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(RESEARCH ARTICLE)

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Preliminary investigation of the chemo diversity of bioactive molecules produced by endophytic fungi isolated from *Manihot utilisima* leaf

Ugochukwu Moses Okezie [1 *], Peter Maduabuchi Eze [2], Festus Basden Chiedu Okoye [3] and Charles Okechukwu Esimone [1]

[1] *Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe, University, Awka, Anambra State, Nigeria.*
[2] *Department of Environmental Health Science, Faculty of Health Sciences and Technology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.*
[3] *Department of Pharmaceutical and Medicinal Chemistry, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.*

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Abstract

Endophytic fungi have demonstrated a harmless relationship living within the internal tissues of several plant hosts and at the same time produce diverse important bioactive compounds having a wide range of pharmaceutical applicability. In this study, nine endophytic fungi, eight from Cassava leaf: *Clg1, Clg2, Clr4, Clr5, Clr6, Clr7, Clr9* and one from Cassava mid-rib: *CRs3* with distinct cultural features were isolated from healthy leaves of *Manihot utilisima* and axenic cultures were fermented on sterile rice medium for 21 days. The antimicrobial and antioxidant activities of the fungal crude extracts were evaluated. Chemical analyses of the metabolic profiles of each fungal extract revealed the presence of nine known compounds with established biological activities. Each fungal extract exhibited antimicrobial activities against at least one Gram positive and Gram negative bacteria with an inhibition zone that ranged from 2 – 6 mm. *Clg2* and *CRs3* fungal extracts demonstrated moderate potential to scavenge free radical with an inhibition of 58 and 60% respectively. Septicine, cyclo(prolylvalyl), pentenedioc acid, neurolenin B, rubrofusarin, p-Hydroxybenzoic acid, protocatechuic acid, citreoisocoumarin, palitantin and pestalio pyrone were the compounds detected in the fungal fermentation products. Our findings reveal that *M. utilisima* leaves harbor endophytic fungi with unique chemodiversity of bioactive secondary metabolites needed for development of new drugs.

Keywords: Endophytic fungi; *Manihot utilisima*, Chemodiversity; Bioactive compounds; Antimicrobial; Antioxidant

1. Introduction

Natural products research has continued to show dominance in the search for newer bioactive molecules capable of disrupting the current trend of microbial resistance to available chemotherapeutic agents caused by various pathogenic organisms as well as the harmful effects of free radicals.

Fungal endophytes are a family of fungi that live within internal tissues of living plants without any negative impact on the host plant [1]. Overtime, this group of microbe have proven to be dependable for novel lead molecules with huge chemodiversity of bioactive molecules.

Manihot esculenta Crantz a source of carbohydrates also contains some important phytoconstituents such as flavonoids, saponins, terpenes, glycosides with diverse pharmacological properties including radical scavenging, antimicrobial, antitumor and anti-inflammatory activities [2; 3]. Decoction made from the leaves and stems of *M. utilisima* are observed

* Corresponding author: Ugochukwu Moses Okeziea
Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe, University, Awka, Anambra State, Nigeria.

to possess anti-inflammatory, anticancer, anti-diarrhea and wound healing properties [4]. Also, the crude extract has been reported to have antibacterial as well as antioxidant activities [5; 6]. However, no data have been reported on the chemodiversity of fungal bioactive secondary metabolites of *Manihot utilisima*, hence this study was embarked upon. In the course of the study, axenic fungal isolates biosynthesized various classes of bioactive molecules that exhibited antioxidant and antimicrobial activities. The aim of the current study was to identify the chemodiversity of secondary metabolites produced by the fungi isolated from healthy leaves of *Manihot utilisima*.

Figure 1 *Manihot utilisima*

2. Material and methods

2.1. Isolation and purification of endophytic fungi

Healthy leaves of the plant under study *M. utilisima* were carefully harvested from a farmland in Agulu, Anambra-Nigeria. They were transported in a plastic bag immediately to the laboratory. Following washing of the samples under a running tap, samples were further surface-sterilized using 70% (for 1 min), and 2.5% (3 min) of ethanol and hypochlorite solutions respectively and then rinsed in sterile double distilled water. The edges were re-exposed and cut into approximately 2 x 1 mm pieces, and the tissues were aseptically and gently stabbed into the sterile malt extract agar (containing 250 mg/mL ciprofloxacin) and incubated at 25°Cfor 4-6 days. Hyphal emerging out of the cultured samples were isolated and multiple sub-culturing were done until axenic cultures were obtained.

2.2. Fermentation and extraction of fungal metabolites

Each axenic culture was aseptically transferred into a sterile rice medium and incubated at 25 °Cfor 21 days without shaking. Fungal fermentation products were homogenized using 500 mL ethylacetate and filtered through a muslin cloth, and the filtrate concentrated at a reduced temperature of 50°Cusing a rotary evaporator (R000101564, ST15 OSA, UK).

2.3. Chemical analysis (Metabolite profiling)

Retention time and Uv spectra were recorded using a high performance liquid chromatography on a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering Germany) and the correlation coefficients were recorded.

2.4. Bioactivity assay

2.4.1. Test organisms

Antimicrobial activities were carried out using commonly implicated pathogenic organisms which includes *Staphylococcus aureus* and *Bacillus subtilis* (as Gram positives); *Escherichia coli* and *Pseudomonas aeruginosa* (as Gram negatives) collected from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka-Nigeria. Cultures were grown on fresh nutrient agar at 37°C for 18-24 hr prior to the assay.

2.4.2. Antibacterial assay

The potential antimicrobial activities of each of the fungal extract was assessed *in vitro* against the four pathogenic bacteria [7]. Each fungal crude extract was assayed at a maximum concentration of 1 mg/mL. Briefly, 2 mg of each crude

fungal extract was reconstituted using 2 mL Dimethysulfoxide (DMSO). Then, each test organism was standardized to 0.5 MacFarland turbidity standard and inoculated on the surface of the sterile Mueller-hinton agar using a sterile swab stick. A sterile cork borer (diameter 6 mm) was used to make hole/well in the agar, and a micropipette was used to transfer 80 µl of each fungal extract into their respective wells. The plates were left on the bench and allowed for pre-diffusion for 15 min before being incubated at 37°Cfor 18-24 hr. After incubation, the potential for antimicrobial activity was observed by measuring using a meter rule, the zone(s) of inhibition produced around the well.

2.4.3. Antioxidant activity

The method described by [8] with moderate modifications was adopted. At a concentration of 1 mg/ml, the potential to scavenge free [2,2-diphenyl-1-picryl hydrazyl (DPPH)] radicals were measured at 490 nm as an index to their antioxidant activity. The reaction mixture included 25 µl of the stock, 25 µl of DPPH (0.1 mol/L) and 150 µl of methanol solution, all in a well. The plates were incubated at 27°Cfor 30 min and the antioxidant potentials were assessed by measuring the absorbance of the mixtures at 490 nm using a UV-vis spectrophotometer (721; PEACE SKY, China). An average of three replicates for each extract concentration was done. Antioxidant activities were expressed as the percentage inhibition of each extract and calculated using the formula: [(Ao – A1)/ Ao] * 100; Ao is the absorbance of the blank solution and A1is the absorbance of the positive control

3. Results

3.1. Isolation, purification and bioactivity of endophytic fungi

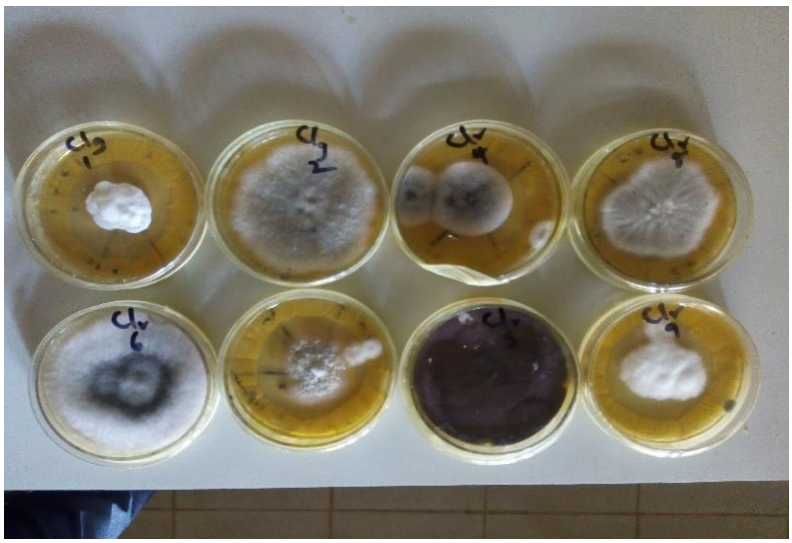


Figure 2 Colony morphology of endophytic fungi isolated from *M. utilisima* leaves on Malt extract agar

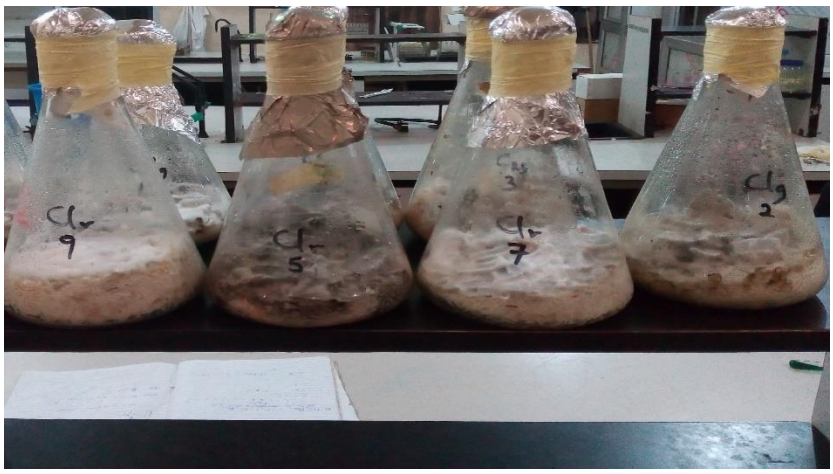


Figure 3 Fermentation of axenic cultures on rice medium

A total of eight endophytic fungi were isolated from healthy leaves of *M. utilisima*. Figure 2. Axenic cultures resulting from multiple aseptic sub-culturing were fermented on local rice medium Figure 3 and each fungus fermentation product was extracted using 500 mL ethylacaetate figure 4.

Figure 4 Fermentation filtrate of isolated fungi isolates

3.2. Antimicrobial activity

The antimicrobial activity demonstrated by the fungal crude extracts showed a moderate activity when compared with the controls Table 1.

Table 1 Zones of inhibition produced by extracts of endophytic fungi associated with *M. utilisima* against pathogenic organisms.

Isolate code	Concentration (1mg/mL) / Inhibition Zone Diameter (mm)				
	B. subtilis	***S. aureus***	***P. aeruginosa***	***E. coli***	***C. albicans***
Clg1	2	0	0	3	0
Clg2	4	0	6	4	0
CRs3	4	0	0	2	0
Clr4	4	0	5	2	0
Clr5	2	0	6	2	0
Clr6	4	0	0	4	0
Clr7	3	0	0	4	0
Clr9	2	0	0	2	0
Cipro 5 µg/mL	17	15	17	19	-
Miconazole 50 µg/mL	-	-	-	-	22

[1]Antimicrobial potential was indicated by inhibition halos produced around the well and measured in millimeters. Commonly implicated pathogens: *B. subtilis, Bacillus subtilis; S. aureus, Staphylococcus aureus; P. aeruginosa, Pseudomonas aeruginosa; E. coli, Escherichia coli; C. albicans, Candida albicans*. Each of the fungal crude extract at 1 mg/mL was assayed against the test organisms. [2] Ciprofloxacin 5 µg/mL and Miconazole 50 µg/mL were used as the positive controls.

3.3. Antioxidant activity

At 1 mg/mL, *Clg2* and *CRs3* produced good radical scavenging activity with an inhibition of 58 and 60% respectively. DPPH radical scavenging potential was observed by a change in the purple color of DPPH to orange.

Table 2 Antioxidant activities of fungal crude extracts

Isolate code	Absorbance (517 nm)	% Inhibition
Clg1	0.938	26
Clg2	0.583	58
CRs3	0.565	60
Clr4	0.78	40
Clr5	0.988	22
Clr6	0.958	24
Clr7	1.054	16
Clr9	1.035	17
Quercitine	0.077	93
DPPH	1.108	-

Key: C= Endophytic fungal extract from Cassava Leaf; Quercitine 1 mg /mL was used as the [positive control].

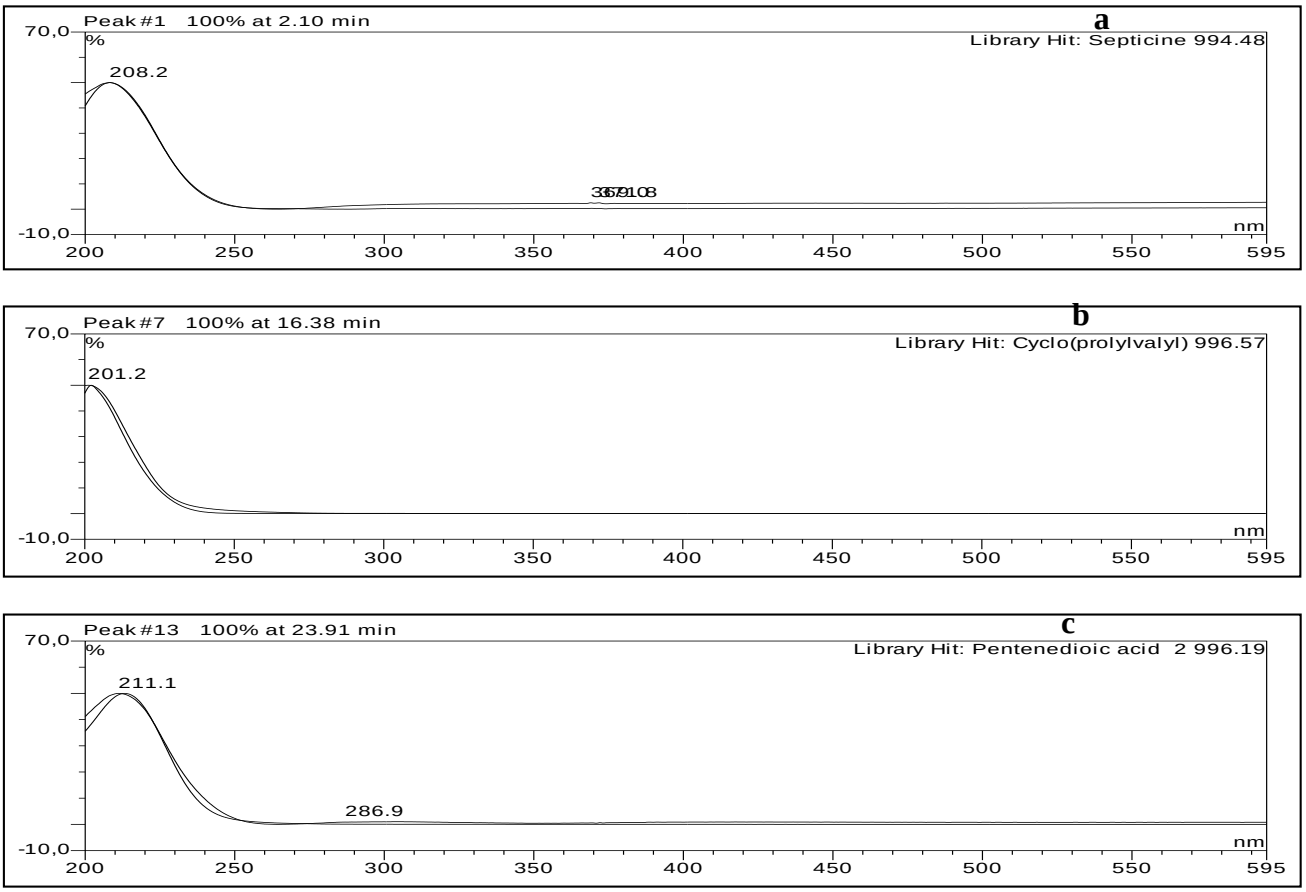
3.4. Detection of fungal Secondary Metabolites

Table 3 Bioactive compounds produced by the endophytic fungi isolates

Compound class / compound name	Retention time (min)	Isolate code	Reported biological activity / Reference
Alkaloids			
Septicine	2.0	*Clg1,*	Antibacterial, [9]; Anti-inflammatory, [10]; Cytotoxicity, [11]
	57.24	*Clg2*	
		Clr5	
		Clr6	
		CRs3	
Peptides			
Cyclo(prolylvalyl)	16.38	*Clg1*	Antibacterial [12], antioxidant, radical scavenging, [13]
	6.30	*Cr3*	
Pentenedioc acid	23.91	*Clg1*	Anticancer, [14]; Antioxidant, Anti-inflammatory, [15]
Neurolenin B	25.54	*Clg1*	Antimalarial, [16]; [17]
Naphtha-γ -pyrone			
Rubrofusarin	33.33	*Clg1*	Weak cytotoxicity, [18]; Antibacterial, [19]; Xanthine oxidase inhibitor, [20]
Phenolic acids			
p-Hydroxybenzoic acid	9.86	*Clg2*	Antimicrobial, [21]
Protocatechuic acid	11.61 *Clr4* 5.46 *Clr6*		Antioxidant [22]; Antimicrobial, Yoswaris *et al.,* [23]

Polyketides			
Citreoisocoumarin	24.41	*Clg2*	Inhibitory activity against α-glucosidase, [24]; Anticancer, [25]
Palitatin	33.94	*Clg2*	Antibacterial; [26]; Antiprotozoal activity, [27]
	32.65	*Clr4*	
	34.23	*Clr5*	
Pentaketide			
Pestalio pyrone	28.88	*Clr6*	Fungistatic and Phototoxic activities, [28]

A preliminary investigation of the secondary metabolic profiles of the endophytic fungal isolates was carried out. This was done in order to detect the active agents present in the fungal crude extracts that may be responsible for the recorded antibacterial and antioxidant activities. The chemical investigation was achieved using HPLC-DAD system. This provided us data on the chemodiversity of secondary metabolites of endophytic fungi isolated from *M. utilisima*. In our study, ten known compounds of different classes were detected in the fungal fermentation products. These includes Septicine, Cyclo(prolylvalyl), pentenedioc acid, neurolenin B. rubrofusarin, p-hydroxybenzoic acid, citreoisocoumarin, palitantin, protocatechuic acid and petalio pyrone G. Some of these compounds have also been previously isolated from several other fungi and reported to possess various biological activities such as antioxidant and antimicrobial activities amongst others (Table 3; Figure 5-11). The various peaks of the ultraviolet spectrum at each retention time for each compound detected was authenticated by comparing with those available in the database in the library hits of UV spectra data at the Intitut fur Pharmazeutische Biolgie und Biotechnologie, Heinrich-Heine-Universitat, Dusseldorf, Germany.



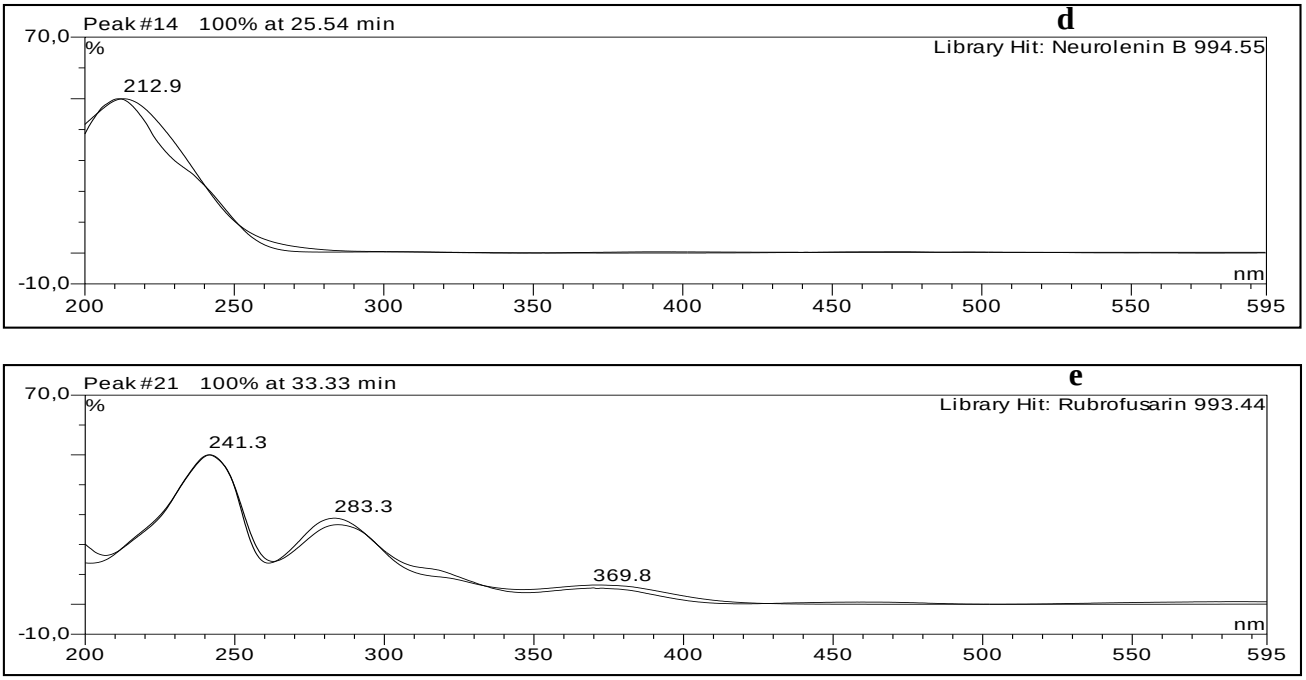
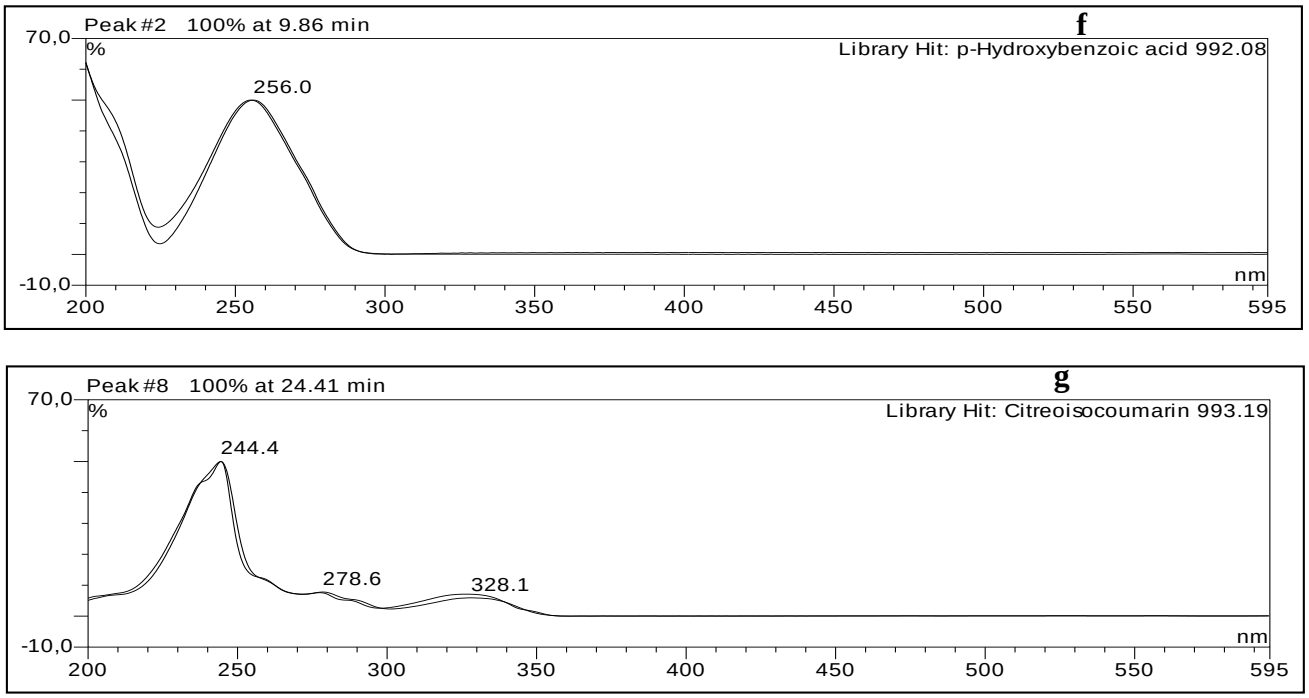


Figure 5 UV spectra of main peaks for *Clg1* extract: Library hit of UV spectra of the compound Absorption maximum UVλmax (methanol): (a) septicine 208.2 nm; (b) cyclo (prolylvalyl) 201.2 nm; (c) pentenedioc acid 211.1 nm; (d) neurolenin B 212.9 nm and (e) rubrofusarin 241.33 and 283.3 nm.



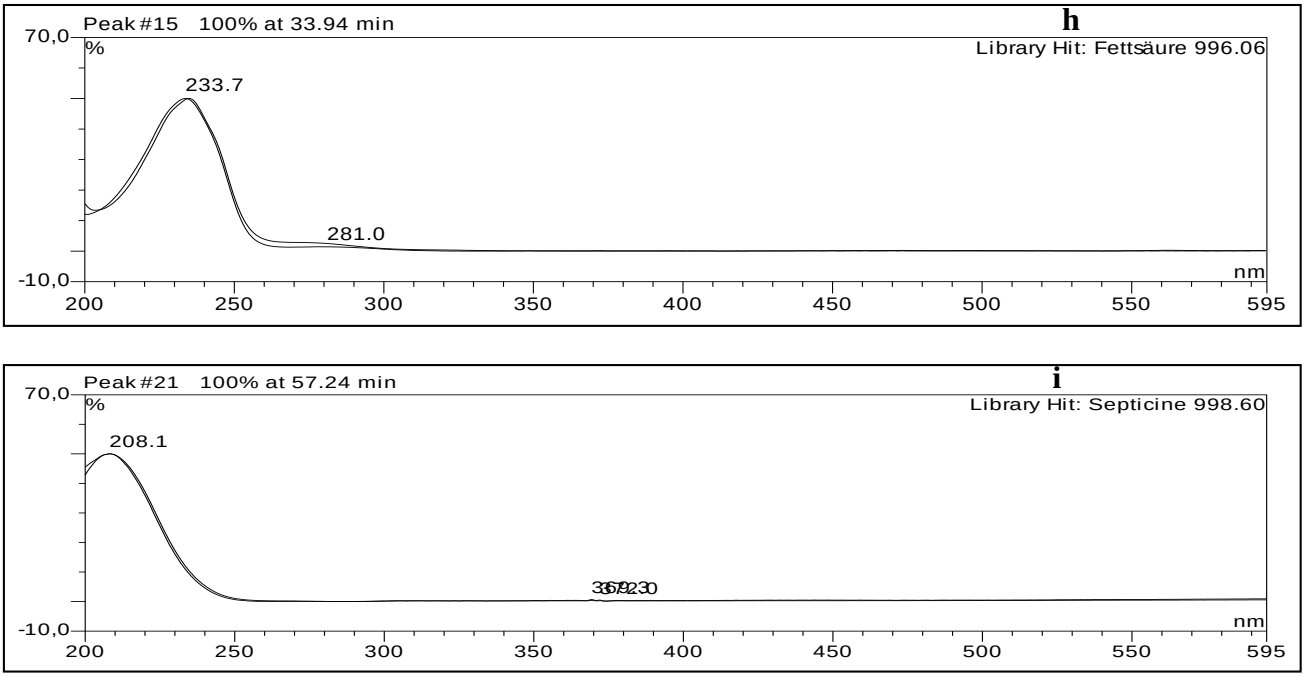


Figure 6 UV spectra of main peaks for *Clg2* extract: Library hit of UV spectra of the compound Absorption maximum UVλmax (methanol): (e) p-Hydroxobenzoic acid 256.0 nm; (g) citreoisocoumarin 244.4 and 278.6 nm; (h) palitantin 233.7 nm; and (i) septicine 208.1 nm.

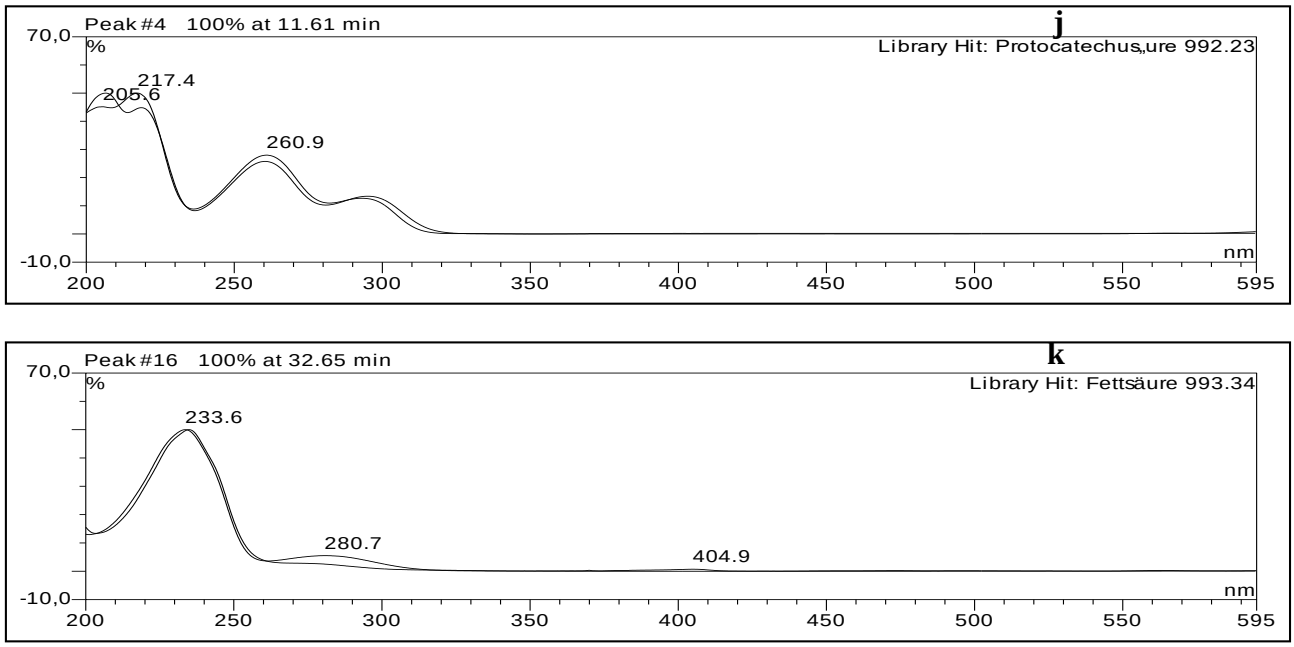


Figure 7 UV spectra of main peaks for *Clr4* extract: Library hit of UV spectra of the compound Absorption maximum UVλmax (methanol): (j) protocatechuic acid 205.6, 217.4 and 260.9 nm; and (k) palitantin 233.6.

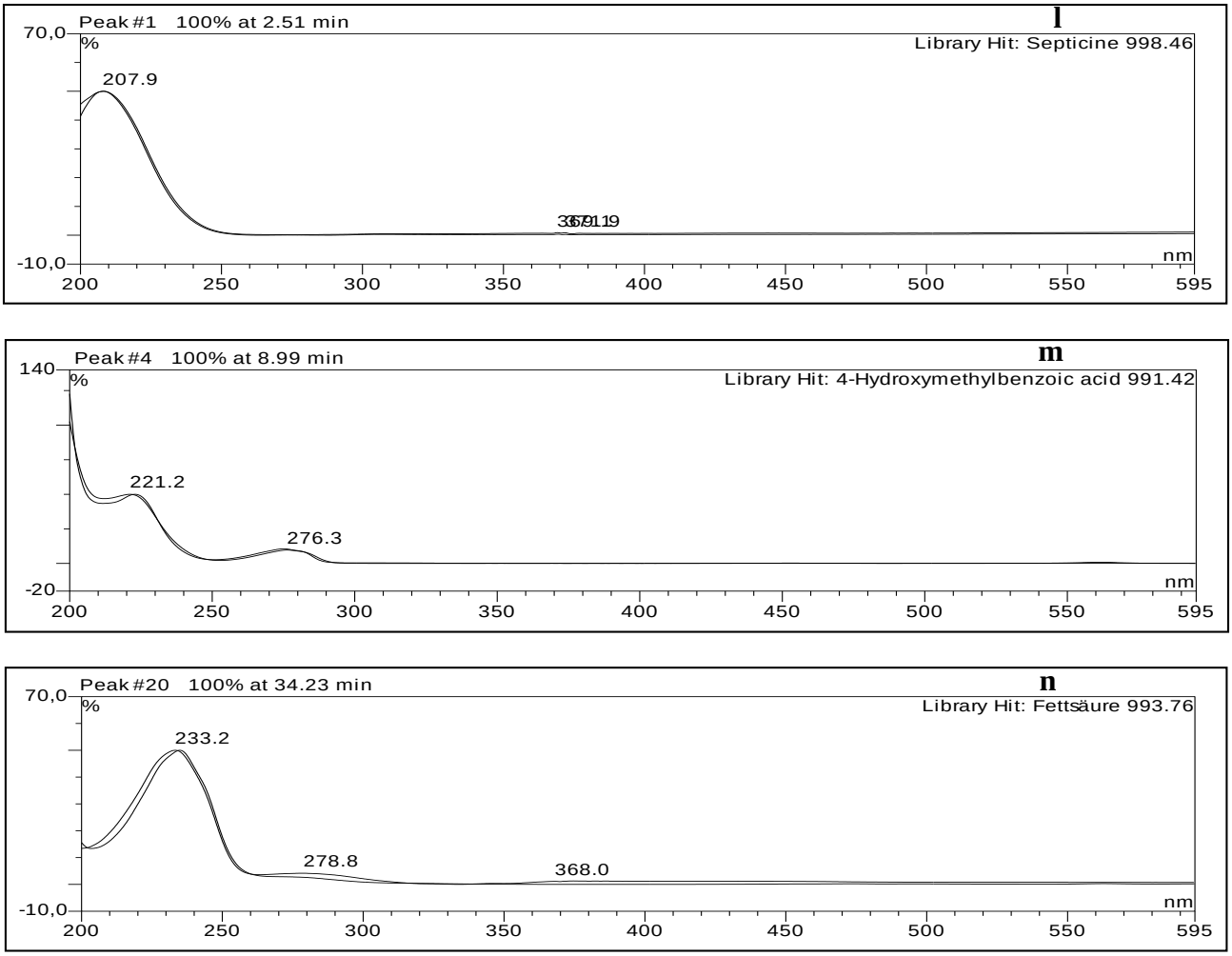
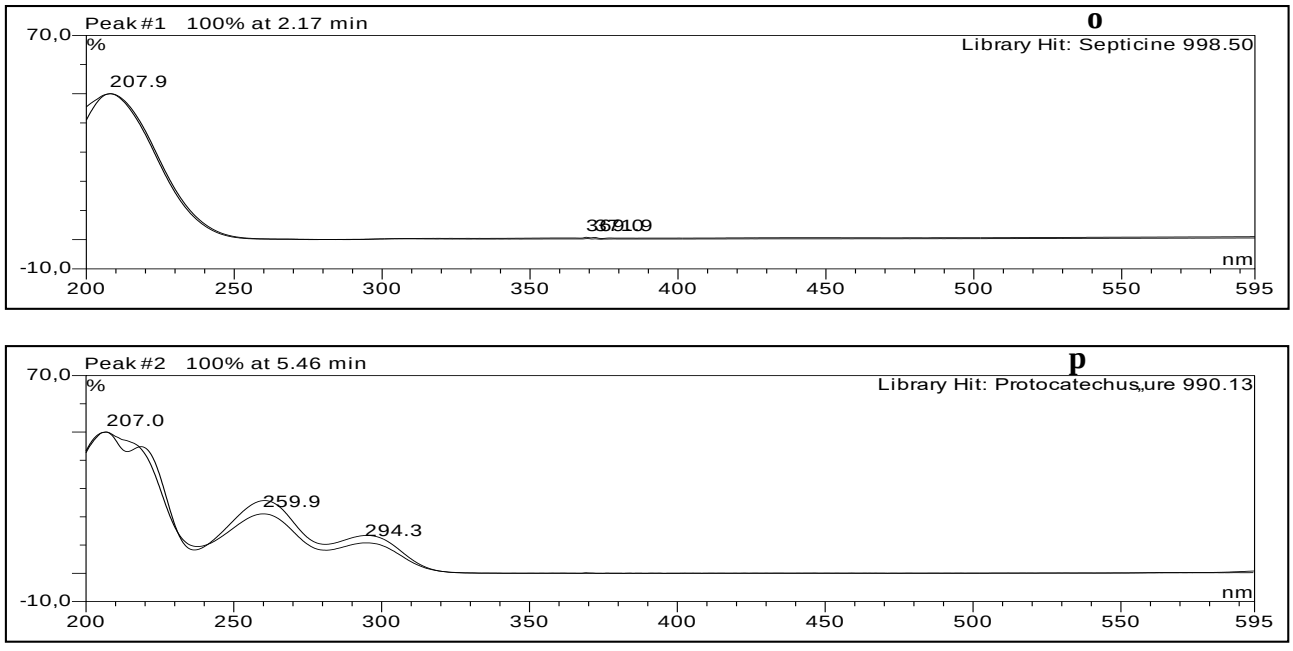


Figure 8 UV spectra of main peaks for *Clrs5* extract: Library hit of UV spectra of the compound Absorption maximum UVλmax (methanol): (l) septicine 207.9 nm; (m) 4-Hydroxymethylbenzoic acid 221.2 nm and 276.3 nm; and (n) palitantin 233.2 nm.



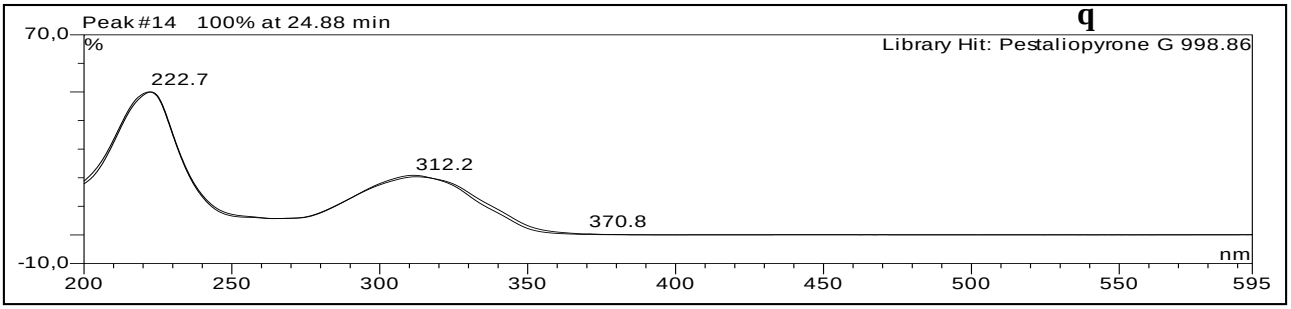


Figure 9 UV spectra of main peaks for *Clr6* extract: Library hit of UV spectra of the compound Absorption maximum UVλmax (methanol): (o) septicine 207.9 nm; (p) protocatechuic acid 207.0 nm, 259.9 nm and 294.3 nm, and (q) pestalio pyrone G 222.7 and 312.2 nm.

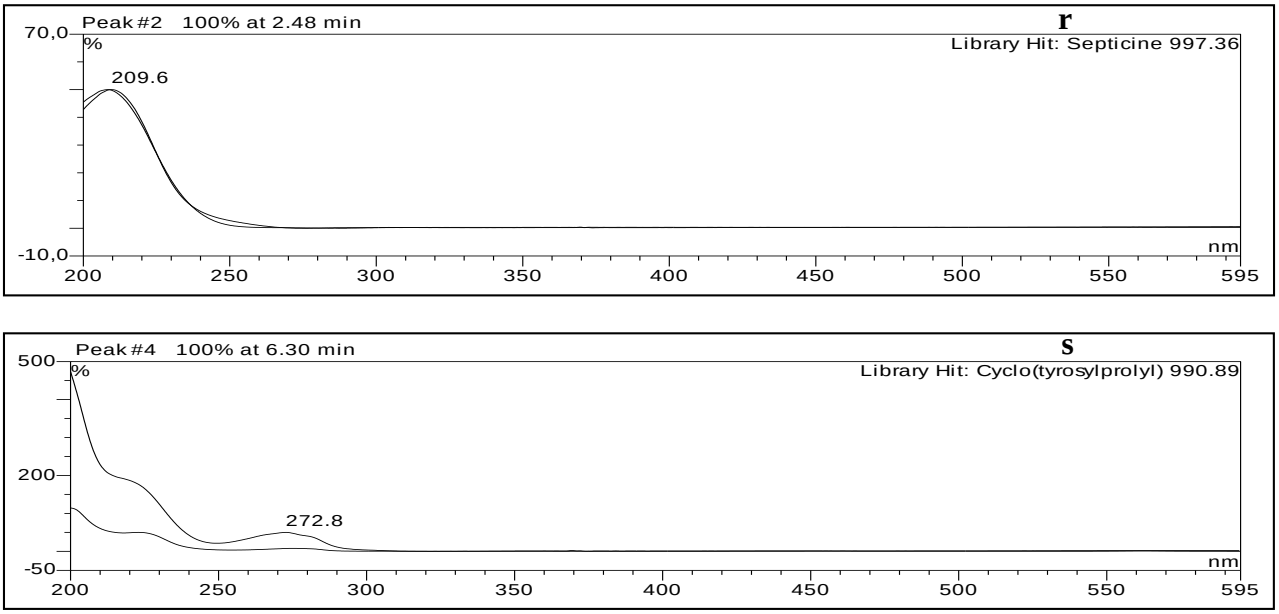
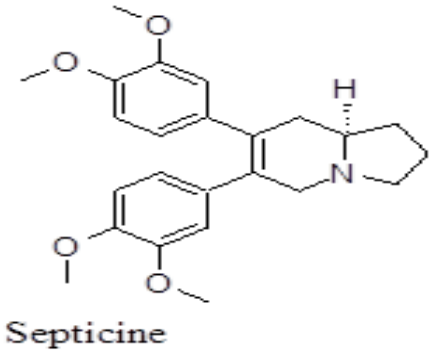
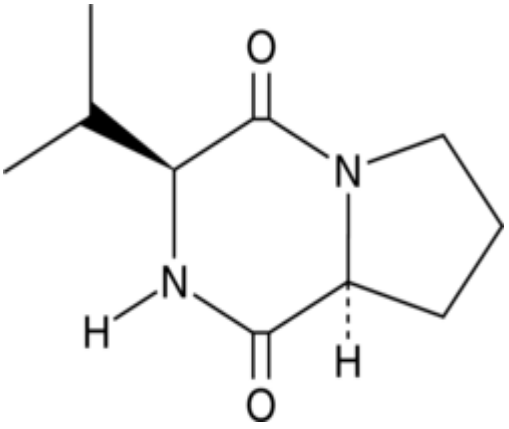


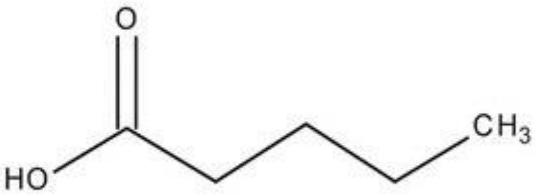
Figure 10 UV spectra of main peaks for *CRs3* extract: Library hit of UV spectra of the compound Absorption maximum UVλmax (methanol): (r) septicine 209.6 nm and (s) cyclo(tyrosylprolyl) 272.8 nm.



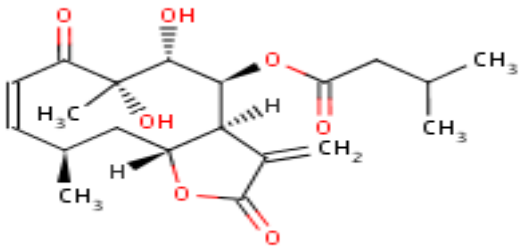
Septicine. Chemical formula: $C_{24}H_{29}NO_4$



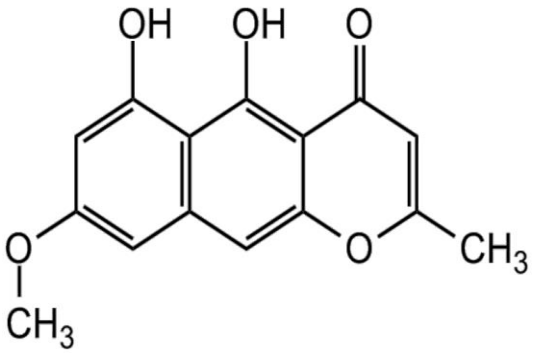
Cyclo (prolylvalyl). Chemical formula: $C_{10}H_{16}N_2O_2$



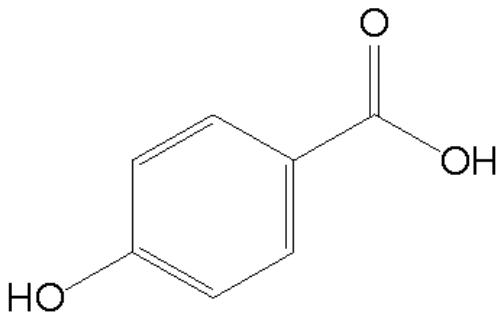
Pentedioc acid. Chemical formula: $C_5H_{10}O_2$



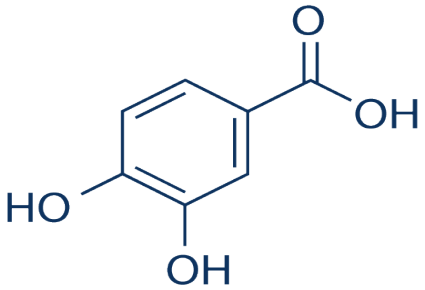
Neurolenin B. Chemical formula: $C_{22}H_{30}O_8$



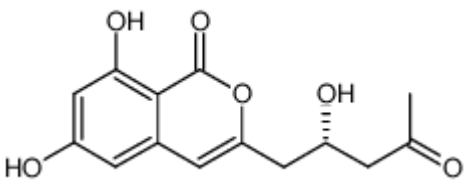
Rubrofusarin. Chemical formula: $C_{15}H_{12}O_5$



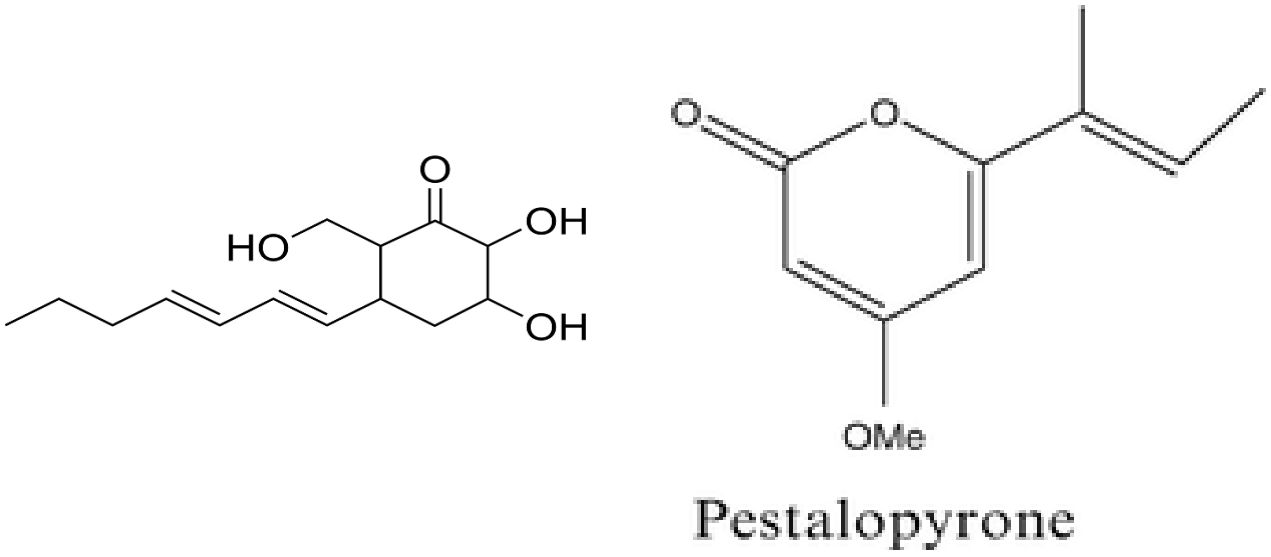
p-Hydroxybenzoic acid. Chemical formula: $C_7H_6O_3$



Protocatechuic acid. Chemical formula: $C_7H_6O_4$



Citreoisocoumarin. Chemical formula: $C_{14}H_{14}O_6$



Palitantin. Chemical formula: $C_{14}H_{22}O_4$ Pestalopyrone. Chemical formula: $C_{10}H_{12}O_3$

Figure 11 Chemical Structures of detected compounds

4. Discussion

In this study, the diversity of endophytic fungi as well as its secondary metabolites were investigated. A total of eight endophytic fungi were isolated from healthy leaves of *M. utilisima*. The different cultural characteristics of each fungus on malt extract agar highlights the diversity of fungal endophytes associated with the plant under study. Similarly, Li *et al.,* [29] reported the isolation of several endophytic fungi from different cassava cultivars.

The chemical analysis and bioassay of the secondary metabolite produced by each fungus after 21 days of fermentation on rice medium were evaluated. Amongst the fungal extracts tested, three extracts (*Clg2, Clr4 and Clr5*) showed good antibacterial activity against *B, subtilis, P. aeruginosa* and *E. coli*. Moreover, all the extracts demonstrated antimicrobial activity against at least one of the test isolates at the tested concentration of 1 mg/mL. The activities of the endophytic fungal extracts against the different test organisms varied. This is evident by the varying inhibition zones produced which ranged between 2 – 4 mm (*B. subtilis*), 5 – 6 mm (*P. aeruginosa*) and 2 – 4 mm (*E. coli*). However, none of the fungal extracts had activity against *S. aureus* and *C. albicans* at the tested concentration. The activities demonstrated by *Clg1, Clg2, CRs3, Clr5, Clr6, Clr7 and Clr9* were observed to be broad spectrum with the Gram negatives being the most inhibited (i.e. sensitive) group among the test organisms used in this work. The sensitivity to the fungal extracts by the Gram negatives in comparison with the Gram positives may be attributed to the difference in their cell wall constituents. Also, the broad spectrum activities demonstrated by the active fungal extracts can be attributed to the combined activities of the bioactive constituents present in each extract. Also, this may be due to the differences in the structural details of detected metabolites, in addition to their hydrophilic nature. Indeed, the external membrane of Gram negative and Gram positive bacteria renders their surfaces highly hydrophilic thus are susceptible to hydrophilic compounds. Most of the compounds detected in the fungal extracts produced by the isolates in this work are hydrophilic including the phenolic and polyketide compounds.

The antioxidant assay revealed *Clr3* and *Clg2* extracts to have good radical scavenging potentials. At 1 mg/mL, *Clr3* and *Clg2* demonstrated good radical scavenging activities with a percentage inhibition of 60 and 58 % respectively, comparable to the control quercitine 93 % (positive control).

Going by the recorded antibacterial and antioxidant activities, the endophytic fungi isolated in this work exhibited potential as sources of bioactive agents needed for the development of new medicines thus, it was necessary to assess the chemodiversity of each fungal extract. Therefore, a preliminary investigation of the secondary metabolic profiles of all the endophytic fungi isolated in this study using HPLC-DAD system was determined according to the clear peaks detected in the chromatograms with specific retention indexes (RI) [Table 3: Figure 5 – 11]. The compounds detected suggests a chemodiversity of secondary metabolites evident by their different structural details (classes). These compounds have also been previously isolated from both fungi and plant extract by several authors and reported to possess various biological activities including antioxidant and antimicrobial activities amongst others (Table 3).

Neurolenin B, a natural product isolated from the Central American plant *Neurolaena lobate* exhibited antimalarial activity against lymphatic *filariasis*. Neurolenin B was observed to be the bioactive component responsible for the recorded activity [16; 17].

Chemical investigation on the fermentation product of *Nectria sp*. HN001 showed the presence of citreoisocoumarin present. This compound demonstrated Inhibitory activity on α-glucosidase [24]

4-Hydroxy-benzoic acid a phenolic compound was isolated from the extract of *Gliocladium roseum* CGMCC 3.3657 [30]. Also, this compound was previously detected in a fungal crude extract that showed antimicrobial activity [21]

Pestalopyrone was isolated from the culture filtrates of *Pestalotiopsis guepinii.* This compound demonstrated phytotoxic activities when applied on some plants as well as antifungal activity [28].

Aspergillus niger isolated from *Cynodon dactylon* produced Rubofusarium B a compound that exhibited cytotoxic and xanthine oxidase inhibition [20].

Cyclo (polylvaly) a secondary metabolite produced by *Streptomyces sp.* strain 22-4 showed activity against phytopathogenic bacteria [12].

Pentedioc acid isolated from the leaf extract of *Bougainvillea x buttiana* (var. Rose) *Holttum* and *Standl* extract (BxbREE) showed antioxidant and anti-inflammatory activities when evaluated [15]; also a derivative of pentanedioic acid amide Synthesized was observed to possess antineoplastic activity *In vitro* and *in vivo* [14].

In a study conducted by Fuska *et al*., [27], palitantin isolated from a submerged culture of *Penicillium frequentans* 60A/7 demonstrated antiprotozoal activity against *Leishmania brasilliensis*. Also, palitantin was isolated from the extract of an enophytic fungus *Aspergillus fumigatiaffnis* and observed to inhibit the growth of *Enterococcus faecalis* UW 2689 and *Streptococcus pnuemoniea* [26].

Finally, Protocatechuic acid (PCA) a highly reactive phenolic compound has been detected in several plant extracts. Several reports indicates PCA to be biologically active with potentials as antimicrobial and antioxidant agents etc. [23]. Similarly, in another study carried out by Nguyen *et al.,* [31] on the inhibitory activity of Protocatechuic acid isolated from extract of *Paenibacillus elgii* HOA73, PCA displayed potent antifungal activity against the selected fungi isolates.

5. Conclusion

In conclusion, in this study we isolated different endophytic fungi from healthy leaves of *Manihot utilisima*. The varieties of fungal endophytes isolated in this work was based on their diverse morphological (cultural) features observed on Malt extract agar. Also, with the aid of a standard chemical (HPLC) analytic protocol, several classes of bioactive secondary metabolites with varying biological activities were detected in the fermentation product of each fungus. Thus, our study provides additional useful data on the reliability of fungal endophytes from *Manihot utilisima* for production of important natural bioactive metabolites with pharmaceutical applications.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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