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Antimicrobial and Antiplasmodial Activities of Endophytic Fungi Associated with *Psidium guajava*

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Abstract

Infections due to antimicrobial-resistant microorganisms have become widespread in recent years. Thus, searching for novel antimicrobial agents to combat such pathogens has become crucial. The current study aimed to evaluate the antimicrobial, antiplasmodial, and immunomodulatory activities of the extracts of endophytic fungi isolated from *Psidium guajava*. Isolation, identification, fermentation, and extraction of the secondary metabolites of the fungal endophytes were carried out following standard procedures. The extracts were subjected to High-Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis to detect their bioactive components. The Antimicrobial activity and Minimum Inhibitory Concentration of the fungal extracts were evaluated against pure cultures of *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Klebsiella pneumonia* using Agar well diffusion and Agar dilution method respectively. The acute toxicity study (LD50) was carried out using Lorke's method. The extracts were tested *in vivo* in mice for antiplasmodial activity against *Plasmodium berghei* and *in vitro* against *Plasmodium falciparum* using Peter and Reyley's curative test method and WHO standardized micro-test system with modification respectively. The immunomodulatory activity of the extracts was evaluated by cyclophosphamide-induced myelosuppression (hematological parameters). Active extracts were further subjected to Delayed-Type Hypersensitivity Response (DTHR) and Haemagglutination Inhibition Assay using Sheep Red Blood Cells as antigens. The result showed *Alternaria* sp. (PGL1, PGL2, PGL3), from *P. guajava*. The HPLC-DAD analysis revealed the presence of bioactive compounds previously reported to have antimicrobial, antiplasmodial, and immunomodulatory properties. The fungal extracts exhibited varying degrees of antimicrobial activity. The LD50 of the fungi extracts was>5000 mg/kg in mice. The extracts at 100 and 200 mg/kg body weight in mice showed varying degrees of antiplasmodial activity. Growth of *P. berghei* was significantly ($p<0.001$) inhibited, curative effect ranges from 59.09 – 100%. Schizont maturation of *P. falciparum* isolates was inhibited and the highest level of inhibition was observed at 1 mg/ml ($p<0.05$). The fungal extracts reversed the effect of cyclophosphamide-induced reduction in total white blood cell counts and % neutrophil. This study showed that the tested plant harbors species of endophytic fungi that contain numerous secondary metabolites. The endophytic fungi showed prophylactic, immunostimulatory, and antiplasmodial activities, which can be exploited to develop antimicrobial, antiplasmodial, and immunomodulatory agents.

Keywords: *Psidium guajava*, antimicrobial, antiplasmodial, endophytes, HPLC_DAD.

INTRODUCTION

Antimicrobial resistance is an urgent global public health threat, killing at least 1.27 million people worldwide and associated with nearly 5 million deaths in 2019 (CDC, 2022). There is a continuing need for new and improved drugs to tackle malaria, which remains a major public health problem, especially in tropical and subtropical regions of the world. The widespread appearance of drug-resistant malaria parasites, even to newly developed second and third-generation therapeutics such as artemisinin and its derivatives, makes the development of novel antimalarial drug treatments all the more urgent (D'Alessandro, 2009). Natural products represent credible sources of new antiplasmodial agents for antimalarial drug development (Ateba, 2018).

Interestingly, during the last 20 years, it has been observed that much of the wealth of microbial biodiversity with novel biochemistry and secondary metabolite production resides in endophytic association with plant tissues (Porras-Alfaro *et al.*, 2011). Endophytes are metabolically more active than their free counterparts due to their specific functions in nature and activation of various metabolic pathways needed to survive in the host tissues (Riyaz-Ul-Hassan *et al.*, 2018). The endophytic fungi of medicinal plants are important because of their capacity to produce structurally and biologically unique natural bioactive compounds (Chowdhary *et al*., 2015; Strobel *et al.*, 2004; Gunatilaka, 2006; Glienke *et al*., 2012). Thus, investigating endophytic fungi from medicinal plants used to treat malaria and microbial infections can lead to new antimalarial and antimicrobial drug discovery. They are known as an important source of various bioactive secondary metabolites which, once isolated and characterized, may also have potential for use in industry, medicine, and agriculture (Strobel *et al*., 2003, Strobel *et al*., 2004, Ujam *et al.*, 2020, Ujam *et al*., 2022).

Psidium guajava (*P. guajava*) is a fructiferous tree from the *Myrtaceae* family. It is animportant food crop and medicinal plant in tropical and subtropical countries, widely used as food (due to its sweet and vitamin-containing fruits) and in folk medicine worldwide (Gutiérrez *et al*., 2008). This plant is a source of various phytochemicals such as saponins, triterpenic acids, and flavonoids (Gutiérrez et al., 2008). Additional pharmacological properties attributed to extracts of *P. guajava* include antioxidant, hepatoprotective, antiallergic, antigenotoxic, antiplasmodial, cytotoxic, cardioactive, anticough, anti-inflammatory, antinociceptive, hypoglycemic, and antidiabetic activities, thus, supporting its uses in traditional medicine (Gutiérrez *et al*.,2008).

MATERIALS AND METHODS

Plant Collection

Healthy and mature leaves of *Psidium guajava* were collected in Enugu State, South-East Nigeria. They were identified at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria where a voucher specimen was deposited under the number PCG474/A/045. Plant material was directly brought to the laboratory in sterile bags and processed within a few hours after sampling.

Test Microorganisms

Clinical microbial strains of *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Salmonella typhi, Aspergillus niger, and Candida albicans* were used to evaluate the antimicrobial activity of the isolated fungal endophyte extracts.

Experimental Animals

Swiss albino rats and mice weighing 130-164 g and 25-28 g respectively of either sex were used in the study. They were obtained from the animal house of the Pharmacology department, University of Nigeria, Nsukka. Animals were housed in standard cages and adequately fed with livestock feed and water *ad libitum* for one week to ensure acclimatization. For ethical reasons, all animals were sacrificed at the end of the study according to American Veterinary Medical Association (AVMA) guidelines (AVMA, 2013), and the experimental protocol was followed according to Guidelines for Care and Use of Laboratory Animals in Biomedical Research (2010).

Standard Drugs

Cyclophosphamides, NONI, Ciprofloxacin, and Miconazole were used as standard drugs.

Malaria Parasite

Chloroquine-sensitive *Plasmodium berghei*, used in the study was obtained from the University of Nigeria, Nsukka, Enugu State Nigeria. Parasite viability was maintained through weekly passage in mice, by aseptic injection of a known amount of parasite into healthy mice every week.

METHODS

Isolation of Endophytic Fungus, Extraction of Its Secondary Metabolites and Identification

The endophytic fungi were isolated from the healthy leaves of *Psidium guajava* following a modified method described by Ujam *et al.*, 2021.

Antimicrobial Evaluation of the Fungal Extract

Antimicrobial screening of the isolated fungal extracts followed our previous method (Ujam *et al*., 2019). The minimum dilution (concentration) of the extract completely inhibiting the growth of each organism was taken as the minimum inhibitory concentration (MIC).

High-Performance Liquid Chromatography-Diode-Array Detection (HPLC-DAD) Assay

The HPLC-DAD analysis was done according to the method described by Ujam *et al*., (2020).

***In vivo* Antiplasmodial Activity of the Endophytic Fungal Extracts**

The in vivo antiplasmodial activity of the extract against blood schizonts of *Plasmodium berghei* was evaluated following Peter and Reyley's curative test method (Peter and Anatoli, 1998). Donor albino mice were infected with chloroquine-sensitive *P. berghei* and a rising parasitemia of 30% was determined using a thin blood film, the blood sample was collected using an EDTA bottle. The collected blood sample was diluted using phosphate-buffered saline (concentration of 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) such that 0.2 ml contained 10,000 infected red blood cells. To avoid variability in parasitemia, all the animals used were infected from the same source. Twenty (20) adult albino mice were used to assess the antiplasmodial effect of the endophytic fungal isolate (PGL1) extract. Animals were inoculated with 10,000 *P. berghei-infected* red blood cells and allowed for three (3) days to establish infection. On day 3 the mice were randomized into four (4) groups of 5 mice each such that the mean parasitemia levels of the groups were almost similar. Groups 1 and 2 were treated with two doses (100 mg/kg and 200 mg/kg) of PGL1 extract respectively while groups 3 and 4 served as the negative and positive controls and were given distilled water (10 ml/kg) and Artemether-lumefantrine respectively (0.3/0.2 mg/kg), while treatment was carried out once daily from day 1 to day 4. On day 4, blood was collected from the tail vein of the mice, and blood films were made using a clean glass slide (Devi *et al*., 2000). The dry blood films were fixed with methanol and subsequently stained with 10% Giemsa for 10 min. They were washed with clean tap water and allowed to air dry. To ensure optimal film quality each film was duplicated. The slides were microscopically examined using x100 magnification in oil immersion (Model Olympus microscope) and the level of parasitemia was assessed. Treatment was continued from day 4 to day 7 and the above procedure was repeated (Dikasso *et al*., 2006). The percentage of curative activity of parasitemia was calculated using the following formula.

$$\%\ Cure\ for\ Parasiteamia\ on\ day\ 3 = \frac{Basal\ parasitaemia\ count - Parasitaemia\ count\ on\ day\ 3}{Basal\ parasitemia\ count}\ x\ \frac{100}{1}\ \ldots\ldots eqn1$$

$$\%\ Cure\ for\ parasitaemia\ on\ day\ 7 = \frac{Basal\ parasitaemia\ count - Parasitaemia\ count\ on\ day\ 7}{Basal\ parasitaemia\ count} x\ \frac{100}{1}\ \ldots\ldots eqn2$$

***In-vitro* Antiplasmodial Activity of the Extracts of the Endophytic Fungal Isolates**

The antiplasmodial assay was carried out based on the *in-vitro* microtechnique method by Riechmann *et al.,* (1978) with little modification as described by Ujam et al.2022.

Statistical Analysis

Results of the study were presented as mean ± Standard error of the mean (SEM) of sample replicate, n=5. Raw data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Turkey's test and independent students. The analysis was done using the statistical package for Social Sciences (SPSS) version 20 for Windows. Statistical significance was established when P<0.05. Graphical illustration was carried out using Microsoft Excel, in 2010.

RESULTS

A total of three fungal endophytes labeled PGL1, PGL2, and PGL3 were isolated from the leaves of *P. guajava*. Results of the preliminary antimicrobial assay of the fungal extracts represented in (Table 1) revealed that at 1 mg/mL, the extract of PGL1 showed antibacterial activity against *S. aureus*, *P. aeruginosa*, and *B. subtilis* with IZD of 4, 11, and 5 mm respectively. The extract of PGL2 showed antibacterial activity against the Gram-negative test bacteria *P. aeruginosa*, *S. typhi*, and *K. pneumoniae* with IZD of 12, 5, and 5 mm respectively. The extract of PGL3 showed antibacterial activity against two out of the five test bacteria- *P. aeruginosa* and *S. typhi* with IZD 7 and 3 mm respectively. The extracts of PGL1 and PGL3 showed antifungal activity against one of the test fungi-*C. albican* with IZD 4, and 5 mm respectively. However, the extract of PGL2 showed no antifungal activity against *C. albican* and *A. niger*. The MIC values of the extract against the test organisms ranged from 0.03125 to 1 mg/mL (Table 2).

Table 1: Antimicrobial activity and zone of inhibition of the endophytic fungal extracts

	Endophytic fungal extract (1 mg/mL)			**Positive control**	**Negative Control**
Test organisms	**PGL1**	**PGL2**	**PGL3**	**Ciprofloxacin (5 µg/mL)**	**DMSO (100% v/v)**
S. aureus	4	0	0	0	0
B. subtilis	0	0	0	7	0
P. aeruginosa	11	12	7	6	0
S. typhi	5	5	3	0	0
K. pneumonia	0	5	0	24	0
				Miconazole (50 µg/mL)	**DMSO (100% v/v)**
C. albicans	3	6	7	14	0
A. niger	0	3	4	21	0

Table 2: Minimum inhibitory concentrations (MICs) of the endophytic fungal extracts

MIC (mg/mL)							
Fungal extracts	***S. aureus***	***B. subtilis***	***S. tyhpi***	***P. aeruginosa***	***K. pnuemoniae***	***A. niger***	***C. albicans***
PGL1	0.25	0.00	0.25	0.06	0.00	0.00	0.25
PGL2	0.00	0.00	0.25	0.03	0.25	1.00	0.25
PGL3	0.00	0.00	0.50	0.06	0.00	0.50	0.125

The in vivo curative test result showed that at 100 and 200 mg/kg concentrations, the extract of PGL1 significantly ($p<0.001$) inhibited the growth of the plasmodium parasite on day 3, at day 7 of the curative experiment. The percentage of curative effect ranges from 82.04 – 90.42. The standard drug artemether-lumefantrine at 120 mg/kg showed a very high curative effect but did not clear the parasites at the end of the treatment. On the other hand, the parasites were seen to increase as the days of treatment progressed with the negative control (distilled water) (Table 3).

The result of the *in-vitro* antiplasmodial assay against *Plasmodium falciparum* is shown in (Table 4). The parasite growth decreases as the concentration of the extracts increases, the negative control (distilled water) had 100% parasite growth. From the experiment, % inhibition of schizont maturation increases as the concentration of the extracts increases. Extract of PGL1 at 1 mg/ml gave 76.67% inhibition of schizont.

Table 3: *In-vivo* antiplasmodial activity of the endophytic fungal extract

Animal Groups	**Treatment**	**Doses (mg/kg)**	**Mean Parasitaemia Count**			**% Cure**	
			B	**D_3**	**D_7**	**D_3**	**D_7**
1	**DW**		43.00 ± 2.00	51.00 ± 1.16	59.00 ± 3.84	-	-
2	**PGL1**	200	47.00 ±0.58	7.00 ± 0.58 [a]	4.50 ± 0.67	82.97	90.42
3		100	44.00 ± 0.58	10.00 ± 1.45 [a]	7.90 ± 1.73	77.27	82.04
4	**AL**		49.00 ± 1.16	4.50 ± 0.87 [a]	3.50 ± 0.87	90.81	92.86

Values are expressed as mean ± SEM, n = 5, * indicates significant difference, *** = a ($p < 0.001$), ** = ($p<0.01$), * = ($p<0.05$); B = Basal, D3 = Day 3 and D7 = Day 7 after inoculation. DW =Distilled Water (Negative control), AL = Artemether-Lumefantrine/20mg-120mg (Positive control)

Table 4: *In-vitro* antiplasmodial activity of the endophytic fungal extract

Concentration of the endophytic fungal extract (mg/mL)				
	0.125	**0.25**	**0.5**	**1.00**
In vitro Schizont Growth Inhibition of *P. falciparum* Isolates by Fungal Extracts (%)				
PGL1	20.00	50.00	53.33	76.67
Positive Control	46.67	66.67	80.00	83.33

$P \geq 0.05$ compared to control; Negative Control = (Distilled water) had 100 % growth. Positive Control = Arthemether-lumefantrine

Figures 1-6 below show the chromatograms and the UV spectra of the detected fungal endophyte extracts as revealed by HPLC-DAD analysis. The compounds are detected with respect to their retention time and are represented alphabetically.

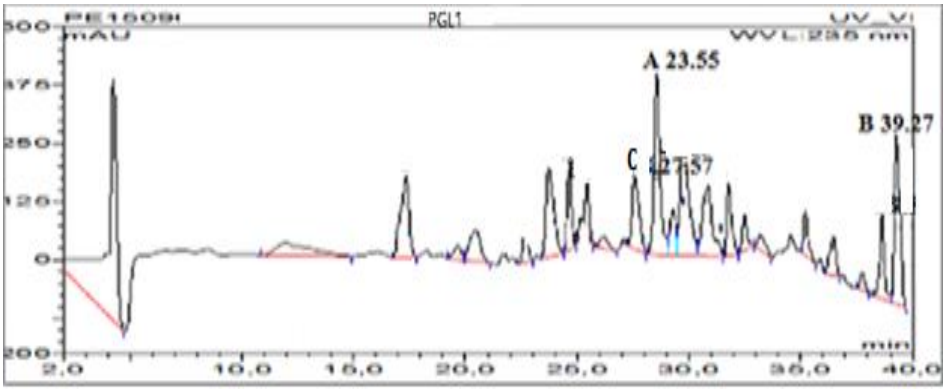
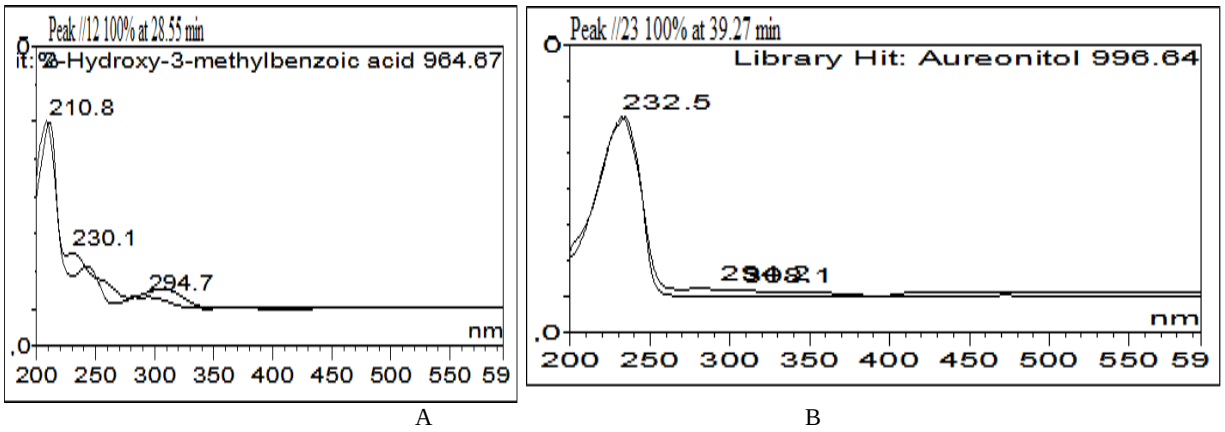


Fig. 1. HPLC-DAD Chromatogram of the isolated Alternaria sp. (PGL1) extract showing the detection of bioactive compounds



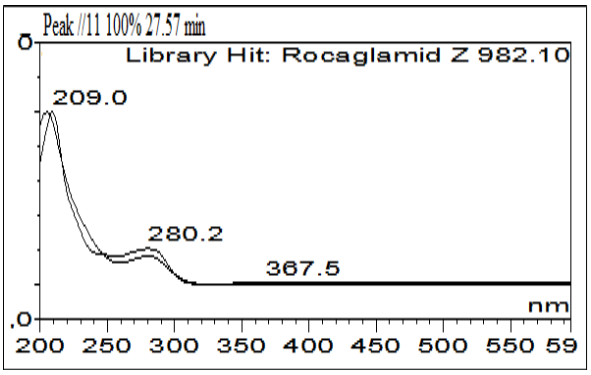


Fig. 2. Ultraviolet (UV) spectra of compounds detected from Alternaria sp. (PGL1) extract. A= 2-hydroxy-3-methyl benzoic acid, B= Aureotinol, C=Rocaglamid

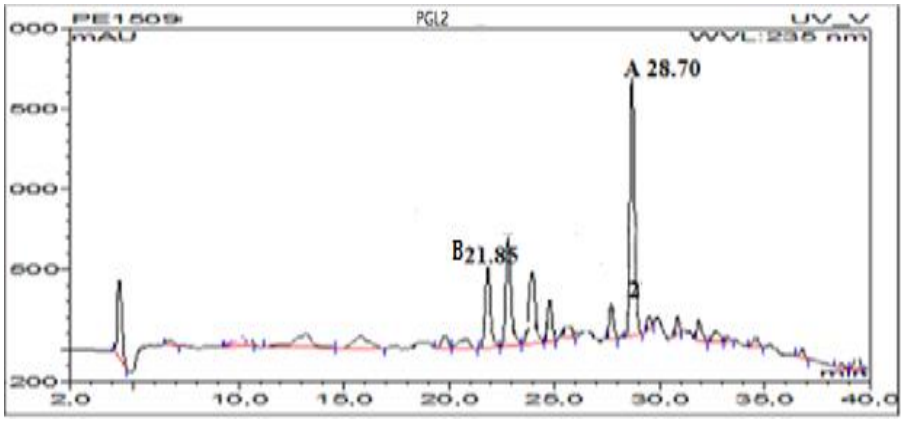
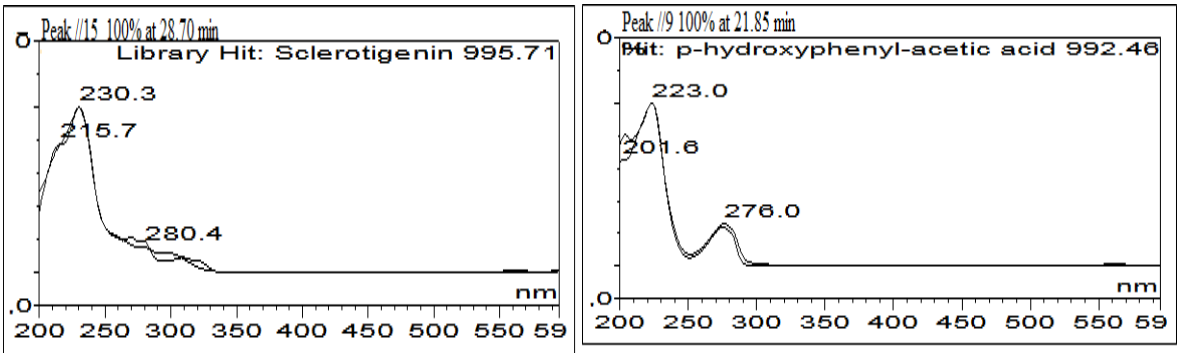


Fig. 3. HPLC-DAD Chromatogram of the isolated Alternaria sp. (PGL2) extract showing the detection of bioactive compounds



A **B**

Fig. 4. Ultraviolet (UV) spectra of compounds detected from Alternaria sp (PGL3) extract. A =Sclerotigenin B = p-hydroxyphenyl-acetic acid

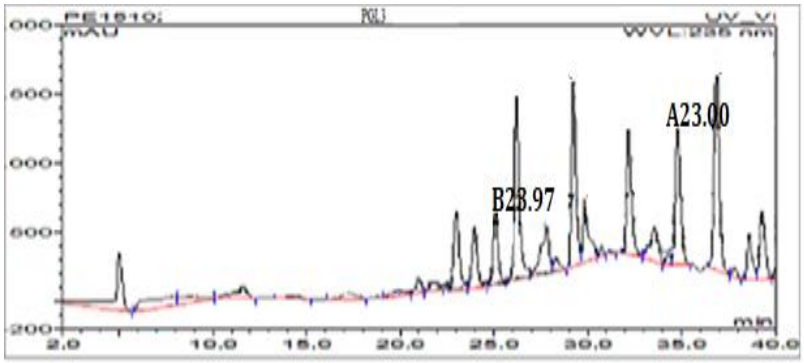
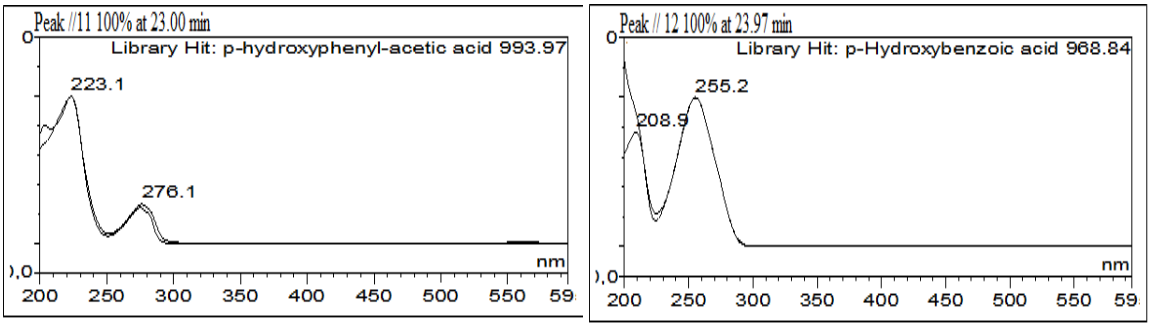


Fig. 5. HPLC-DAD Chromatogram of the detected compounds of Alternaria sp (PGL3) extract showing detection of bioactive compounds A, B



A B

Fig. 6. Ultraviolet (UV) spectra of compounds detected from Alternaria sp. (PGL3) extract. A= p-hydroxyphenyl acetic acid B = p-hydroxybenzoic acid

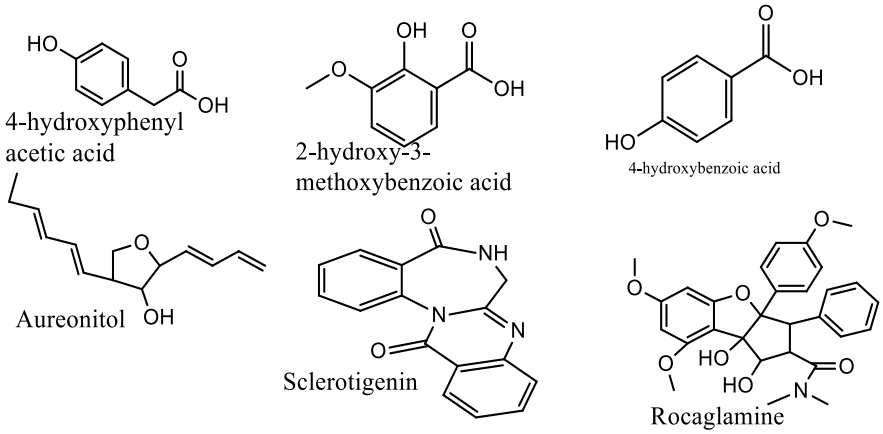


Figure 7: Chemical structure of some bioactive compounds detected from Alternaira species

DISCUSSIONS

Currently, the world population is facing devastating chronic diseases that affect humans. The resistance of pathogens to commercial antibiotics is increasing, thus limiting the therapeutic potential and effectiveness of antibiotics. Consequently, the need to search for novel, affordable, and nontoxic natural bioactive compounds from endophytic fungi in developing new drugs with multifunction mechanisms to meet human needs is essential.

The fungal extract exhibited potent antimicrobial activity against the tested bacteria and Fungi (Tables 1 and 2). Secondary metabolites of the two endophytic fungi (PGL1, PGL2, and PGL3) investigated in this study displayed considerable antiplasmodial activity (Tables 3 and 4). The biological activities exhibited by the endophytic fungal extracts could be said to be due to the different constituents of the extracts. HPLC analysis of the fungal extracts revealed the presence of 2-hydroxy-3-methoxybenzoic acid, naamine F, viridicatol, P-hydroxybenzoic acid, palitantin, hemibastadin in PGL1 which were previously reported to have antimicrobial activity (Yang *et al*., 2015; Sacramento *et al.*, 2015; Mousa *et al.*, 2015, Astelbauer *et al*., 2012). P-hydroxyphenyl acetic acid, 4-methoxybenzaldehyde, benzyl-pyridin A, methiosetin, sulochrin were among the many bioactive compounds identified in the extract of the fungi PGL2. These compounds according to (Xu *et al*., 2010; Ohashi *et al*., 1999) have antimicrobial properties. The antimicrobial activity observed may be because of the presence of ixoside, bastadin, p-hydroxybenzoic acid, and septicine which have been shown to have antimicrobial activities in previous studies (Franklin *et al.*, (1996; Manuja *et al*., 2013; Lee *et al.*, 2011).

In addition, *Alternaria* sp. have been isolated as an endophytic fungi of different plant species and their antimicrobial properties have also been studied by some authors. Methanol extract of *Alternaria* sp. isolated from *Biota orientalis* showed antimicrobial activity against tested bacterial and fungal strains (Subbulakshmi *et al*., 2012). Similarly, Hellwig et al. (2002) reported that the altersetin, a natural product isolated from *Alternaria* sp. showed potent antibacterial activity against several pathogenic gram-positive bacteria. Raviraja *et al.*, (2006) isolated fifteen species of endophytic fungi from leaf, stem, and bark samples of medicinal plants growing in three different locations of the Western Ghats of India, among the list, *Alternaria* sp. 2-hydroxy-3-methoxybenzoic acid: was first isolated by Ali and colleagues (1998), from the *Stocksia brahuica* plant.

Aureonitol was isolated from the fungus *Chaetomium* Kuntze ex-Fries found in soil and cellulose-containing substrate (Ellis *et al.*, 2007). Among the secondary metabolites produced by this genus, aureonitol, a tetrahydrofuran (THF) derivative ((Jervis and Cox, 2008), is an abundant metabolite. Aureonitol has been isolated from different species of the genus *Chaetomium*, from pure cultures *in vitro* and in association with the plant *Helichrysum aureonitens* in nature (Jervis and Cox, 2008).

Rocaglamide was reported by Janprasert and colleagues (1992) as a highly substituted benzofuran isolated and identified as the active insecticidal constituent in the twigs of the Chinese rice flower bush, *Aglaia odorata*. Besides, rocaglamide was also reported as a novel antileukemic 1H-cyclopenta[b]benzofuran isolated from *Aglaia elliptifolia* by King and colleagues (1982).

The crude extract of PGL1 showed antimicrobial, immunomodulatory, and antiplasmodial activities in this study. Sclerotigenin was first isolated from sclerotia of *P. sclerotigenum* and was found to have antiinsectan activity, which was why Joshi *et al.,* (1999) suggested sclerotigenin to play a role in the longevity of sclerotia (Zhang *et al.,* 2007). P-hydroxyphenyl acetic acid has been reported to have antimicrobial and immunomodulatory activities. 4-methoxybenzaldehyde possesses antimicrobial properties (Dalvi and Garge, 2011). 22-Dehydrocampesterol is a seed germination stimulant (Evidente *et al.,* 2011). In this study, PGL2 crude extract showed antimicrobial activity and moderate immunomodulatory activities.

Sclerotigenin has anti-insectant activity (Figure 7). Fusaristatin A has anticancer activity according to Abdalla and Matasyoh, (2014). Manuja *et al.,* 2013 reported that p hydroxybenzoic acid has anti-inflammatory, antimicrobial, and anti-sickling activities. Bastadin, macrocyclic derivatives of bromotyrosine, were first isolated from the tropical verongid sponge *Iantbella basta* (Dexter and Garson, 1993) and it has been reported to have anti-inflammatory and antimicrobial (Franklin *et al.,* 1996). These observed effects probably could be due to the presence of the identified phytoconstituents.

These endophytes can serve as a ready source for large-scale production of these bioactive compounds for pharmaceutical or industrial applications. The discovery of novel therapeutic molecules from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens and the declining number of potent, safe andnontoxic drugs available against infectious diseases and cancer.

CONCLUSION

The study demonstrated that endophytic fungi of *Psidium guajava* produce secondary metabolites with biological properties. These endophytic fungi can be a good source of novel therapeutic compounds that may play a vital role in the development of drugs for the effective treatment of diseases.

Conflict of Interests: The authors declare no conflict of interest.

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