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Gyrinops walla Gaertn and an associated endophyte *Fusarium dlaminii*: A new sources of α and β amyrins

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Abstract

Amyrins and their derivatives are pentacyclic triterpenes with well-known pharmacological properties including antimicrobial activities. The crude ethyl acetate extract (EA) of an endophytic fungus we isolated from the bark of *Gyrinops walla* Gaertn. showed strong, broad-spectrum antimicrobial activity against a number of human pathogens; both Gram-positive and Gram-negative bacteria including a clinical isolate of methicillin-resistant *Staphylococcus aureus* and *Candida glabrata* in Kirby-Bauer disc diffusion test. The active extract analysed by Gas Chromatography-Mass Spectrometry evident the presence of α and β Amyrin. The same two compounds were also detected in the EA extract of the inner stem bark. The sequence analysis of the DNA of the ITS regions and NCBI BLASTn search confirmed the fungus as *Fusarium dlaminii* (GenBank accession no. MW633321). This is the first report of fungal-derived α amyrin while *G. walla* is reported for the first time as a new source of both the α and β amyrins.

Keywords: A amyrin, β amyrin, endophyte, *Fusarium dlaminii*, *Gyrinops walla*

Introduction

Development of drug resistance in human pathogenic bacteria, fungi and viruses claim for new therapeutic agents for effective treatment of diseases in humans. The condition can be worsened globally with the excessive use of sanitizers due to the COVID-19 pandemic. The *in vitro* production of natural drugs by endophytes includes anticancer, antimicrobial, antidiabetic and immunosuppressant compounds [15]. Occasionally, some of these compounds are the same as those produced by the respective hosts. One of the strategies to select a host plant to isolate a promising candidate is to tap plants with records of traditional medicines for human use [19]. Triterpenoids are reported to possess promising pharmacological activities such as anticancer, anti-inflammatory, antiviral, antibacterial, antifungal, antidiuretic and giardicidal [11]. The α and β amyrin are pentacyclic triterpenes which have been isolated from several medicinal plants and oleo-resin obtained by bark incision of some species of the Burseraceae family. For example, the α and β Amyrin are main constituents of the resin of *Protium paniculatum* [1]. Both *in vitro* and *in vivo* studies have shown that amyrins have important pharmacological activities against various health-related conditions such as inflammation, microbial infections and cancers [8]. *Gyrinops walla*, an endogenous resinous plant growing in Sri Lanka and India is used in the ancient Ayurveda medicinal practices; the bark for the casting of broken bones while other parts are used in treatment for toothaches, nail infections, serpent stings and many other ailments [2,5]. Although antimicrobial activity of plant-derived natural resins from several families has been reported [17]; no published information is available on potential antimicrobial activities of *G. walla*. However, overexploitation of *G. walla* in the perfume industry for its resin has caused it to be declared as an endangered species [11]. An option to safeguard the plant biomass is to screen the associated endophytes for bioactive metabolites as some endophytes produce similar bioactive compounds as their hosts. In spite of this, we conducted a preliminary screening of *G. walla* bark extract as well as the extracts of its endophytic fungi for antimicrobial activity.

Materials and Methods

Isolation of endophytic fungi from bark of G. walla

Stem bark samples of the *G. Walla* plant were collected from the home garden in Kurunegala, Sri Lanka (7°35'16.4"N, 80°07'12.0"E). The bark pieces (approximately 1x1cm) successively surface sterilized using 10% (v/v) NaOCl (5 min), sterile distilled water (1 min, 3 washes), 70% (v/v) ethanol (1 min) and sterile distilled water (1 min, 3 washes). Edges of the pieces were trimmed, blot dried and transplanted on potato dextrose agar (PDA) medium, amended with Streptomycin (100 mg/l) and incubated at 28 ºC until the outgrowth of endophytic fungi was observed. Surface sterility was checked by tissue imprints; pressing the surface sterilised tissue on a replicate PDA plate for 10 min. The pure cultures were established by transferring fungal hyphal tips that emerged from the cut surface of master plates to fresh PDA plates and were maintained in the refrigerator at 4 ºC. Stock cultures were prepared by suspending spores of the isolated fungi in 20% glycerol and stored in (-80) ºC refrigerator for future use.

Identification of CPT from endophyticfungusThe S-1 culture of all endophytes in solid media was preliminarily screened for the production of CPT by TLC. CPT from endophytic fungi was extracted using chloroform: methanol 4:1(v:v) at a rate of 0.1:10 (w:v). 12 the extract was centrifuged at 10,000 rpm for 10 min at 10

OC. The resultant supernatant was collected and evaporated to dryness at RT. The concentrate was re-dissolved in methanol (HPLC grade) and filtered by 0.45 µm filters. Extracts were preliminary anlyanalyzed for the presence of

CPT using TLC and further confirmed by HPLC and LC-MS.

Detection and Identification of CPT from endophyticfungusThe S-1 culture of all endophytes in solid media was preliminarily screened for the production of CPT by TLC. CPT from endophytic fungi was extracted using chloroform: methanol 4:1(v:v) at a rate of 0.1:10 (w:v). 12 the extract was centrifuged at 10,000 rpm for 10 min at 10 OC. The resultant supernatant was collected and evaporated to dryness at RT. The concentrate was re-dissolved in methanol (HPLC grade) and filtered by 0.45 µm filters. Extracts were preliminary anlyanalyzed for the presence of CPT using TLC and further confirmed by HPLC and LC-MS.

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Preliminary antimicrobial assay (AMA) by agar culture plug diffusion test

The co-culture method using the agar culture plug diffusion technique [6] was used to determine the antagonism among isolated fungi and test pathogens in Muller Hinton agar (MHA) for pathogenic bacteria and MHA supplemented with 2% glucose (MHAG) for *Candida* spp. Pathogens tested were *Candida glabrata* (ATCC 90030), *C. parapsilosis* (ATCC 22019), *C. albicans* (ATCC 10231), *C. tropicalis* (ATCC 13803), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 25853), *Staphylococcus aureus* (ATCC 25923) and Methicillin-resistant *S. aureus* (MRSA); a clinical isolate, kindly supplied by the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. *Candida* species were maintained in Yeast Extract Peptone Dextrose agar (YPDA) and the pathogenic bacteria were maintained in Nutrient Agar (NA) medium. Suspensions of overnight grown test pathogens were made in sterile distilled water and the optical density (OD) at 600 nm was adjusted to 0.1. Lawns of test pathogens were made separately using sterile cotton buds immersed in the suspensions on the MHA and MHGA as appropriate. Agar culture plugs (6 mm in diameter) from the growing edge of a plate culture of endophytic fungi were placed on the lawns and incubated at 37 ºC for 24 hours. A zone of inhibitions (ZOI) surrounding the culture for further investigations.

Ethyl acetate extraction of endophytic fungus, B.14 and Kirby-Bauer assay

Two PDA plates of 14-day old cultures of B.14 were cut into small pieces (~ 0.5 x 0.5 cm) along with agar and extracted in 50 ml of ethyl acetate (EA). The organic fraction was separated by filtering through No. 1 Whatman filter paper. The extraction repeated twice more using 50 ml of solvent at each time, all three ethyl acetate fractions were pooled and evaporated for dryness in a rotary evaporator. The dark brown residue (10 mg) was constituted in 10 ml of ethyl acetate (EAF).

Standard Kirby-Bauer disc diffusion assay was carried on MHA or MHAG. As the preliminary AMA in co-culture indicated that *C. glabrata, E. coli, S. aureus* and MRSA were the most susceptible pathogens (based on ZOIs), only these pathogens were used for assessing the antimicrobial property of the EAF by disc diffusion assay. Pathogen suspensions were made as described earlier, but the lawns were made by inoculating 500 µl of the suspension. The sterile filter paper discs impregnated with 20 µl of EAF were placed on the lawn and incubated at 37º C for 24 hours. Positive controls were Ketoconazole for *C. glabrata*, Gentamycin for *E. coli* and Chloramphenicol for both *S. aureus* and MRSA while EA was used as the negative control. The diameters of ZOIs were measured. The test was done in triplicates. The CLSI, (Table 1) standard was used to interpret the antibiogram to classify the test organisms as resistant, intermediate or susceptible.

Identification of the endophytic fungus, B.14

Tentative identification of the fungus was done using colony morphology on Malt Extract Agar (MEA) and microscopic

characteristics of vegetative and reproductive structures of the fungus by preparing slide cultures. Confirmation of the identity of the fungus was done by molecular analysis of the ITS (internal transcribed spacer) region of rDNA containingITS1, ITS2 and 5.8S rRNA gene. Isolation of fungal genomic DNA was carried out as described by [3]. The DNA pellet was washed in ice-cold 70% ethanol, air-dried and suspended in 50 µl of sterile deionized water. The PCR amplification was carried out in a Thermocycler (Biorad T-100 Thermocycler, California, USA) and primers used were; forward-ITS1; TCCGTAGGTGAACCTGCGG and reverse-ITS4; CCTCCGCTTATTGATATGC [18]. The amplified DNA was sent to Macrogen Company Ltd (South Korea) for sequencing. Sequences of the extracted DNA were aligned using NCBI's BLASTn search, limited to the sequences from ex-type material for the newly generated ITS sequence of B.14.

The ethyl acetate extraction of plant bark

A fresh sample of *G. walla* stem bark (heartwood) was washed and dried at the 40 ºC until a constant weight was obtained. It was ground to a coarse powder in a grinder and extracted with ethyl acetate. One gram of the powder was extracted in 25 ml of EA. The extraction was done thrice, the pooled extract was concentrated using the rotary evaporator and residue (30 mg) was dissolved in 1 ml solvent. It was centrifuged at 12,000 rpm for five minutes and the supernatant (EAB) was analysed by Gas Chromatography-Mass Spectrometry (GC-MS).

Gas chromatography-mass spectroscopic analysis of EAB and EAF

The compositions of the EAF and EAB were determined by the GC-MS analyses, according to a protocol described by [21] with some modifications. An Agilent 7890A GC coupled to an Agilent 5975C MS was used in the analysis. The separation column was an Agilent HP-5MS with 5% Phenyl Methyl Siloxanes (30 m x 250 µm x 0.25 µm). Pure helium was used as the carrier gas, upto a flow rate of 1 ml/min. Split-less mode injections were performed at 250 °C. Exactly1 µl of the crude EAF and EAB were injected separately. The injector chamber was maintained at 250ºC.The oven temperature program was as follows. The temperature was maintained at 50 °C for 1 min, and increase at a rate of10ºC/min to 155ºC for 15 min and then 155 ºC to 280ºC for 30 min with a total run time were 72.125 min. The ionization voltage, at the MS was70 eV and the NIST MS database was used to identify the MS fragmentation patterns.

Results

Isolation of endophytic fungi from G. walla and preliminary antimicrobial assay (AMA)

A total of six endophytic fungi were isolated from the bark samples of *G. walla* and only two of these isolates showed antimicrobial activity against human pathogens tested during the preliminary investigation of the AMA using the co-culture technique. As the endophytic fungus; B.14 antagonised all eight tested pathogens, it was selected for further investigations.

Identification of the endophytic fungus B.14

The fungus grown on MEA medium showed reddish-orange colony, the mycelium with branched, septate and hyaline hyphae. It produced oval or kidney-shaped microconidia and ellipsoid, elongated macroconidia with 2-3 septa (Figure 1) thus identified as a *Fusarium* isolate. The ITS region of the isolate of *Fusarium* sp. was successfully amplified and obtained an amplicon with the expected size of approximately 550 bp and the NCBI's BLASTn search limited to the sequences from ex-type material for the newly generated ITS sequence of B.14 revealed that it is closely related to *Fusarium dlaminii* (GenBank MW633321) with 99.81% sequence similarity.

Antimicrobial assay for ethyl acetate extract of the fungus (EAF)

The results of culture metabolites reflected the co-culture results, exhibiting strong inhibition of the growth of all four pathogens; *C. glabrata*, *E. coli*, *S. aureus* and MRSA tested (Table 2 and Figure 2). According to the CLSI interpretative standards, the three bacterial species tested; *E. coli*, *S. aureus* and MRSA were susceptible while *C. glabrata* was moderately susceptible to the EAF.

Gas Chromatography-Mass Spectroscopic analysis of EAB and EAF

The analysis of the bioactive EAF of PDA plate culture revealed the presence of four major compounds; of which two compounds X and Y were identified as β amyrin and α amyrin respectively by comparison of the MS fragmentation pattern of the substances of the EAF with NIST mass spectra libraries (Figure 3). The, α, and β amyrins though have similar mass spectral data, were distinguished by relative intensities of the peaks at 203 [4]. Similarly, GC-MS analysis of the EAB revealed that the heartwood of *G. walla* also produces these amyrin isomers (compound P and Q) (Figure 4) in addition to several types of aroma principles that are commonly found in the agarwood.

Discussion

The α and β amyrins are detected commonly in medicinal plants, and many research evident that amyrin isomers in plant extracts and pure compounds exhibit antimicrobial, antioxidant, anti-inflammatory activity under both *in virto* and *in vivo* conditions [8]. Recently, it was documented that the alpha, beta-amyrin also prevent hyperglycemia, hyperlipidemia, and body weight gain in mice experiments [13]. Although we have not tested the antimicrobial activity of pure compounds, it is possible these compounds present in the EAF might serve as antimicrobial agents or precursors of such agents as α and β amyrins are precursors of Ursolic and Oleanolic acids respectively, and these acids are well known for their antimicrobial activities thus, used commercially in cosmetics and health products [9].

In the present study, the identification of these two amyrins in the EAF of *F. dlaminii* was unexpected. And this stimulated the subsequent investigation of the host bark extract, as amyrins are usually synthesized in resinous plants [8]. The pentacyclic triterpenoids and their natural derivatives are mainly obtained from plants however, their relatively low concentrations and time-consuming, labour-intensive extraction procedures are the major drawbacks [20]. On the other hand, it is not feasible to extract them from plants that are categorised as red-listed or endangered. Therefore, microbial synthesis of these compounds and their further biotransformation using microorganisms are gaining much popularity during the last decade [10]. In spite of this,

quantification of amyrins produced by *F. dlaminii*, optimizing extraction procedure and culture conditions have been identified as important tasks in future work. There is only a single report is available on a triterpenoid; Wallenone extracted from leaf extract of *G. walla* [14]. Further, there is also a single report on β-amyrin isolated from a fungus; *Aspergillus niulance* [7]. Therefore, the endophytic *F. dlaminii* we isolated here is a new source of fungal-derived α-amyrin while *G. walla* is also a new source of α and β amyrins.

Figures and Tables

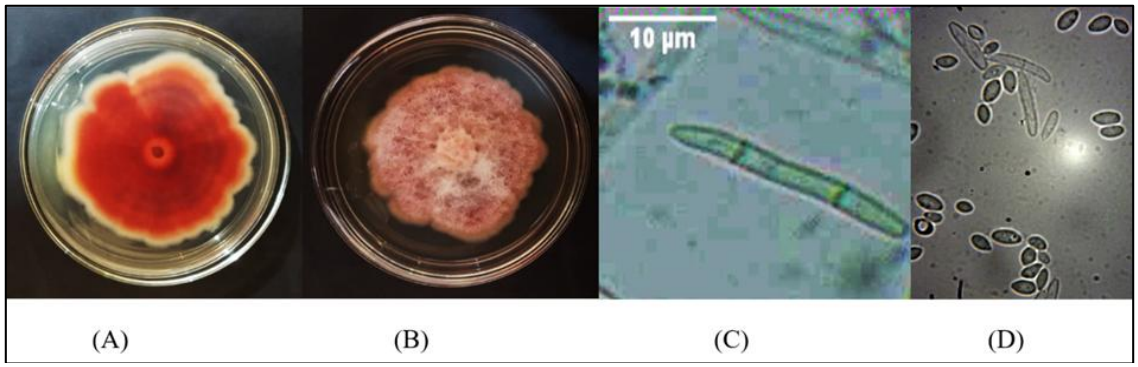


Fig 1: (A) Upper surface and (B) bottom surface view of the colony on MEA after 7 days of growth at 28 ºC. (C) Two-septate macroconidia (D) Oval shaped microconidia and mono septate, elongated macroconidia of endophytic *Fusarium dlaminii*

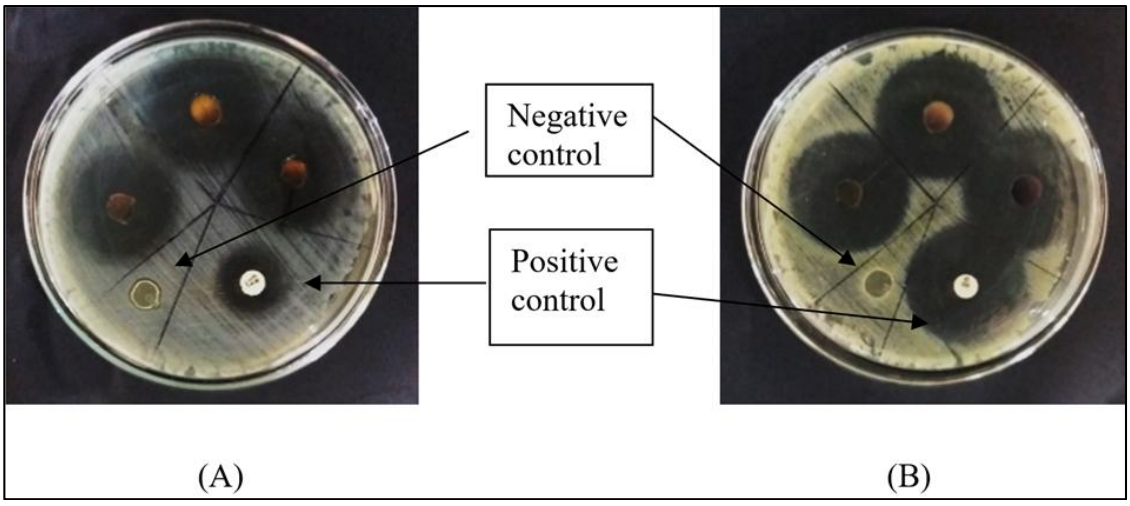
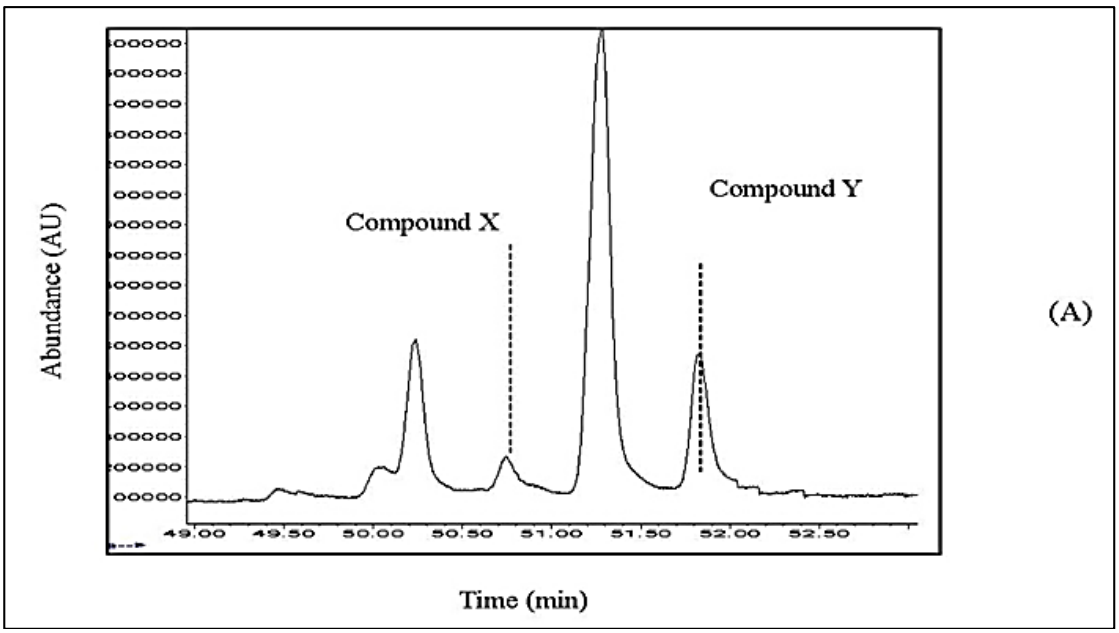


Fig 2: Antimicrobial potential of *Fusarium dlaminii* (A) Agar culture plug assay against *Escherichia coli* (B) *Staphylococcus aureus* in co-cultures on MHA medium (24 h after incubation at 37 ºC). Positive controls (A) Gentamycin 10 µg/disc and (B) Chloramphenicol 30 µg/disc. The negative control for both (A) and (B) was ethyl acetate (solvent)



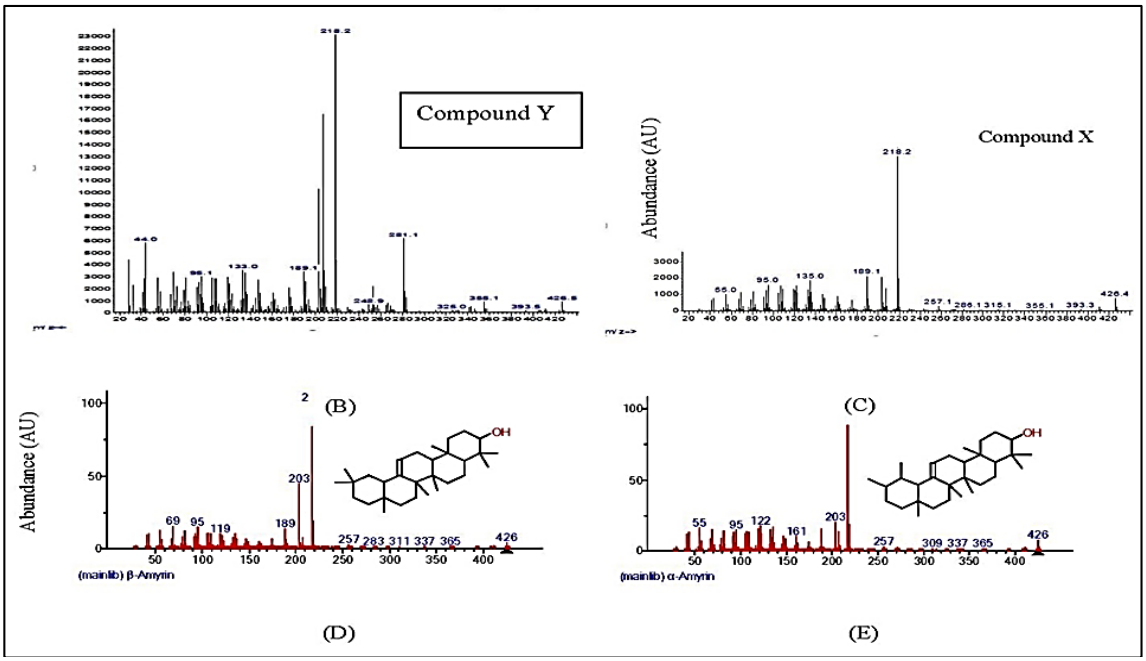


Fig 3: (A) GCMS chromatogram of Ethyl acetate extraction of *Fusarium dlaminii* (EAF) (B) Mass spectrum of β amyrin in the EAF(C) Mass spectrum of α amyrin in the EAF (D) Mass spectrum of β amyrin in the NIST database (E) Mass spectrum of α amyrin in the NIST database

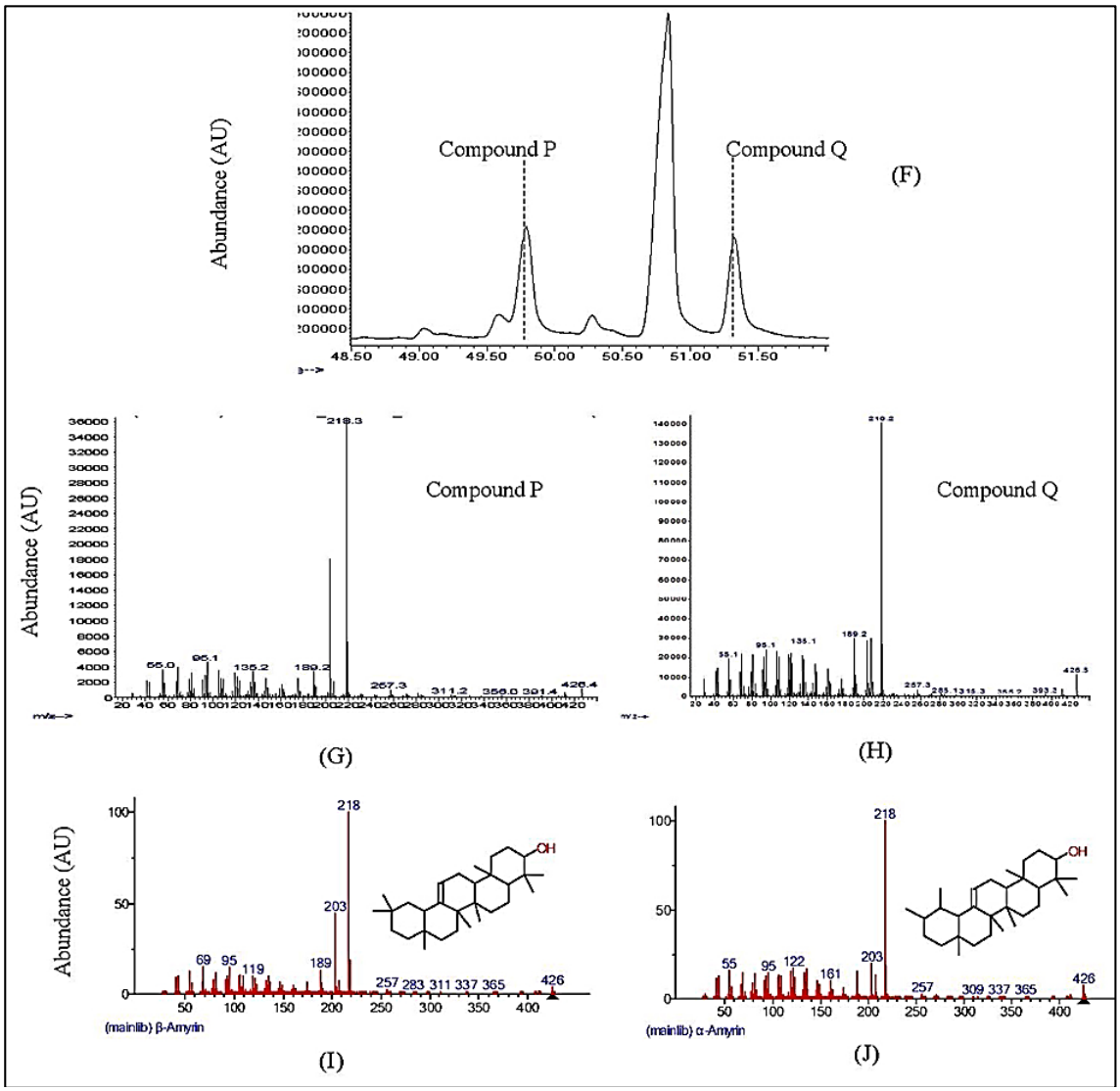


Fig 4: (F) GCMS chromatogram of the ethyl acetate extraction of *Gyrinops walla* bark (EAB). (G) *Masss pectrum* of β amyrin in the EAB.(H) Mass spectrum of α amyrin in the EAB(J) Mass spectrum of β amyrin in the NIST database (J) Mass spectrum of α amyrin in the NIST database

Table 1: Interpretative standards of zone diameter for disc diffusion technique

Anti-microbial Agent	Disc Content	Zone diameter (To the nearest whole millimeter)		
		Resistant	Intermediate	Susceptible
Chloramphenicol	30 µg	≤ 12	13-15	≥ 16
Gentamycin	10 µg	≤ 12	13-14	≥ 15
Ketoconazole	10 µg	≤ 20	21-27	≥ 28

Table 2: *In vitro* antimicrobial activity of the ethyl acetate extract of *F. dlaminii* (EAF) cultured on PDA for 14-day as assessed by standard Kirby-Bauer disc diffusion

	Mean diameter (mm) + SD of inhibition zone			
	C. glabrata	***E. coli***	***S. aureus***	**MRSA**
PDA Plate culture-EA fraction	22.0 ± 1.6	23.0 ± 1.7	26.2 ± 1.3	24.5 ± 2.2
Chloramphenicol (30 µg)	-	-	16.0 ± 0.0	16.0 ± 0.0
Gentamycin (10 µg)	-	15.0 ± 3.0	-	-
Ketoconazole (10 µg)	28.0 ± 0.0	-	-	-

Conclusions

The ethyl acetate extracts of *G. walla* and its associated endophytic fungus, *F. dlaminii* exhibit antimicrobial activity. The active extracts contain both α and β amyrins. *F. dlaminii*s is identified as a rare resource of α amyrin.

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Ethical Approval

Not applicable

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