, R22P, R33P) stored in the refrigerator were collected and transferred into the test tubes. Two-fold serial dilution was done on the stock solution of 500 mg/ml concentration to get 250 mg/ml. The same dilution procedure was done to get 125 and 62.5 mg/ml, respectively. Under aseptic conditions using the agar well diffusion method, Mueller-Hinton agar and Potato dextrose agar were prepared for bacterial and fungal isolates, respectively. A marker was used to divide the petri dishes into 4 sides. The test microorganism strains were spread evenly on the surface of the agar plate using a sterile swab. A sterile cork borer of 0.6 cm diameter was used to bore a well on the agar plates. The 0.1ml of fungal extracts of varying concentrations were dispensed into the wells using sterile 1ml syringes. The agar plates were incubated at 37 °C for bacteria and 25 – 30 °C for fungi for 24 - 48 hours. The inhibition zone diameters (IZD) formed were measured in cm for all the plates after incubation using a meter rule. The procedure was done in triplicate to determine the mean of the IZD of the bacteria.

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

Minimum inhibitory concentration is determined after incubation as the lowest possible concentration to inhibit the visible growth of the test microorganism. MICs are usually used to verify the resistance of the test microorganism in the laboratory [15]. The minimum inhibitory concentration refers to the *in vitro* thresholds at which bacterial strains become susceptible or resistant to an antibiotic [16]. Thus, the

MIC was obtained using the lowest concentration enough to inhibit isolates.

The Minimum Bactericidal Concentration is the lowest antimicrobial dilution concentration that stops the microorganisms from growing on the agar plate after incubation for 24 - 48 hours. It is implied that only nonviable organisms are present when an organism fails to grow on the plate [17].

Further incubation of the agar plates at 37 °C for bacteria and 25 – 30 °C for fungi for 24 - 48 hours, respectively. The Minimum fungicidal concentration and minimum bactericidal concentration were determined, and the results were recorded.

In vivo Acute Inflammatory Assay

The stock solution of the fungi extracts was diluted to 100 mg/ml, 50 mg/ml and 25 mg/ml concentrations. The mice were marked at head, tail, trunk, TTR (tail and trunk), HTL (head and tail), HTR (head and trunk), TRF (tail and right forelimb), RF (right forelimb), LF (left forelimb), BF (both forelimb), HBF (head and both forelimb), TBF (tail and both forelimb), HTTR (head, tail and trunk), and were placed in three (3) different cages (A, B, C) for the test mice and the control mice was placed in cage labelled (E). The mice were allowed to rest for 2 hours, and 0.2 ml of the secondary metabolite extracts was administered to the test mice. Drinking water was given as the negative control to the mice, while 0.2 ml of 50 mg diclofenac was given as the positive control with the aid of oral gavage. Carrageenan was then injected into the left hind paw area of all the mice to induce inflammation. At 0 minutes, a Vernier calliper was used to measure the diameter of the paw inflammation and the result was recorded. This procedure was then repeated at 30 minutes, 1 hour, 2 hours, and 4 hours.

Toxicology Assay

The toxicology assay was done for a period of 14 days. At day 1, the mice were allowed to rest for 2 hours and 0.2 ml of the fungi secondary metabolites at 50 mg/ml and 100 mg/ml concentrations was administered to the mice. The blood samples of the mice were collected and the following parameters of baseline analysis was obtained; the liver enzyme assay in mg/ml (ALP, ALT, AST), the kidney enzyme biomarker in mg/ml (creatinine, urea), red blood cell (10^4 cells/mm^2), white blood (10^6 cells/mm^2), hemoglobin (g/dl), PCV % (packed cell volume), DLC % (differential leukocyte count); N (neutrophil), B (basophil), E (eosinophil), L (leucocyte), M (monocyte). The mice were transferred back to their cages with adequate food and water. On day 2, the mice were allowed to rest for a few hours, and 0.2 ml of the fungi secondary metabolites at 50 mg/ml and 100 mg/ml concentrations was administered to the mice. The mice were transferred back to their cages with adequate food and water. The same procedure was carried out on the mice from 3 to 13 days, respectively. At day 14, the mice were allowed to rest for 2 hours and 0.2 ml of the fungi secondary metabolites at 50 mg/ml and 100 mg/ml concentrations was administered to the mice. The blood samples of the mice were collected, and parameters for the final analysis were obtained.

RESULTS

Endophytic Fungi Isolation and Morphological Identification

After isolation, three (3) endophytic fungi isolates (Figure 1) were obtained and coded as R12, R22P, and R33P. R12, R22P, and R33P were all gotten from the root of *P. americana* Mill. plant, and all had different morphological appearances.

Fermentation and Yield of Secondary Metabolites

Following the fermentation process, R12, R22P and R33P secondary metabolites were extracted from the root endophytic fungi of *P. americana* Mill. of which the yield was estimated. The results in Figure 2 showed that R12 has the highest yield at 1.4 g, while both R22P and R33P have the same yield at 1.3 g.

The Effect of the Fungal Metabolites on Bacteria and Fungi

The results in Figure 3 showed that the R12 fungi metabolite had strong antimicrobial activity against *E. coli.* No antimicrobial activity was recorded for *K. pneumoniae* and *S. lutea*. The highest concentration of the R12 metabolite, 500 mg/ml, gave the best antimicrobial activity across the bacterial test organisms. The lowest concentration of the metabolite, 62.5 mg/ml, only had an antimicrobial activity on *E. coli*. Across the bacteria, *E. coli* showed antimicrobial activity on all the different concentrations of the R12 metabolites used. *P. aeruginosa* showed antimicrobial activity on all the metabolite concentrations except the lowest concentration. *S. aureus* showed antimicrobial activity only on metabolite concentrations A and B. *S. marcescens* showed antimicrobial activity only on metabolite concentration A. *B. subtilis* showed

antimicrobial activity on all the metabolite concentrations except the least concentration.

The results in Figure 4 showed that the R22P fungi metabolite had strong antimicrobial activity against E. coli. No antimicrobial activity was recorded for *P. aeruginosa, K. pneumoniae, S. lutea*, and *B. subtilis*. The highest concentration of the R22P metabolite, 500 mg/ml, gave the best antimicrobial activity across the bacteria test organisms. The concentrations of the metabolites 125 mg/ml and 62.5 mg/ml had the least antimicrobial activity across the bacterial test organisms. E. coli showed antimicrobial activity on all the different concentrations of the R22P metabolites used. *S. aureus* showed antimicrobial activity on all the metabolite concentrations used. *S. marcescens* showed antimicrobial activity only on metabolite concentrations A and B.

The results in Figure 5 showed that the R33P fungi metabolite had strong antimicrobial activity against B. subtilis. Antimicrobial activity was recorded across the bacterial test organisms. The highest concentration of the R33P metabolite, 500 mg/ml, gave the best antimicrobial activity against the bacteria test organisms. The lowest concentration of the metabolite, 62.5 mg/ml, had no antimicrobial activity on *K. pneumoniae* and *S. lutea*. Across the bacteria, *E. coli, B. subtilis, S. marcescens, S. aureus,* and *P. aeruginosa* showed antimicrobial activity on all the different concentrations of the R33P metabolites used. *Klebsiella pneumoniae* showed antimicrobial activity at all metabolite concentrations except the lowest concentration. *S. lutea* showed antimicrobial activity only on metabolite concentrations A and B.

The results in Figure 6 showed that the R12 fungi metabolite had strong antimicrobial activity against R12 fungi isolates. No antimicrobial activity was recorded on R33P fungi isolates. The highest concentration of the R12 metabolite, 500 mg/ml, gave the best antimicrobial activity across the fungi organisms. The lowest concentration of metabolites, 62.5 mg/ml, had no antimicrobial activity on R33P and *Candida albicans*. Across the fungi isolates, R12, R22P and *C. albicans* showed antimicrobial activity on all the different concentrations of the R12 metabolites used. *C. albicans* showed antimicrobial activity only on the metabolite concentrations A and B.

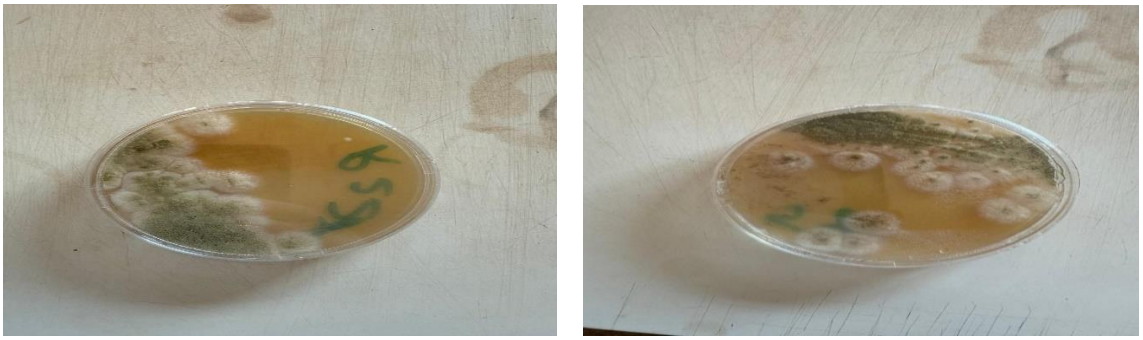
The results in Figure 7 showed that the R22P fungi metabolite had strong antimicrobial activity against R33P fungi isolates. No antimicrobial activity was recorded on *C. albicans*. The highest concentration of the R22P metabolite, 500 mg/ml, gave the best antimicrobial activity across the fungi organisms. The lowest concentration of the metabolite, 62.5 mg/ml, had no antimicrobial activity on R22P and *C. albicans*. Across the fungi isolates, R12, R22P, and R33P showed antimicrobial activity on all the different concentrations of the R22P metabolites used. R22P showed antimicrobial activity only at metabolite concentration A.

The results in Figure 8 showed that the R33P fungi metabolite had strong antimicrobial activity against R12 fungi isolates. No antimicrobial activity was recorded on R33P fungi isolates. The highest concentration of the R33P metabolite, 500 mg/ml, gave the best antimicrobial activity across the fungi organisms. The lowest concentration of metabolites, 62.5 mg/ml, had no antimicrobial activity on R33P and *C. albicans*. Across the fungi isolates, R12, R22P and *C. albicans* showed antimicrobial activity on all the different concentrations of the R33P metabolites used. *C. albicans* showed no antimicrobial activity, only on the metabolite concentration D.

Determination of the MIC of fungi metabolites on the bacteria and fungi organisms.

The results of the study, as shown in Table 1, show that the fungi metabolite R12 does not affect the fungi isolate R33P, but has an MIC at 62.5 mg/ml for both R12 and R22P, and at 125mg/ml for *C. albicans*. The fungi metabolite R22P does not affect *C. albicans,* but has an MIC at 62.5 mg/ml for both R33P and R12, and at 500 mg/ml for R22P. The fungi metabolite R33P does not affect the R33P isolate but has an MIC at 15.625 mg/ml for both R22P and R12 isolates, and at 62.5 mg/ml for *C. albicans.*

The results of the study, as shown in Table 2, the fungi metabolite R12 do not affect *K. pneumoniae*, but has an MIC at 500 mg/ml for *S. marcescens*, at 250 mg/ml for *S. aureus*, at 125 mg/ml for *B. subtilis*, and at 62.5 mg/ml



A B

C

Figure 1. The different endophytic fungi isolates, A, B, and C, represent the R22P endophytic fungi isolate, the R12 endophytic fungi isolate, and the R33P endophytic fungi isolate, respectively.

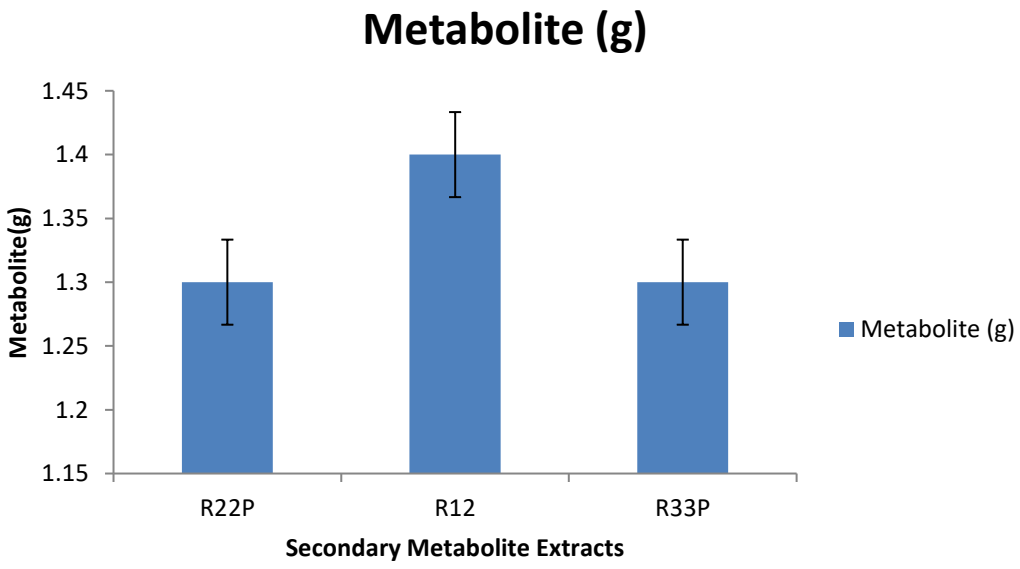


Figure 2. The yield of the secondary metabolites.

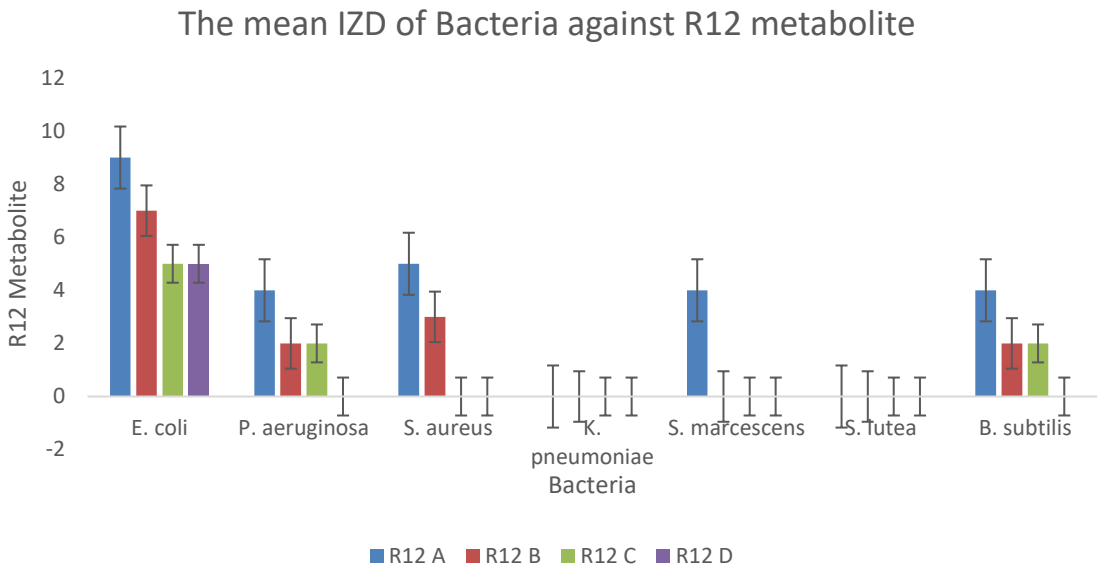


Figure 3. The mean IZD of bacteria against the R12 secondary metabolite. Keys: A = 500 mg/ml, B = 250 mg/ml, C = 125 mg/ml, D = 62.5 mg/ml.

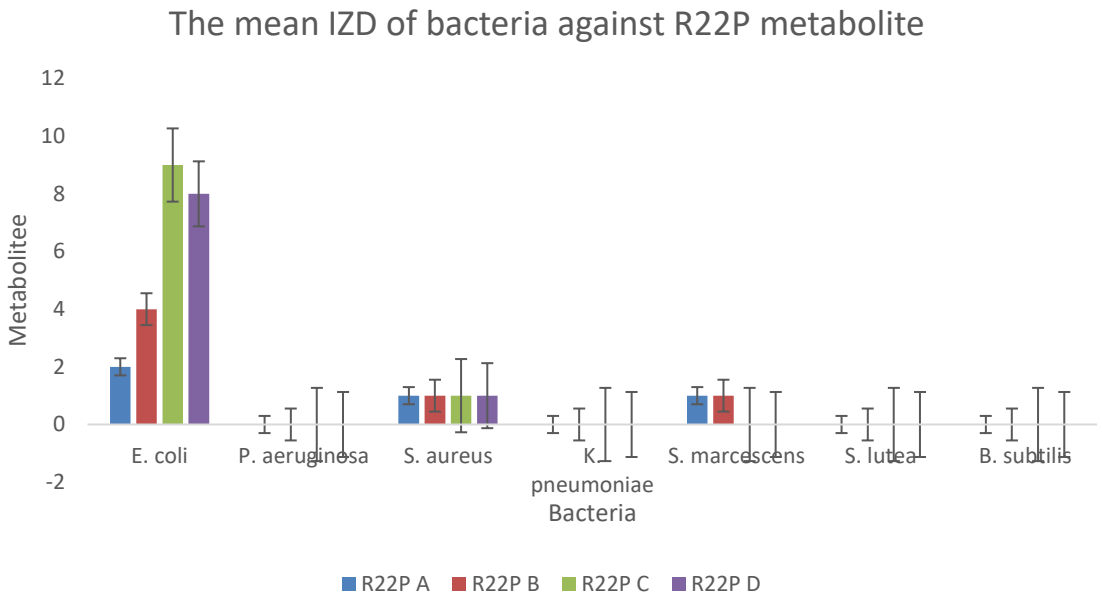


Figure 4. The mean IZD of bacteria against the R22P secondary metabolite. Keys: A = 500 mg/ml, B = 250 mg/ml, C = 125 mg/ml, D = 62.5 mg/ml.

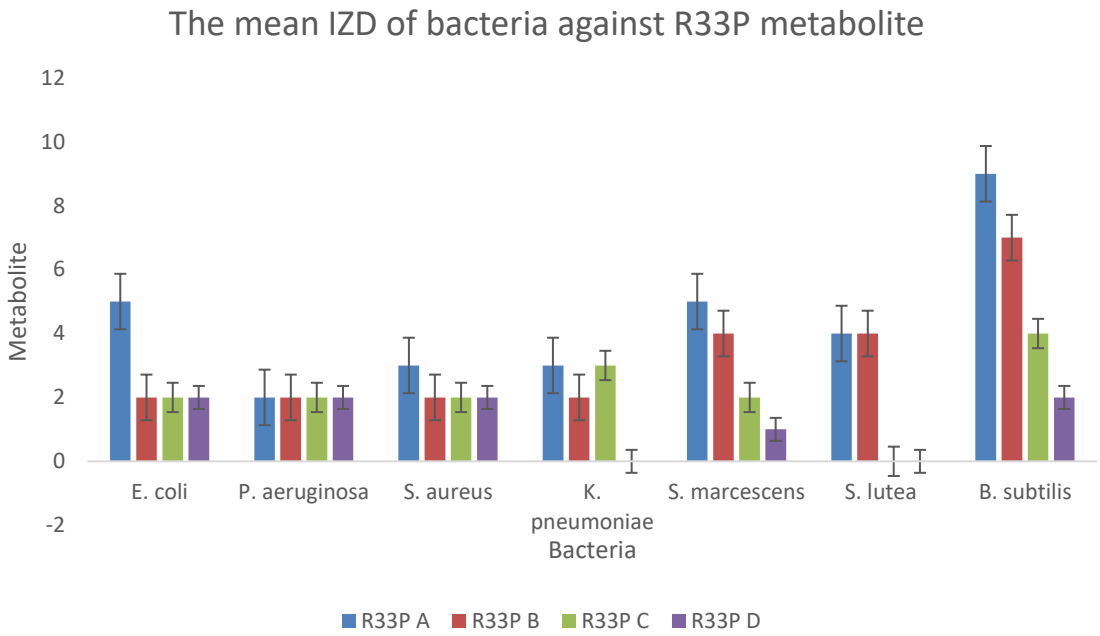


Figure 5. The mean IZD of bacteria against the R33P secondary metabolite. Keys: A = 500 mg/ml, B = 250 mg/ml, C = 125 mg/ml, D = 62.5 mg/ml.

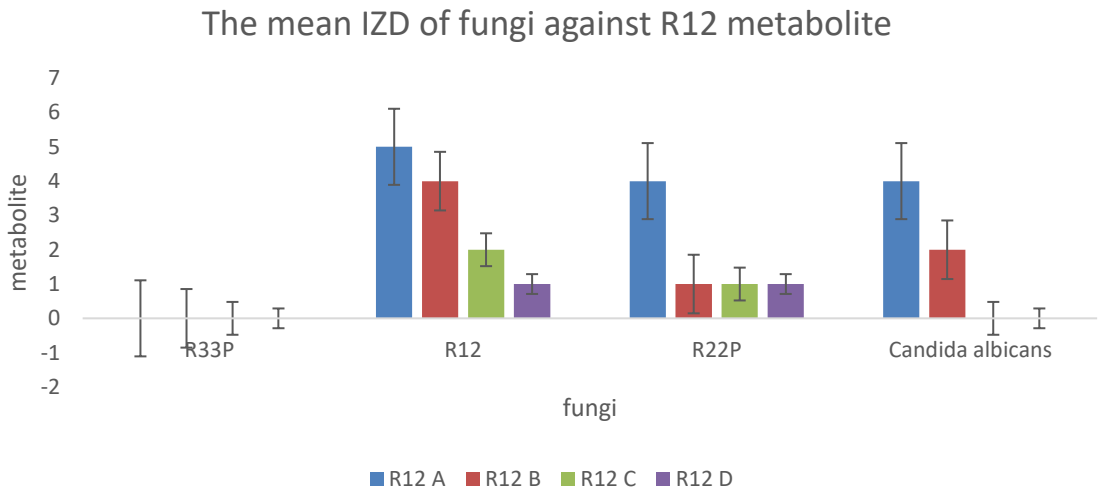


Figure 6. The mean IZD of fungi against the R12 metabolite. Keys: A = 500 mg/ml, B = 250 mg/ml, C = 125 mg/ml, D = 62.5 mg/ml.

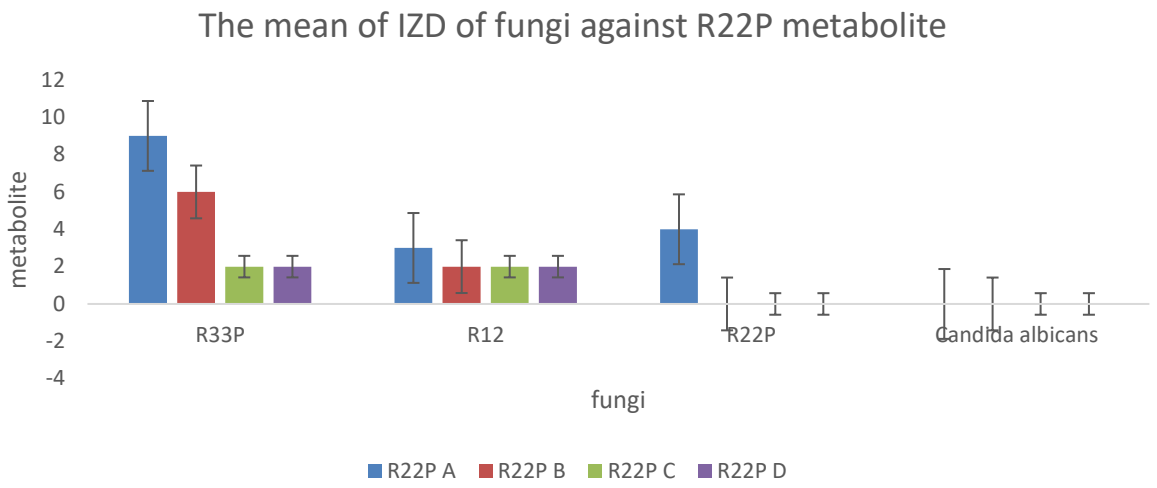


Figure 7. The mean IZD of fungi against the R22P metabolite. Keys**:** A = 500 mg/ml, B = 250 mg/ml, C = 125 mg/ml, D = 62.5 mg/ml.

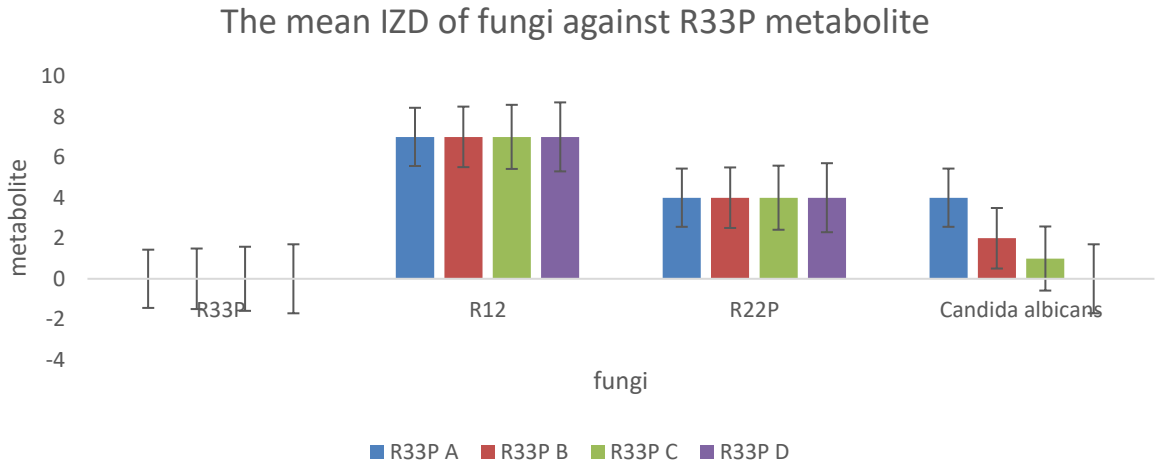


Figure 8. The mean IZD of fungi against the R33P metabolite. Keys: A = 500 mg/ml, B = 250 mg/ml, C = 125 mg/ml, D = 62.5 mg/ml.

Table 1. Different MICs of the fungi metabolites on the fungi isolates.

Metabolites (mg/ml)	R33P	R12	R22P	*C. albicans*
R12	-	62.5	62.5	125
R22P	62.5	62.5	500	-
R33P	-	15.625	15.625	62.5

Table 2. Different MICs of the fungi metabolites on the bacteria

Metabolites (mg/ml)	*E. coli*	*P. aeruginosa*	*S. aureus*	*K. pneumoniae*	*S. marcescens*	*S. lutea*	*B. subtilis*
R12	62.5	31.25	250	-	500	-	125
R22P	15.625	-	62.5	-	250	-	-
R33P	62.5	62.5	31.25	125	62.5	250	62.5

Table 3. Different MBCs of fungal metabolites against bacteria

Metabolites (mg/ml)	*E. coli*	*P. aeruginosa*	*S. aureus*	*K. pneumoniae*	*S. marcescens*	*S. lutea*	*B. subtilis*
R12	62.5	250	500	-	500	-	125
R22P	500	-	500	-	500	-	-
R33P	62.5	250	125	125	250	62.5	125

Table 4. Different MFCs of fungal metabolites on fungal isolates

Metabolites (mg/ml)	R33P	R12	R22P	*C. albicans*
R12	-	125	500	250
R22P	125	62.5	500	-
R33P	-	125	31.25	250

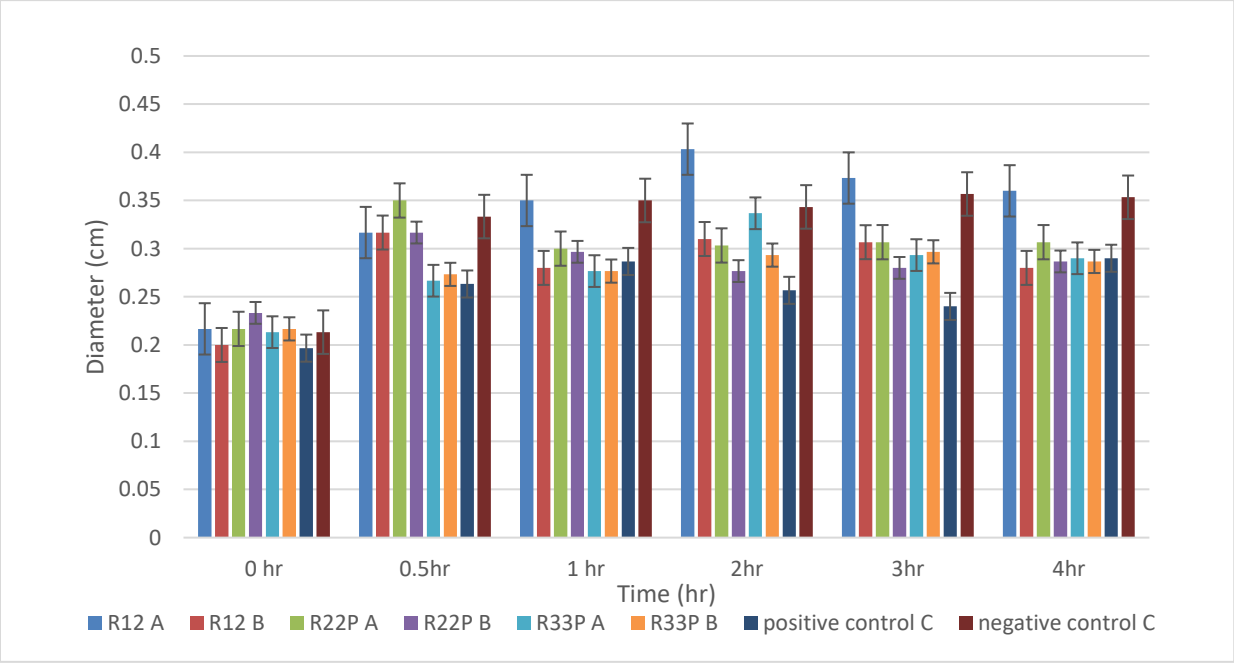


Figure 9. The control and fungi metabolites extracts at different concentration effect on inflammation at different time intervals. Keys: A = 50 mg/ml, B = 100mg/ml, C = 10mg/ml

Table 6: Baseline analysis of the fungi metabolite toxicity effect on the test mice.

Metabolites	Concentration (mg/ml)	Liver enzyme assay (mg/dl)			Kidney Enzyme Biomarker (mg/dl)		Blood analysis				DLC %				
		ALT	ALP	AST	Creatinine	Urea	RBC (10^6 cells/mm2)	WBC (10^4 cells/mm2)	Hb (g/dl)	%PCV	N	Ba	E	L	M
R12	A	28.67 ± 2.49	34.33 ± 6.02	73.33 ± 4.11	1.13 ± 0.19	72.33 ± 2.87	4.43 ± 0.22	4.857 ± 0.12	14.3 ± 0.24	44 ± 1.63	61.33 ± 1.70	3.33 ± 0.94	2.33 ± 0.47	31 ± 2.16	2 ± 0.82
	B	31.33 ± 2.49	32.67 ± 2.87	68 ± 7.79	1.15 ± 0.22	69 ± 2.45	4.377 ± 0.12	4.536 ± 0.09	14.33 ± 0.12	45 ± 1.63	60 ± 0.82	3.67 ± 0.47	3 ± 0.00	30 ± 2.16	2.67 ± 0.94
R22P	A	29.33 ± 2.49	30.33 ± 1.25	75.33 ± 2.49	0.96 ± 0.06	70.67 ± 3.86	4.36 ± 0.22	4.691 ± 0.47	14.33 ± 0.05	44.67 ± 1.25	59 ± 4.24	3.67 ± 1.25	3 ± 0.82	29 ± 0.82	3.33 ± 0.47
	B	32.33 ± 4.64	35.33 ± 0.94	69.33 ± 2.05	1.08 ± 0.07	70.33 ± 8.65	4.347 ± 0.12	4.531 ± 0.21	14.27 ± 0.19	44.33 ± 1.70	61 ± 1.63	3.67 ± 0.47	2.33 ± 0.47	30.67 ± 1.70	2.33 ± 0.47
R33P	A	32.67 ± 3.30	31 ± 2.16	72.67 ± 5.79	1.07 ± 0.08	66.33 ± 3.09	4.743 ± 0.34	4.313 ± 0.03	14.3 ± 0.08	44.33 ± 1.25	61 ± 2.16	4 ± 1.41	2.67 ± 1.25	28.67 ± 3.85	3.67 ± 1.25
	B	31.67 ± 2.05	34.67 ± 3.30	75 ± 1.41	1.063 ± 0.10	72 ± 7.48	4.7547 ± 0.28	4.437 ± 0.08	14.4 ± 0.16	44.67 ± 2.05	60.67 ± 2.87	3.33 ± 0.94	2 ± 0.00	30.33 ± 3.40	3.33 ± 0.47
control	C	30.8 ± 2.05	33.67 ± 6.01	72 ± 4.55	1.05 ± 0.11	69.67 ± 2.05	4.775 ± 0.08	4.32 ± 0.13	14.37 ± 0.05	44.33 ± 1.25	61.33 ± 1.70	4 ± 0.00	2.33 ± 0.47	29 ± 0.82	3 ± 1.41

Keys: ALT = Alanine transaminase, ALP =Alkaline phosphatase, AST = Aspartate transaminase, WBC = White Blood Cells, RBC=Red Blood Cells, PCV = Packed Cell Volume, HB = Hemoglobin, DLC = Differential Leucocyte Count, N = Neutrophils, Ba = Basophils, E = Eosinophils, L = Lymphocytes, M = monocytes, A = 50 mg/ml, B = 100 mg/ml, C = 10 mg/ml

Table 7. Final analysis of the fungi metabolite toxicity effect on the test mice

Metabolites	Concentration (mg/ml)	Liver enzyme assay (mg/dl)			Kidney enzyme biomarker (mg/dl)		Blood analysis				DLC %				
		ALP	ALT	AST	Urea	Creatinine	RBC (10^6 cells/mm2)	WBC (10^4 cells/mm2)	%PCV	Hb (g/dl)	N	Ba	E	L	M
R12	A	31.33 ± 1.70	34 ± 4.32	74 ± 4.08	77.67 ± 2.62	1.083 ± 0.11	4.794 ± 0.23	4.607 ± 0.22	45 ± 1.63	14.43 ± 0.12	59.33 ± 2.62	3.67 ± 0.47	1.67 ± 0.47	31.33 ± 3.09	4 ± 0.82
	B	36 ± 3.27	35 ± 2.16	78.67 ± 3.68	81.33 ± 1.70	1.213 ± 0.04	4.534 ± 0.25	4.327 ± 0.28	44.67 ± 0.94	14.37 ± 0.17	61.33 ± 1.70	3 ± 1.63	2.33 ± 0.47	29.67 ± 0.47	3.67 ± 0.47
R22P	A	32.33 ± 2.87	33.67 ± 4.64	72.33 ± 4.50	68.33 ± 1.25	1.227 ± 0.05	4.851 ± 0.18	4.293 ± 0.13	44.33 ± 1.25	14.3 ± 0.08	60.33 ± 1.25	5.33 ± 1.25	2.67 ± 0.47	26.33 ± 0.47	5.33 ± 0.47
	B	31.67 ± 3.68	34.67 ± 3.40	73.33 ± 4.99	66.33 ± 4.92	1.857 ± 0.64	5.160 ± 0.76	4.42 ± 0.19	45 ± 2.16	14.5 ± 0.14	60.67 ± 1.70	5 ± 0.00	3 ± 0.82	27.33 ± 1.25	4 ± 0.82
R33P	A	29.33 ± 2.49	38.33 ± 1.25	73.67 ± 6.02	74.67 ± 6.24	1.263 ± 0.11	4.907 ± 0.58	4.287 ± 0.08	44.33 ± 1.25	14.4 ± 0.22	60.67 ± 0.94	2.67 ± 0.47	3 ± 1.41	28.33 ± 1.25	4 ± 0.82
	B	30.67 ± 4.99	33.33 ± 2.49	74.33 ± 4.50	73 ± 3.74	1.26 ± 0.33	4.883 ± 0.14	4.313 ± 0.15	43.67 ± 2.05	14.37 ± 0.33	58.67 ± 2.49	4.33 ± 1.25	2.33 ± 0.47	30.67 ± 1.89	3.33 ± 0.94
Control	C	29.33 ± 2.05	34.67 ± 4.03	75 ± 2.16	73.67 ± 3.68	1.043 ± 0.09	4.896 ± 0.13	4.4 ± 0.14	44.33 ± 1.25	14.37 ± 1.25	59 ± 2.16	3.67 ± 0.47	2.33 ± 0.47	31.33 ± 2.49	3 ± 0.82

Keys: ALT = Alanine transaminase, ALP = Alkaline phosphatase, AST = Aspartate transaminase, WBC = White Blood Cells, RBC = Red Blood Cells, PCV = Packed Cell Volume, HB = Hemoglobin, DLC = Differential Leucocyte Count, N = Neutrophils, Ba = Basophils, E = Eosinophils, L = Lymphocytes, M = monocytes, A = 50 mg/ml, B = 100 mg/ml, C = 10 mg/ml.

for *E. coli*, and at 31.5 mg/ml for *P. aeruginosa*. The fungi metabolite R22P does not affect *P. aeruginosa, K. pneumoniae, S. lutea, B. subtilis*, but has an MIC at 62.5 mg/ml for *S. aureus*, at 15.625 mg/ml for *E. coli* and at 250 mg/ml for *S. marcescens*. The fungi metabolite R33P has an MIC of 62.5 mg/ml for *E. coli, P. aeruginosa*, *S. marcescens* and *B. subtilis,* at 250 mg/ml for *S. lutea*, at 125 mg/ml for *K. pneumoniae,* and at 31.25 mg/ml for *S. aureus.*

Determination of the MBC and MFC of fungi metabolites on the bacteria and fungi organisms

The results of the study, as shown in Table 3, the fungi metabolite R12 do not affect *K. pneumoniae* and *S. lutea*, but has MBC at 500 mg/ml for *S. marcescens* and *S. aureus*, at 125 mg/ml for *B. subtilis*, at 31.25 mg/ml for *E. coli*, and at 250 mg/ml for *P. aeruginosa*. The fungi metabolite R22P does not affect *P. aeruginosa, K. pneumoniae, S. lutea, B. subtilis*, but has MBC at 500 mg/ml for *S. aureus*, *E. coli* and *S. marcescens*. The fungi metabolite R33P has an MIC at 62.5 mg/ml for *E. coli* and *S. lutea,* and *B. subtilis,* at 250 mg/ml for *P. aeruginosa* and *S. marcescens, and* at 125 mg/ml for *K. pneumoniae* and *S. aureus.*

The results of the study, as shown in Table 4, show that the fungi metabolite R12 does not affect the fungi isolate R33P, but has MFC at 125 mg/ml for R12, at 500 mg/ml for R22P, and at 250mg/ml for *C. albicans*. The fungi metabolite R22P does not affect *C. albicans,* but has MFC at 62.5 mg/ml for the R12 isolate, at 125 mg/ml for the R33P isolate, and at 500 mg/ml for the R22P isolate. The fungi metabolite R33P does not affect R33P but has MFC at 31.25 mg/ml for R22P isolate, at 125 mg/ml for R12 isolate and at 250 mg/ml for *C. albicans.*

The *in vivo* effect of the fungi metabolite on the inflammation of the test mice

The results of the study, as shown in Figure 9, the fungi metabolite R12 showed an increase in inflammation from 0 hours to 2 hours and a slight decrease from 3hours to 4 hours for the 50 mg/ml dose. The fungi metabolite R12 showed an increase in inflammation from 0 minutes to 2 hours and a slight decrease from 3 hours to 4 hours for the 100 mg/ml dose. The fungi metabolite R22P showed a slight increase in inflammation from 0 minutes to 30 minutes and a decrease from 1 hour to 4 hours for the 50 mg/ml dose. The fungi metabolite R22P showed an increase in inflammation from 0 minutes to 30 minutes and a slight decrease from 1 hour to 4 hours for the 100 mg/ml dose. The fungi metabolite R33P showed an increase in inflammation from 0 minutes to 2 hours and a slight decrease from 3hours to 4 hours for the 50 mg/ml dose. The fungi metabolite R33P showed an increase in inflammation from 0 minutes to 2 hours and a slight decrease from 3hours to 4 hours for the 100 mg/ml dose. The positive control showed an increase in inflammation from 0 minutes to 1 hour and a decrease from 2 hours to 4 hours for the 10 mg/ml dose. The negative control showed no significant increase or decrease in inflammation from 0 minutes to 4 hours for the dose administered.

***In vivo* Toxicity Effect of the Fungal Metabolite on Mice**

The results in Table 6 show the liver, kidney and blood function of the mice at day 1 of administering the fungi metabolites and the control test using drinking water. The different concentration of the fungi metabolites was used to determine the liver enzyme assay, the kidney enzyme biomarker and the blood analysis of the test mice.

The results in Table 7 show the liver, kidney and blood function of the mice at day 14 of administering the fungi metabolites and the control test using drinking water. The different concentration of the fungi metabolites was used to determine the liver enzyme assay, the kidney enzyme biomarker and the blood analysis of the test mice.

DISCUSSION

P. americana is a well-documented medicinal plant with extensive physiological, nutritional, and pharmacological relevance, with various parts, including roots, leaves, bark, and fruits, reported to possess therapeutic properties [6]. The bioactivity of this plant is largely attributed to its rich reservoir of secondary metabolites, which serve as important sources of biologically active compounds with antimicrobial, anti-inflammatory, and antioxidant properties [6]. In recent years, attention has shifted toward plant-associated endophytes as alternative and sustainable producers of such bioactive molecules, often mirroring or even surpassing the metabolic capabilities of their host plants.

In the present study, endophytic fungi were successfully isolated from the roots of *P. americana*, supporting previous reports that medicinal plants harbour diverse endophytes with significant biotechnological potential [18]. The use of a rice-based solid-state fermentation system supplemented with potato dextrose broth provided a nutrient-rich substrate that enhanced fungal growth and secondary metabolite production. This approach aligns with established fermentation strategies known to improve metabolite yield through optimised carbon and nitrogen availability [19]. Ethyl acetate was employed as the extraction solvent due to its intermediate polarity, which enables efficient recovery of a wide spectrum of phytochemicals, including flavonoids, phenolics, tannins, and terpenoids, all of which are known contributors to antimicrobial activity [7].

The observed variation in metabolite yield among the isolates may reflect intrinsic differences in fungal metabolic capacity, growth kinetics, and adaptation to the fermentation substrate. Such variability is commonly reported among endophytic fungi and is influenced by both genetic and environmental factors [20]. Despite relatively comparable yields, significant differences were observed in the bioactivity profiles of the extracts, highlighting that metabolite quantity does not necessarily correlate with bioactivity, but rather with the specific chemical composition of the extracts.

The antimicrobial assays demonstrated that the fungal metabolites exhibited varying degrees of activity against both Gram-positive and Gram-negative bacteria, as well as fungi.

Notably, the R33P metabolite displayed the broadest antimicrobial spectrum, inhibiting all tested organisms at varying concentrations. This suggests the presence of potent and possibly multiple bioactive compounds with diverse mechanisms of action. In contrast, the R12 metabolite showed limited activity against *P. aeruginosa* and *S. lutea*. The resistance observed in *P. aeruginosa* is consistent with its well-documented intrinsic and acquired resistance mechanisms, including efflux pumps, low membrane permeability, and enzymatic degradation of antimicrobial agents [21]. These features make it one of the most challenging pathogens to treat and a critical target in antimicrobial research.

The MIC results further substantiated the antimicrobial potential of the extracts, with R33P demonstrating strong activity across multiple bacterial strains. The particularly low MIC observed against *S. aureus* (31.25 mg/ml) suggests a high level of susceptibility, which may be attributed to differences in cell wall structure between Gram-positive and Gram-negative bacteria. Similarly, the low MIC of R22P against *E. coli* (15.625 mg/ml) indicates potent antibacterial activity even at minimal concentrations, reinforcing the therapeutic promise of these metabolites. The determination of MBC and MFC values confirmed the bactericidal and fungicidal properties of the extracts, indicating that the metabolites are not merely inhibitory but capable of eliminating microbial cells. This is a critical consideration in the context of antimicrobial resistance, where bactericidal agents are often preferred for effective treatment.

The antifungal activity observed against *C. albicans* and other fungal isolates further highlights the broad-spectrum nature of these metabolites. Endophytic fungi are known to produce structurally diverse compounds, including polyketides, alkaloids, and peptides, which contribute to their antifungal efficacy [22]. The differential activity observed among the isolates may be attributed to variations in metabolite composition and concentration.

CONCLUSION

This study provided insight into the antimicrobial effects, anti-inflammatory effects, and toxicological effects of the secondary metabolites of endophytic fungi isolated from the roots of *P. americana* (Mill.). The results of this research indicate that the bioactivities and anti-inflammatory properties of each fungal isolate varied. R12 and R33P demonstrated the best antimicrobial efficacy against the bacteria, while R22P demonstrated the best antimicrobial efficacy against the fungi. These differences in performance may be due to the availability of nutrients, the specific requirements of fungi and bacteria, the presence of other microorganisms, the pH of the environment, the presence of inhibitory substances, and the different concentrations of the fungi metabolites. In addition, the acute *in vivo* anti-inflammatory study shows the positive effect and efficacy of the fungi metabolites against inflammation. In the toxicity assay carried out, there is no significant difference between the baseline analysis and the final analysis. This observation suggests that the fungi isolate's ability to produce antimicrobial, anti-inflammatory, and less toxic compounds.

The *in vivo* anti-inflammatory assessment revealed that all extracts exhibited measurable anti-inflammatory effects, with R33P demonstrating the most pronounced activity, comparable to the standard drug diclofenac. This suggests that the metabolites may interfere with inflammatory pathways, potentially through inhibition of prostaglandin synthesis or modulation of cytokine production. Such findings are consistent with previous reports on plant-derived and endophyte-derived metabolites exhibiting anti-inflammatory properties [18,23]. However, the relatively lower activity observed with R12 indicates that not all endophytic metabolites possess equivalent pharmacological potential.

Toxicological evaluation revealed mild but notable alterations in biochemical and haematological parameters. The observed elevation in urea and creatinine levels suggests possible renal stress or impaired kidney function, which may be linked to metabolite-induced toxicity or dehydration. Similarly, increased levels of liver enzymes (ALT, AST, and ALP) indicate potential hepatocellular injury or metabolic disruption. These findings are important, as they highlight the need for careful dose optimisation and further purification of the active compounds to minimise toxicity. The haematological changes, including increased red blood cell counts and decreased white blood cell counts, may reflect physiological stress or immunomodulatory effects of the metabolites. The reduction in WBC levels may suggest a suppressive effect on immune function, like certain conventional antibiotics known to induce neutropenia.

This study reinforces the growing body of evidence that endophytic fungi associated with medicinal plants are valuable sources of bioactive secondary metabolites with significant antimicrobial and anti-inflammatory potential. However, the observed toxicological effects underscore the importance of further studies focused on compound isolation, structural characterisation, and mechanistic evaluation. Such investigations are essential to fully harness the therapeutic potential of these metabolites while ensuring safety and efficacy in drug development.

The results show that *P. americana* (Mill.) plays a crucial role in determining the growth and antimicrobial metabolite production of the endophytic fungi. The study highlights the importance of understanding that plants produce endophytic fungi, which also produce bioactive compounds that have effects against fungi and bacterial microorganisms, as well as anti-inflammatory activities. Further work should be carried out on the *P. americana* (Mill.) plant to isolate fungal endophytes from the different plant parts and to fully characterise them and understand the mechanisms behind their interactions.

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AUTHORS' CONTRIBUTION

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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