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ABSTRACT:

Endophytic microorganisms are recognized as potential source of novel chemical molecules that might be useful in the treatment of infectious diseases. In this study, six medicinal plants (*Morinda citrifolia*, *Plumeria rubra*, *Artocarpus altilis*, *Musa nana*, *Sansevieria trifasciata* and *Saccharum officinarum*) traditionally. They are used for treatment or management of symptoms such as cough, fever and skin diseases, were investigated for the antimicrobial activity of metabolites produced by endophytic microorganisms. Seven endophytes were isolated using the mycological media, potato dextrose agar (PDA). Three of the endophytes were identified as fungi from the *Aspergillus* species, three as actinomycetes and one as a gram negative bacterium. All endophytic isolates were subjected to anti-bacterial, anti-tuberculosis (TB), anti-fungal, anti-HIV, and cytotoxicity assays using micro-broth dilution assay technique in sterile 96-well plates. All isolates displayed antimicrobial activity inhibiting at least one of the test pathogens at the concentration of 100µg/ml. Of the 7 endophytic isolates, 6 isolates were active against TB, 7 were active against the bacterial test pathogens, and 2 were active against fungi while none were active against HIV. From the 7 isolates, 3 were non-toxic to mammalian cells at a concentration of 100µg/ml.

KEYWORDS: Antimicrobial activity, endophytes, *Morinda citrifolia, Plumeria rubra, Artocarpus altilis*, *Musa nana*, *Sansevieria trifasciata* and *Saccharum officinarum*.

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INTRODUCTION:

Nearly all plant species host endophytes, but few of these microorganisms have been characterized [1]. Antibiotics, antimycotics, immunosuppressant, and anticancer compounds are only a few examples of what endophytes have been found to produce in the recent past [2]. Thus, the prospects of finding new drugs that may be effective candidates for treating newly emerging diseases in humans, plants, and animals are great. A recent study noted that endophytes from tropical regions produced significantly more bioactive secondary metabolites than those from temperate parts of the world [3].

Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals [4]. Studies done by Strobel and colleagues have demonstrated endophytic microbes possessing antibacterial, antifungal, anti-tuberculosis, antiviral and anticancer agents [5, 6]. To name a few that have provided modern medicine with valuable new cures include penicillin from the fungus *Penicillium notatum*, bacitracin from the bacterium *Bacillus subtilis* and taxol, an important chemotherapeutic agent, is synthesized by an endophyte of the Pacific Yew tree [6]. A newly described species of *Pestalotiopsis*, an endophytic fungus *Pestalotiopsis jesteri*, from the Sepik River area of Papua New Guinea (PNG) produces jesterone and hydroxy-jesterone which exhibit antifungal activity against a variety of plant-pathogenic fungi [7]. An additional secondary metabolite identified as ambuic acid, is an antifungal agent which has been recently described from several isolates of *P. microspora* found as representative isolates in many of the world's rainforests including PNG [8]. PNG is not only located in the tropical region but rich in biodiversity and harboring a variety of medicinal plants. These may be a potential source of drug discovery from endophytes. The current study was carried out to determine the antimicrobial activity of secondary metabolites biosynthesized by endophytes obtained from some medicinal plants in the Central Province.

METHODOLOGY:

Collection of Plant Samples and Isolation of Endophytes

Six plants (*Morinda citrifolia, Plumeria rubra, Artocarpus altilis, Musa nana, Sansevieria trifasciata* and *Saccharum officinarum*) were selected based on their use in (Table 1) traditional medicine for the treatment of cough, fever and various infections [9, 10]. Plant parts such as leaves, stem, and petioles were collected in the field, stored in sterile plastic bags, and subsequently processed for isolation of endophytes.

Table 1: Medicinal plants used in the study and their traditional uses

Plant species	Family	Plant material	Medicinal use	Reference
Morinda citrifolia	Rubiaceae	Leaves & petiole	Cough & TB	[9]
Plumeria rubra	Apocynaceae	Leaves	Scabies and stings from insect bites	[10]
Artocarpus altilis	Moraceae	Leaves	Cough & tonsillitis	[10]
Musa nana	Musaceae	Stem	Fever	[10]
Sansevieria trifasciata	Agavaceae	Leaves	Ringworm & fungal disease	[10]
Saccharum officinarum	Poaceae	Leaves	Sore throat	[10]

The collected samples were washed thoroughly with sterile distilled water and air dried. The materials were then surface sterilized by immersing them sequentially in 70% ethanol for 3min and 0.5% NaOCl for 1min and rinsed thoroughly with sterile distilled water [11]. The excess water was dried under laminar airflow chamber. Using a sterile scalpel outer tissues were removed and inner tissues of 0.5cm2 size were dissected and placed on petri-plates containing Potato Dextrose Agar (PDA) media [12, 13]. The plates were then incubated at 25±2°C until growth appeared. The explants were observed once a day for the growth of endophytic microorganisms. Growths from the plated explants were immediately transferred into PDA slant and maintained at 4°C. The endophytic isolates were identified using staining techniques based on their morphological and reproductive characters using standard identification manuals [14, 15]. Isolates that possessed typical characteristics of fungal growths were identified using lacto phenol cotton blue stain while those having bacterial characteristics were identified using gram staining. All the isolates were maintained in Potato dextrose agar slant and sterile water. The experimental procedures were carried out in the Molecular Biomedicines and Biodiscovery Laboratory (MBBL) in the School of Medicines and Health Sciences (SMHS), University of Papua New Guinea (UPNG).

Lignin Degradation Assay via Poly-R agar clearance (Poly-R agar)

Lignin modifying enzyme (LME) Basal Medium (LBM) was prepared by supplementing with 0.02 % w/v polymeric dye Poly-R 478 (Poly-R; Sigma) and 1.6 % w/v agar and autoclaved. Then 1.0 ml of a separately sterilized 20 % w/v aqueous glucose solution was added to each 100 ml of growth medium prepared and aseptically transferred to Petri dishes. After inoculation of test fungus on the Petri dishes, they were incubated at 25 oC in darkness and

examined daily for 10 days. The production of LME was recorded as clearance of violet colored medium [16].

Cultivation and Extraction

The endophytes were cultivated in Potato dextrose broth (Difco) by placing agar blocks of actively growing pure culture (3mm in diameter) in 250ml Erlenmeyer flasks containing 100ml of the medium [12]. The flasks were incubated at 25±1 ° C for 3 weeks with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile cheesecloth to remove the mycelia mats for fungal isolates. The endophytic metabolites were extracted by sequential solvent extraction procedure using hexane, dichloromethane, ethyl acetate and methanol as solvents. Equal volumes of the filtrate and the solvents was taken in a separating funnel and shaken vigorously for 10 min. The solution was then allowed to stand, and the solvent was collected. The organic solvents were evaporated and the resultant residue was dried in vacuum evaporator to yield the crude extracts. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) at 100µg/ml for antimicrobial bioassay [17].

Evaluation of Antimicrobial Activity

Assays that were carried out to determine antimicrobial activity of endophytic crude extracts included anti-bacterial assay, anti-fungal assay, anti-tuberculosis (TB) assay and an anti-HIV assay. Antimicrobial assays were carried out using micro broth dilution assay technique in sterile 96- microtiter plate [18]. Four common human pathogens were used in the present study. The test pathogens included a Gram-positive bacteria - *Bacillus subtilis* (Bs), two Gram-negative bacteria - *Escherichia coli* (Ec) and *Francisella novicida* (Fn), a fungal pathogen - *Candida albicans* (Ca) and a TB pathogen - *Mycobacterium tuberculosis*. All the test pathogens were obtained from College of Pharmacy, Department of Pharmacology University of Utah, USA.

Antibacterial and antifungal assay

Bacterial and fungal strains were assayed according to CLSI/NCCLS method M07-A5 in Mueller-Hinton II broth [19]. The micro-broth dilution assay technique was carried out using 96-microtitre plates. The positive control and endophytic extracts were dissolved in DMSO to produce stock solutions at 100 µg/ml. Ampicillin was used as the control for *E. coli*, gentamycin was used as the for control *B. subtilis*, kanamycin was used as the control for *F. novicida* and econazole was used as the control for *C. albicans* DMSO was used as a negative control. Initially, the human pathogen cultures were dispensed in 200 µL of Muella Hinton II Broth medium into a 96-well culture plates at 100,000 cells per well. Then 1.0 µL of the control and 1.0 µL of the extract were added in duplicate wells. After 24 hours incubation at 37oC, absorbance at 570 nm was measured using a Biorad Model 450 microtiter plate reader (Biorad, Hercules, CA). All data

were corrected against media-only blank wells. The percent inhibition was derived as the fraction of the sum of the test wells over the sum of control wells subtracted from unity and multiplied by 100. This was calculated using the formula below:

$$\% \text{ inhibition} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

Anti-tuberculosis (MTT) Assay

Inhibition of M. tuberculosis H37Ra growth was quantified using a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay modified from previously published methods [20, 21]. Rifampacin and plant extracts were dissolved in DMSO to produce stock solutions at 100µg/ml. DMSO was used as a negative control. M. tuberculosis cultures were dispensed in 200 µL of ADC (albumin-dextrose complex) enriched 7H9 medium into a 96-well culture plates at 100,000 cells per well. One µL of DMSO (control) or DMSO containing drug or extract was added in triplicate wells. After four days incubation at 37oC, 11 µL of sterile MTT [5 mg/mL in PBS (phosphate buffered saline)] was added and incubated overnight. Viable M. tuberculosis metabolizes the MTT to an insoluble purple formazan product that was solubilized by the addition of 50 µL of a solubilization solution [5% SDS (sodium dodecyl sulfate) w/v, 50% DMF (dimethylformamide) v/v, 45% H2O v/v]. Absorbance was read at 570 nm and all data were corrected against media-only blank wells. The percentage inhibition was calculated using the formula as shown in the antibacterial and antifungal assay.

Cytotoxicity Assay

CEM-TART (human T- cell lymphoblastic leukemia) cells were maintained in Rosewell Park Memorial Institution (RPMI) culture medium supplemented with 20% fetal bovine serum and standard concentrations of penicillin and streptomycin (10,000 U and µg/liter respectively). Doxorubicin (1 µg/mL) was used as the positive control. On the first day cells were grown in suspension and seeded in a 96-well plate (2 x 104 cells/well). After 24 hours the medium was exchanged [22]. Solutions of the crude extracts were prepared in DMSO and the final concentrations (100µg/ml) were achieved by direct dilution into the cell medium. The samples or the vehicle were added and incubated for a period of 48 hours. After this interval, 50 µL of a MTT solution (5 mg/mL in PBS) were added to each well and the cells were incubated for an additional 3 hours. Subsequently, the medium was removed, cells were washed two times with PBS, and 300 µL of isopropanol–HCL (0.04mol/L) were added. Cell viability was determined by absorbance measurements at 570 nm. The color intensity

is correlated with the number of healthy living cells [23]. Cell survival was calculated using the formula:

$$\% \text{ survival} = \frac{100 \times (\text{abs. of treated cells} - \text{abs. of culture medium})}{\text{abs. of untreated cells} - \text{abs. of culture medium}}$$

where "abs." means absorbance

Anti-HIV Assay

The cytoprotection assay used in this study was developed by Kiser and co-workers [24]. In brief, CEM-TART and the 1A2 subline cells were maintained in RPMI culture medium supplemented with 20% fetal bovine serum and standard concentrations of penicillin and streptomycin (10,000 U and µg/liter respectively). When grown at, 5% CO2, and maintained at a concentration between 5 x l04/ml and 2 x l06/ml, the cells doubled approximately every 24 hours. For infection with virus, 2 x106 CEM-TART cells were pelleted by centrifugation and resuspended in 0.5 ml of virus stock (freshly thawed). After 1 hr incubation at 37°C the cells were pelleted and resuspended at 2 x l05/ml for growth. Virus stock was prepared by centrifugation of a virus-producing culture of CEM-TART cells, 7 to 9 days after infection, and simply freezing aliquots of virus-containing supernatant. Following overnight incubation, the cells were counted and dispensed into a 96 well micro titer culture dish at 20,000 cells/well in 1.5 ml culture medium. Serial dilutions of test extracts were added to this (concentrations spanning 5 logs from 50ug/ml to 0.05µg/ml). Cultures were examined microscopically daily, and quantified every other day to generate cell growth inhibition curves. Cells were counted by removing 10 µl of cells and mixing with an equal volume of MTT dye.

Cultures are treated in quadruplicate with azidothymidine (AZT) as a positive control. Significant cytoprotection is evident at AZT concentrations of 0.5 to 5 µg/ml. Concentrations of AZT above 10 µg/ml are cytotoxic. This modified assay uses MTT exclusion as an alternative means of quantifying CEMTART and CEM-1A2 cell growth. The T-cell leukemia subline 1A2 is more sensitive than the TART line to cell killing by HIV infection and therefore it was selected as the primary cell line for this assay [24, 25]. When the total cell population was quantified daily over 4 days, it was found that the number of cells in non-infected and infected cultures decreased after approximately 10 days. The

DMSO concentration was less than or equal to 1% culture volume. Only HIV-IM Δtat rev strain was utilized. Absorbance was read at 570 nm and all data were corrected against media-only blank wells. The percentage inhibition was calculated using the formula as shown antibacterial and antifungal assay.

RESULTS:

Identification of organisms

The actinomycetes species were isolated from the petiole and of *M. citrifolia*, and the leaves of *M. citrifolia* and *P. rubra*. The *Aspergillus* species were isolated from the leaves of *A. altilis, S. trifasciata* and *S. officinarum* while the Gram – bacteria was isolated from the stem of M. nana.

Enzymatic Activity of Lignin Degradation Assay

Only fungal organisms were subjected to the lignin degradation assay because they are known to be good degraders of lignin. All fungal isolates indicated negative reaction thus no production of lignin degrading enzymes was observed. In the present study, the most active crude extract against the bacterial pathogen *E. coli* was the ethyl acetate extract from the *Aspergillus* sp. isolated from the leaf of *S. officinarum* having a total inhibition of 73% which was compared to the positive control, ampicillin which had a total inhibition of 100% (table 2).

B. subtilis were highly susceptible to all of the crude extracts, especially the methanol extracts. The most active crude extract was from the methanol extract of an actinomycete isolated from the leaf of *M. citrifolia* having a total inhibition of 118% which was compared to the positive control, gentamycin which had a total inhibition of 104% (table 2). *F. novicida* was also sensitive to most of the extracts. The most active crude extracts was from the methanol extract of a gram negative bacterium isolated from the stem of *M. nana* having a total inhibition of 91% compared to the positive control, kanamycin which had a total inhibition of 100 % (table 2).

One fungal pathogen was tested for sensitivity to the endophytic crude extracts. The most active crude extract was from the dichloromethane extract of an *Aspergillus* sp. isolated from the leaf of *A. altilis* having a total inhibition of 91%, which compared to the positive control, econazole that had a total inhibition of 88.16% (table 2). The anti-tuberculosis assay demonstrated that the most active endophytic crude extract was from the methanol extract of a gram negative bacterium isolated from the stem of M. nana with a total inhibition of 91% compared to the positive control, rifampacin, which had a total inhibition of 100.66% (table 2).

Antimicrobial Assay

Table 2: Antimicrobial activity data of the titled controls and endophytic crude extracts at 100µg/ml by the broth dilution assay technique after 24 hours.

Percentage inhibition (%)

	Bacteria												Fungi				Mycobacterium				Virus			
Controls	**E.c**				**B.s**				**F.n**				**C.a**				**MTB**				**HIV**			
Negative Control	No Percentage Inhibition																							
Ampicillin	100				—				—				—				—				—			
Gentamycin	—				104				—				—				—				—			
Kanamycin	—				—				100				—				—				—			
Econazole	—				—				—				88				—				—			
Rifampacin	—				—				—				—				100				—			
Azidothymidine	—				—				—				—				—				77			
Crude extract	**H**	**D**	**E**	**M**	**H**	**D**	**E**	**M**	**H**	**D**	**E**	**M**	**H**	**D**	**E**	**M**	**H**	**D**	**E**	**M**	**H**	**D**	**E**	**M**
Mcp	12	13	4	15	23	21	30	97	75	61	32	68	4	84	54	20	75	61	32	68	36	20	35	16
Mcl	4	2	9	1	18	36	2	16	45	81	60	66	7	13	18	17	45	81	60	66	30	22	31	14
Prl	4	21	14	9	83	48	1	107	39	83	49	67	16	15	17	19	39	83	49	67	16	31	15	35
Aal	14	15	20	5	24	12	1	114	40	39	12	90	2	91	5	17	40	39	12	90	30	16	27	7
Mns	5	29	8	11	22	113	2	95	68	91	77	91	14	13	17	1	68	39	77	91	57	61	41	53
Stl	25	43	20	4	1	104	1	116	67	79	74	83	12	18	17	11	67	39	74	83	58	50	48	9
Sol	51	28	73	16	114	113	87	113	68	13	45	74	13	18	48	15	68	13	45	74	51	45	54	3

E.c-*Escherichia coli*, B.s-*Bacillus subtilis*, F.n - *Francisella novicida* , C.a- *Candida albicans,* MTB – *Mycobacterium tuberculosis*, HIV - Human Immune Deficiency Virus
H – hexane, D – dichloromethane, E – ethyl acetate, M – methanol
Mcp – *Morinda citrifolia* (petiole), Mcl - *Morinda citrifolia* (leaf), Prl – *Plumeria rubra* (leaf), Aal – *Artocarpus altilis* (leaf), Mns – *Musa nana* (stem). Stl - *Sansevieria trifasciata* (leaf), Sol - *Saccharum officinarum* (leaf)
% inhibition activity - A $\geq$ 70, 30 $\leq$ Q < 70, I < 30 [A – active, Q – questionable, I – inactive]

Table 3: Cytotoxicity activity data of endophytic crude extracts at 100 µg/ml by the broth dilution assay technique after 24 hours

	Percentage survival (%)			
Control		**Human T cells**		
Concentration	**1µg/Ml**	**0.1µg/mL**	**0.01 µg/mL**	**0.001µg/mL**
Negative Control		No Percentage Inhibition		
Doxorubicin	1.78	7.1	22.4	99.9
Crude extract	**Hexane**	**Dichloromethane**	**Ethyl acetate**	**Methanol**
Mcp	30	2	5	3
Mcl	71	75	55	98
Prl	110	62	7	31
Aal	71	60	1	5
Mns	4	57	1	15
Stl	3	34	8	2
Sol	40	2	1	85

Mcp – *Morinda citrifolia* (petiole), Mcl - *Morinda citrifolia* (leaf), Prl – *Plumeria rubra* (leaf), Aal – *Artocarpus altilis* (leaf), Mns – *Musa nana* (stem). Stl - *Sansevieria trifasciata* (leaf), Sol - *Saccharum officinarum* (leaf)
% survival activity - Active < 30% Survival, Questionable 30 < 70% Survival, Inactive > 70% Survival

None of the endophytic crude extracts tested were HIV active, thus all inhibited less than 50 % of AZT in HIV infected cells (table 2). The positive control used to compare the potency of the crude endophytic extracts was AZT.

The cytotoxicity results indicate extracts of three isolates have limited toxicity to human T cells. The highest number of Human T cells survived in the crude extract of the hexane extract from an Actinomycete isolated from the leaf of *P. rubra* having a percentage survival of 110% (over 100% because the artifact of clearing of medium) followed by methanol extract of an Actinomycete isolated from the leaf of *M. citrifolia* having a percentage survival of 98 % and having a percentage survival of human T cells at 85 % was the methanol extract of an aspergillus's sp. isolated from the leaf of *S. officinarum* (table 3). The effectiveness of the endophytic crude extracts was compare to four different concentrations of doxorubicin and their number of surviving human T cells as indicated in table 3.

DISCUSSION:

Medicinal plants are reported to harbor bioactive endophytes. These microorganisms are currently considered as a source of novel secondary metabolites for medical, agricultural and/or industrial exploitation. Endophytes are thought to preclude bioactive compounds because they are occupying literally millions of

unique biological niches [1]. Very few metabolites from endophytes are reported in literature. Much work needs to be done in this field.

Six potential medicinal plants used locally by communities in the Central Province were selected for endophytic studies (Table 1). All the plant species were found colonized with endophytic microorganisms (fungi and bacteria). The endophytes were isolated using mycological media potato dextrose agar (PDA). Altogether 7 endophytic microorganisms were isolated from the six medicinal plants, out of which 3 were identified as fungi from the *Aspergillus* species, 3 as Actinomycetes and 1 as a gram negative bacteria.

The lignin degradation assay indicated a negative reaction by all fungal isolates thus there was no production of lignin degrading enzymes. Decolonization of the polymeric dye Poly-R 478 by fungi has been positively correlated with production of the lignin degrading enzymes including laccase [16]. This simple test gives clear results since decolonization of the violet dye can be easily observed.

In the present study, ethyl acetate extract from the *Aspergillus* sp. isolated from the leaf of *S. officinarum* exhibited a total inhibition of 73% of *E. coli* in which appeared to be the most active extract as showed in table 2. From literature, there are no findings of endophytic fungus isolated from *S. officinarum* with antibacterial properties [26].

B. subtilis was highly susceptible to all of the crude extracts, especially the methanol extracts. According to literature, methanol is known to be a far more consistent extraction of antimicrobial substances from compared to other solvents such as water, ethyl acetate, dichloromethane, butanol, etc. [27]. The most active crude extract was from the methanol extract of an Actinomycete isolated from the leaf of *M. citrifolia* having a total inhibition of 118% of *B. subtilis* (table 2). From literature, endophytes isolated from *M. citrifolia* had demonstrated broad antimicrobial activity such as having antibacterial and antifungal properties [28].

The most active crude extract against *F. novicida* was from the methanol extract of a gram negative bacterium isolated from the stem of *M. nana* demonstrating a total inhibition of 91% as seen in table 2. From literature there are no reported findings of endophytes isolated from *M. nana* demonstrating antibacterial properties. The most active crude extract was the dichloromethane extract of an *Aspergillus* sp. isolated from the leaf of *A. altilis* that appeared to have a total percentage inhibition of 91% against *C. albicans* as observed in table 2. The total percentage inhibition of econozole by *C. albicans* was lower compared to the crude extract as indicated in the results in table 2. Endophytes

have been isolated from *A. altilis* however no antifungal activity has been indicated but there had been demonstration of antitumor activity [29].

According to the present study, the anti-tuberculosis assay demonstrated that the most active endophytic crude extract was from the methanol extract of a gram negative bacterium isolated from the stem of *M. nana* having a total inhibition of 91% by the *M. tuberculosis* (table 2). Interestingly, there is no reported study of endophytes being isolated from M. nana.

None of the endophytic crude extracts tested were active against HIV; all inhibited less than 50 % of AZT in HIV infected cells. The cytotoxicity results indicate extracts of three isolates have minimal toxicity to human T cells. These were the hexane extract from an Actinomycete isolated from the leaf of *P. rubra*, the methanol extract of an Actinomycete isolated from the leaf of *M. citrifolia*, and the methanol extract of an *Aspergillus* sp. isolated from the leaf of *S. officinarum* (table 3).

Thus three endophytic extracts that appeared non -toxic to mammalian or human T cells also demonstrated antimicrobial and anti-TB properties. These are candidates that need to be further tested and developed for compound potential pharmaceuticals. The other crude endophytic extracts that were considered more toxic to Human T cells could be considered as anticancer agents and therefore can be subjected to further anticancer assays to quantify the existing results.

CONCLUSION:

Results obtained in this study indicate that endophytic microorganisms isolated from PNG medicinal plants produced some crude extracts that possess antimicrobial potentials especially against pathogenic bacteria and fungi. It was also observed that some endophytic microorganisms produced cytotoxic extracts that could be considered as anticancer agents. The crude extracts of the three isolates that were not toxic to mammalian cells should be subjected for chemical analysis for compound purification and characterization. Our data supports the general scientific opinion that endophytic microorganisms of medicinal plants are potential sources of bioactive compounds.

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