ISOLATION AND ANTIBACTERIAL ACTIVITY OF THE TERPENOID COMPOUNDS FROM *CEPHALOSPORIUM* SP.

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Abstract

Endophytic fungal strain *Cephalosporium* sp. was isolated from the woods of *Hesperethusa crenulata* (Roxb.) Roem. For extraction, separation, isolation of ten-liter fermentation and antimicrobial activity of the fermented broth on eight test organisms were conducted at Microbiology Lab, Department of Botany, University of Yangon. Antimicrobial activity of the fermented broth indicated highly activity against eight test organisms. After fermentation, the filtrate was extracted on Ambilite XAD 16 resin column with methanol. The methanol extract showed good antimicrobial activity than on *Aspergillus flavus*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Micrococcus luteus*, *Salmonella typhus* and *Staphylococcus aureus.* Separation and isolation of the bioactive compounds from the methanol extract were carried out by using silica 34 gel columns with various solvent systems at Microbiology Lab, Department of Botany, University of Yangon. The seven compounds (A to G) were isolated from the methanol extract of 10 L fermentation. Among them, three terpenoid compounds were mentioned in this paper. Among the isolated compounds, the compound A, B and C were terpenoid compounds and they indicated antibacterial activity on five test organisms. Therefore, these compounds are good to inhibit diarrhea and fever on man as well as crown gall and leaf blight diseases on plants.

Keywords: *Cephalosporium* sp., Fermentation studies, Terpenoids

Introduction

Plant endophytic fungi were found in each plant species examined, and there are over one million fungal endophytes existed in the nature (Petrini, 1991). Plant endophytic fungi have been recognized as an important and novel resource of natural bioactive products with potential application in agriculture, medicine and food industry (Strobel, *et al*., 2004). Many scientists have been interested in studying fungal endophytes as potential producers of novel and biologically active compounds (Stierle *et al*., 1993).

In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully discovered from the endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids and phenols (Zhang *et al*., 2006). Terpenes play an important role as the signal compounds and growth regulators (Narayan *et al.*, 2017).

The objectives of this study are to extract the bioactive compounds from fermented broth of *Cephalosporium* sp. isolated from the woods of *Hesperethusa crenulata* (Roxb.) Roem., to isolate the bioactive compounds from methanol extract of fermented broth, to study the characterization of the isolated compounds and to evaluate antibacterial activity of the isolated compounds.

Materials and Methods

Fermentation of isolated fungal strain *Cephalosporium* sp.

The small piece (1cm^2) of fungus from the plate culture of *Cephalosporium* sp. was inoculated into 300 mL of conical flask containing 180 mL of sucrose/yeast extract seed medium.

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The flask was incubated at 30°C for two days as seed culture. Two days old seed culture (180 mL) was transferred into ten flasks of 2 L conical flask containing 1 L fermentation medium (15 mL seed culture in each flask). These flasks were incubated on shaker at 100 rpm for a week at room temperature (Strobel and Sullivan, 1999). These fermented broths from 10 flasks were tested for antimicrobial activity on *Aspergillus flavus*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Micrococcus luteus*, *Salmonella typhus* and *Staphylococcus aureus.*

Antimicrobial activity by paper disc diffusion assay

Broth culture (50 µL) of test organisms (*Aspergillus flavus*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Micrococcus luteus*, *Salmonella typhus* and *Staphylococcus aureus*) was added to 100 ml assay medium sucrose /yeast medium (SY) and then poured into plates. After solidification, paper discs infused with broth samples were applied on the test plates and incubated at 30°C for 24 hrs. When clear zones (inhibitory zones) showed around the paper discs, they were measured. The paper disc size is 6.0 mm. These inhibitory zones showed the presence of the bioactive compounds that inhibit the growth of the test organisms (Davis and Stout, 1971).

Extraction of the bioactive compounds from fermented broth

After testing antimicrobial activity, 10 L fermented broth was filtered with the filter paper. The mycelia were filtered and eluted with acetone while the filtrate was applied on an Amberlites XAD 16 resin column. The resin column was washed with water, followed by five liters of methanol (Figure 1). The acetone extract and methanol extract were evaporated on water bath at 50-55 °C. These extracts were tested for antimicrobial activities on *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Malassezia furfur*, *Salmonella typhi* and *Staphylococcus aureus* (Grabley *et al.*, 1999).

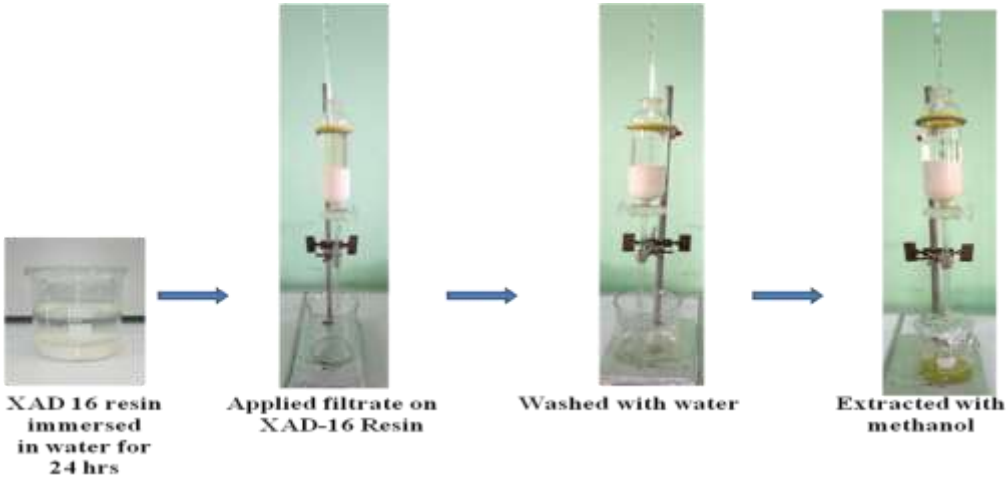


Figure 1 Extraction on Amberlites XAD-16 Resin

Separation, Isolation of terpenoid compounds

This chemical portion was conducted at Microbiology Laboratory, Department of Botany, University of Yangon. The bioactive compounds from fungal strain *Cephalosporium* sp. were separated and isolated by using various solvent systems (PE: DCM (1:1, 1:2,1:3, 1:4, 1:5, 1:7, 1:9, 1:10, (DCM 100%), DCM: MeOH (100%, 97:3, 93:7, 90:10, (EA, 100%), EtOAc: MeOH (95:5, 9:1, 8:2, 6:4), (MeOH 100%)) on 34 silica gel (100g) column. The fractions were collected and the collected fractions were combined according to their behavior on TLC plate.

Silica gel column chromatography

According to TLC result, silica gel column chromatography was carried out. The silica 34 gel (100 g) was dissolved in dicholoromethane thoroughly and then poured into the column. After the level of the silica gel surface in the column was stable, the methanol extract was added through the column. The column was eluted with petroleum ether: dicholomethane (1:1, 1:2, 1:3, 1:4, 1:5, 1:7, 1:9, 1:10, (DCM, 100%), DCM: MeOH (100%, 97:3, 93:7, 90:10, (MeOH 100%) and then nine fractions were collected. The column size was 3.5 cm × 42 cm and flow rate was 2 mL per minute as shown in Figure 2 (Grabley *et al.*, 1999).

The eluting solvent	-	PE:Dicholomethane (1:1, 1:2, 1:3, 1:4, 1:5, 1:7, 1:9, 1:10) DCM 100%, DCM:MeOH (100%, 97:3, 93:7, 9:1) MeOH, 100%
Column Size	-	3.5cm × 42 cm
Flow rate	-	2ml/min

Figure 2 Silica 34 gel column

Characterization of the isolated compounds from *Cephalosporium* sp.

The isolated compounds were characterized by spectroscopic techniques such as UV and FT-IR spectra, and their behaviour on TLC plates. The spectra were undertaken at Universities Research Centre, University of Yangon. The spectral assignments were assigned according to Robert and Francis (2014).

Antimicrobial activity of the isolated compounds from *Cephalosporium* sp.

The three terpenoid compounds were tested their antimicrobial activities on eight pathogenic microorganisms *Agrobacterium tumefaciens, Aspergillus flavus, Candida albicans, Escherichia coli, Malassezia furfur, Salmonella typhi, Staphylococcus aureus* and *Xanthomonas oryzae* by paper disc diffusion assay.

Results

Antimicrobial activity of 10 L fermentation of *Cephalosporium* sp.

In this study, the fermented broths from the ten fermentation flasks showed highly antimicrobial activity against eight test organisms as shown in Table 1.

Table 1 Inhibitory zones (mm) of fermented broths from 10 L fermentation

Fermentation flask	*Asper. flavus*	*Bacillus subtilis*	*Candida albicans*	*E. coli*	*M. furfur*	*Micro. luteus*	*Sal. typhi*	*Staphy. aureus*
Flask 1	19	19	20	19	20	19	19	22
Flask 2	21	19	21	20	24	20	18	23
Flask 3	20	20	20	19	19	18	18	21
Flask 4	22	18	21	20	23	18	20	27
Flask 5	20	18	18	22	22	18	17	23
Flask 6	21	25	23	22	22	17	21	24
Flask 7	13	11	14	18	17	12	12	16
Flask 8	24	26	25	23	27	24	23	29
Flask 9	17	21	19	22	20	18	16	19
Flask 10	16	16	15	20	18	15	14	18

10 -12 mm = weak activity, 13 - 17 mm = high activity, >18 mm = very high activity

Isolation of the bioactive compounds from *Cephalosporium* sp.

The one hundred and eight small fractions were collected from silica gel 34 column with various solvent systems. According to their R_f values and colour spots on TLC plates under UV 254 nm and sprayed with reagent, they were combined into nine large fractions: F1 (1 & 2), F2 (3 & 4), F3 (5-12), F4 (13-41), F5 crystals (42-47), F6 (48-51), F7 (52-68), F8 (69-90) and F9 (91-108) as shown in Figure 3.

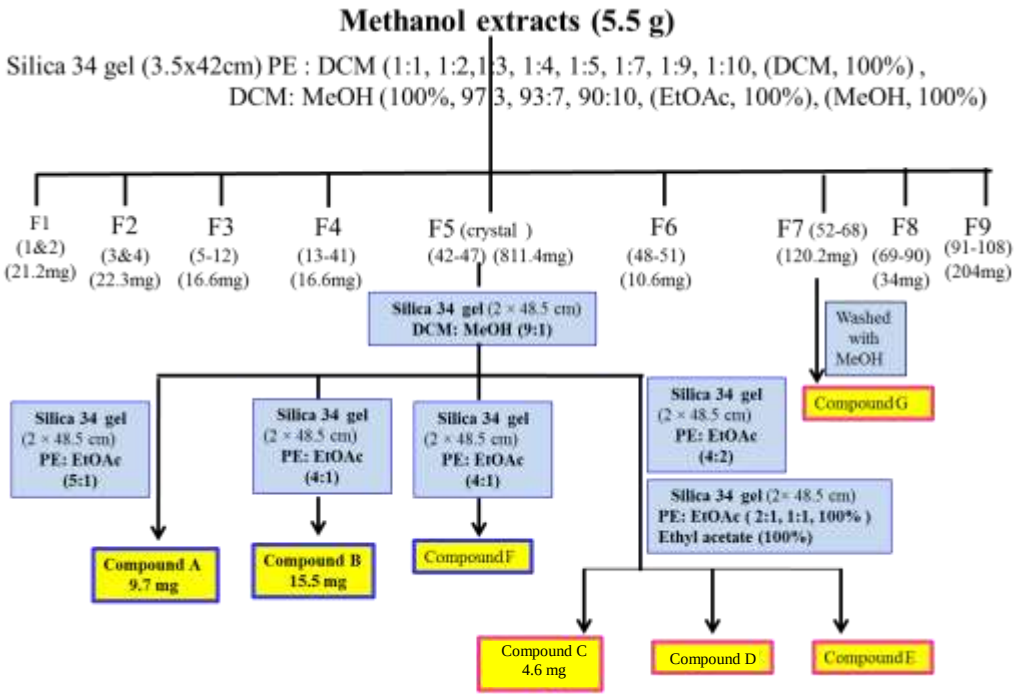
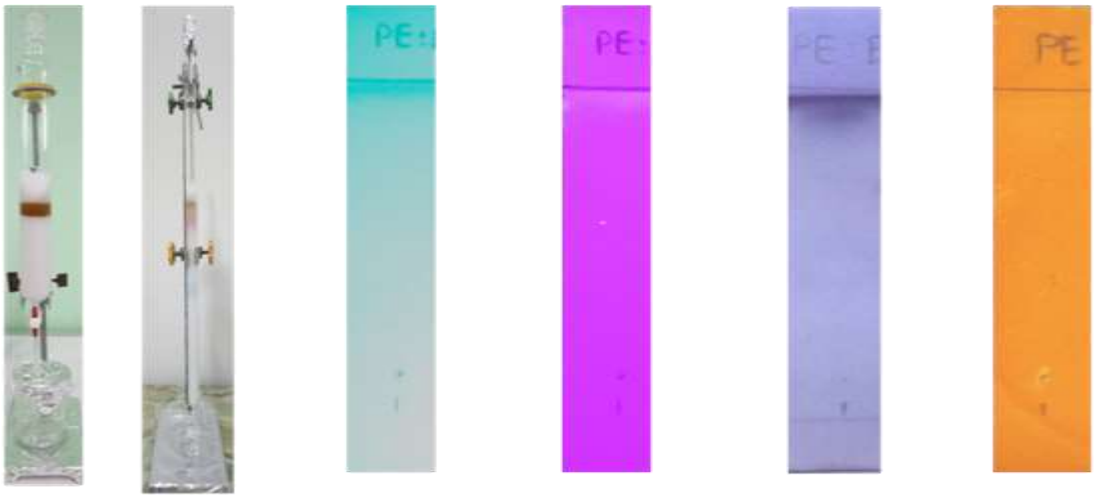


Figure 3 Isolation procedure of bioactive compounds

Physico-chemical properties of the isolated compound A



Silica 34 gel columns **UV 254 nm** **UV 365 nm** **With anisaldehyde** **With Dragendroff**

The eluting solvent = DCM: MeOH (9:1)
= PE: EtOAc (5:1)
Flow rate = 2ml / min
R_f = 0.93 (PE: EtOAc 1:1)
Spot colour = dark blue (Anisaldehyde reagent)

Figure 4 The TLC profile of isolated compound A

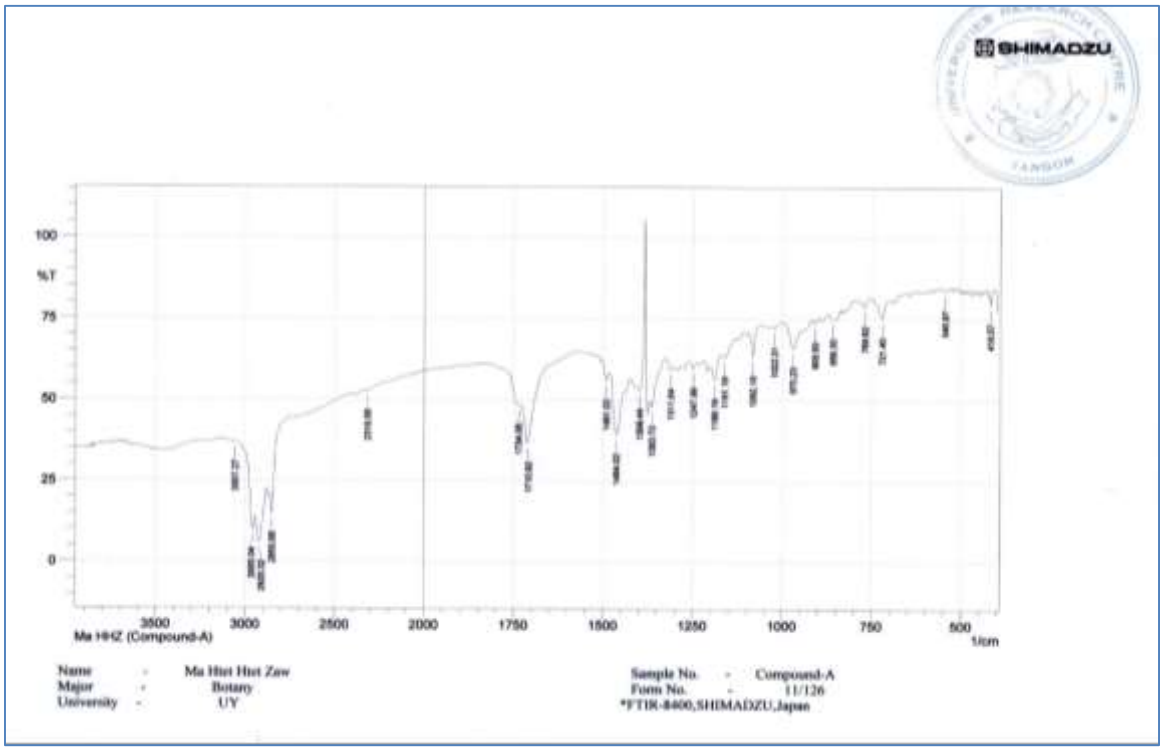


Figure 5 FT-IR spectrum of the isolated compound A

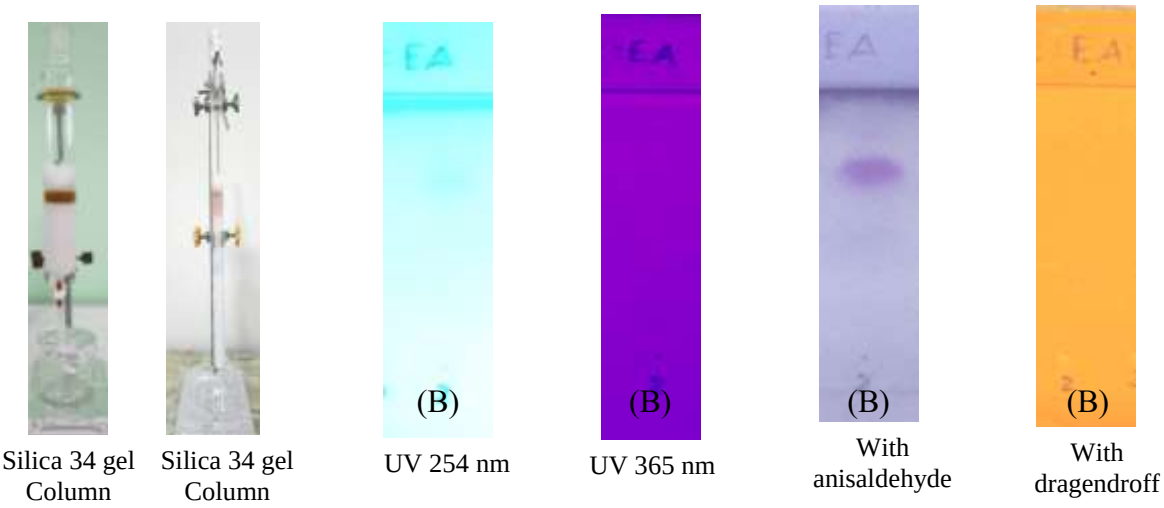
Table 2 FT-IR spectral data assignment of the isolated compound A

Wave number (cm^{-1})	Assignment
3450	O-H stretching vibration of hydroxyl group
2955, 2850	C-H stretching of -CH_3 and -CH_2- group
1710	C=O stretching vibration of carbonyl group
1491,1464, 1363	C-H bending of -CH_3 and -CH_2- group
1464, 970	O-H bending vibration of hydroxyl group
1247, 1181, 1082, 1022	C-O-C stretching vibration (ether)

The compound A was isolated from the fraction 5, it is UV inactive. It has R_f (0.93) (PE: EtOAc, 1:1). It gave a dark pink colour with anisaldehyde reagent firstly. Then, the colour changed into dark blue (Figure 4).

In its IR spectrum (Figure 5), O-H stretching vibration of hydroxyl group was observed at 3450 cm^{-1}. CH_3 and CH_2 groups (C-H stretching) were found at 2955 cm^{-1} and 2850 cm^{-1}. C = O stretching vibration of carbonyl group was shown at 1710 cm^{-1}. CH_3 and CH_2 groups (C-H bending) were observed at 1491 cm^{-1}, 1464 cm^{-1} and 1363 cm^{-1}. O-H bending vibration of hydroxyl group was seen at 1464 cm^{-1} and 970 cm^{-1}. C-O-C stretching vibrations (ether) were found at the wave numbers 1247 cm^{-1}, 1181 cm^{-1}, 1082 cm^{-1} and 1022 cm^{-1}. This substance is good soluble in petroleum ether or dichloromethane. It is a terpenoid compound according to behaviour on TLC plate and IR spectral data.

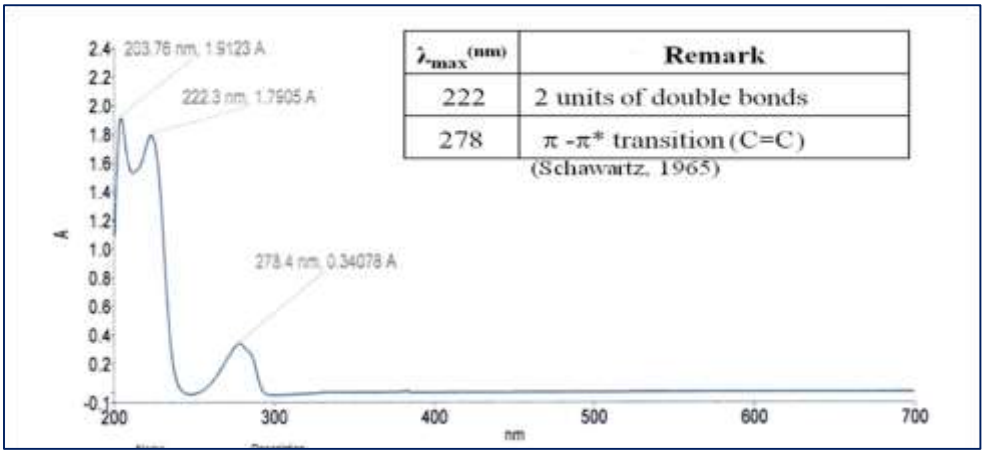
Physico-chemical properties of the isolated compound B



Silica 34 gel Column | Silica 34 gel Column | UV 254 nm | UV 365 nm | With anisaldehyde | With dragendroff

The eluting solvent = DCM: MeOH (9:1)
= PE: EtOAc (4:1)
R_f = 0.73 (PE: EtOAc 1:1)
Spot colour = light colour (under UV 254nm)
= primary colour is dark pink with anisaldehyde reagent but later changed purple colour

Figure 6 The TLC profile of isolated compound B



λ_{max} (nm)	Remark
222	2 units of double bonds
278	π -π* transition (C=C)

(Schawartz, 1965)

Figure 7 UV spectrum of the isolated compound B

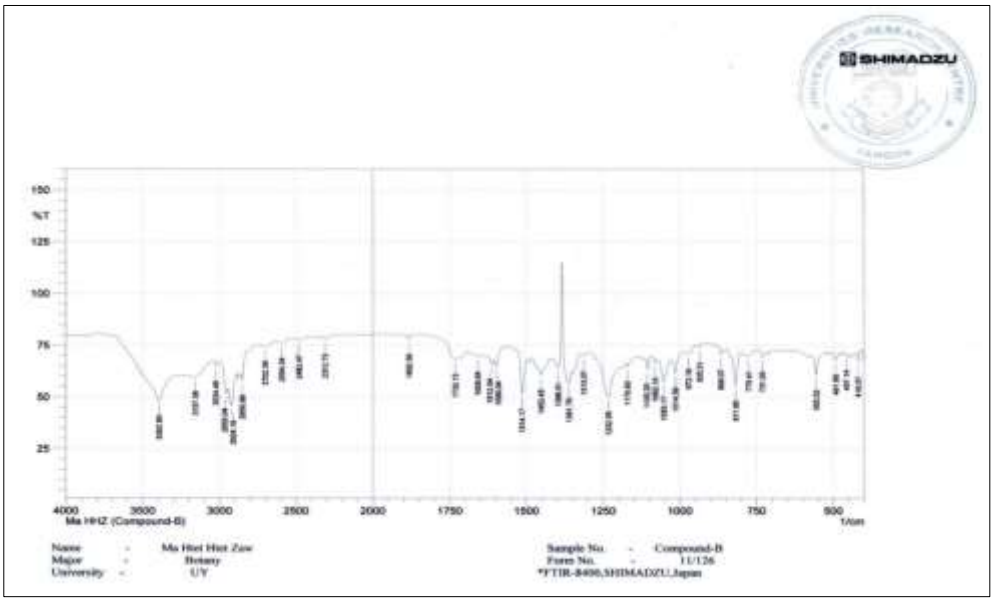


Figure 8 FT-IR spectrum of the isolated compound B

Table 3 FT-IR spectral data assignment of the isolated compound B

Wave number (cm^{-1})	Assignment
3392	O-H stretching vibration in phenolic group
2924, 2850	C- H stretching vibration of -CH_3 and -CH_2 -
1732	C=O stretching vibration
1658, 1514, 1452	C=C stretching vibration (aromatic)
1014, 1053, 1105	C-O stretching vibration of C-O-C
1452, 972	C-OH and O-H bending of aromatic ring

The compound B was isolated from the fraction 5 as a UV active substance under absorbing band at 254 nm. It has R_f 0.73 (PE: EtOAc, 1:1). It gave a dark pink colour with anisaldehyde reagent firstly. Later, the colour changed into purple spot (Figure 6). This substance is good soluble in dichloromethane or methanol. Its UV spectrum showed the two units of double bonds (C=C) at 222 and 278 nm as shown in Figure 7.

In its IR spectrum (Figure 8), O-H stretching vibration of hydroxyl group was observed at 3392 cm^{-1}. C-H stretching vibrations of CH_3 and CH_2 groups were found at 2924 cm^{-1} and 2850 cm^{-1}. C=O stretching vibration was shown at 1732 cm^{-1}. C=C stretching vibrations (aromatic) were found at the wave numbers 1658 cm^{-1}, 1514 cm^{-1} and 1452 cm^{-1}. C-O stretching vibrations of C-O-C were found at 1014 cm^{-1}, 1053 cm^{-1} and 1105 cm^{-1}. C-OH and O-H bending vibrations of aromatic ring were seen at 1452 cm^{-1} and 972 cm^{-1}. It is a terpenoid compound according to behaviour on TLC profile, UV and IR spectral assignments.

Physico-chemical properties of the isolated compound C

Silica 34 gel Column | Silica 34 gel Column | Silica 34 gel Column | With anisaldehyde

The eluting solvent = DCM: MeOH (9:1)
= PE: EtOAc (2:1, 1:1, 100%) Ethyl acetate (100%)
Flow rate = 2ml / min
R_f = 0.36 (PE: EtOAc 1:1)
Spot colour = Inactive
Purple colour (Anisaldehyde reagent)

Figure 9 The TLC profile of isolated compound C

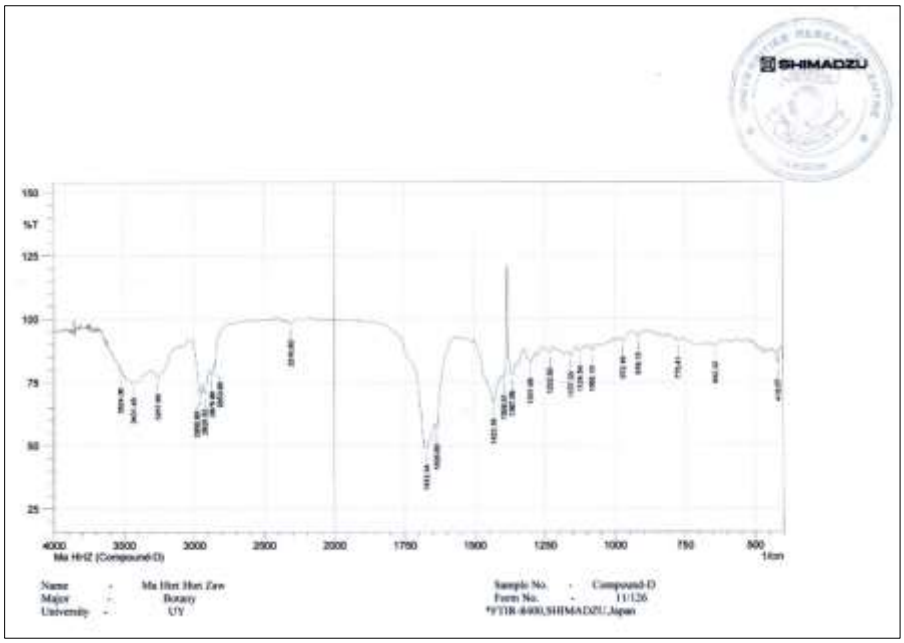


Figure 10 FT-IR spectrum of the isolated compound C

Table 4 FT-IR spectral data assignment of the isolated compound C

Wave number (cm^{-1})	Assignment
3431, 3257	O-H stretching vibration in phenolic group
2958, 2875	C-H stretching of -CH_3, -CH_2- and -CH
1672	C=C stretching vibration (aromatic C=C)
1433, 1396	C-H bending vibration of -CH_3, -CH_2- and -CH
1157	C-O-C stretching vibration of ether

The compound C was also isolated from the fraction 5, and it is UV inactive. It has R_f 0.50 (PE: EtOAc 1:1). It gave a pink colour with anisaldehyde reagent (Figure 9). This substance is good soluble in petroleum ether and ethylacetate.

In its IR spectrum (Figure 10), O-H stretching vibration in phenolic group was observed at 3431 cm^{-1} and 3257 cm^{-1}. The wave numbers 2958 cm^{-1} and 2875 cm^{-1} showed the presence of C-H stretching vibrations for CH_3 and CH_2 group. C=C stretching vibration (aromatic C=C) was shown at 1672 cm^{-1}. C-H bending vibrations of CH_3 and CH_2 and CH were observed at 1433 cm^{-1} and 1396 cm^{-1}. C-O-C stretching vibration of ether was found at the wave number 1157 cm^{-1}. It is a terpenoid compound according to its behaviour on TLC profile and IR spectral assignments.

Antibacterial activities of the terpenoid compounds

The compounds A, B and C showed weakly antibacterial activity on *Agrobacterium tumefaciens, Escherichia coli* and *Salmonella typhi* while the compound A also showed moderately antibacterial activity on *Staphylococcus aureus* and *Xanthomonas oryzae.*

Discussion and Conclusion

Endophytic fungal strain *Cephalosporium* sp. was isolated from the wood of *Hesperethusa crenulata* (Roxb.) Roem., and utilized for extraction, separation, isolation and antimicrobial activity of the bioactive compounds. In antimicrobial activity of 10 L fermentation, all fermentation flasks showed high activity against eight test organisms. In the extraction, the fermented broths were applied on XAD 16 resin column followed by elution with methanol. Mohamed Shaaban *et al*., (2013) also used XAD-16 resin to extract the filtrate using followed by elution with methanol. The methanol extract showed highly activity against eight test organisms.

The three isolated compounds were the terpenoid compounds that showed antimicrobial activity on *Agrobacterium tumefaciens*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Xanthomonas oryzae.* Gerlach *et al*., (2018) reported that the use of sulfuric anisaldehyde reagent greatly improves the detection of terpenes. Miao *et al.,* (2012) stated that harziane diterpenes were isolated from endophytic fungus. Liu *et al.,* (2013) mentioned that new sesterterpenoids were isolated from different endophytic fungi. Yumei *et al*., (2004) reported that the secondary metabolites of *Cephalosporium* sp. showed activity against *M. luteus, B. subtilis, S. aureus, S. typhi, E. coli* and *P. aeruginosa*.

In conclusion, the seven compounds were isolated from the methanol extract of *Cephalosporium* sp. The three from the seven compounds were the terpenoid compounds that possessed antibacterial activity on five test organisms. Therefore, these compounds inhibited diarrhea, fever, and skin infection on man as well as crown gall disease and leaf blight disease on plants.

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