STRUCTURE ELUCIDATION OF AN ISOLATED COMPOUND FROM THE HENLIN HOT SPRING DERIVED THERMOPHILIC BACTERIAL STRAIN AND ANTIBACTERIAL ACTIVITY

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Abstract

The thermophilic bacterial strain No. 21 which was obtained from the Department of Biotechnology, Mandalay Technical University, showed antagonistic activity against *S. aureus* (MRSA). Ethyl acetate crude extract was prepared from the strain fermentation broth. A pure Organic compound (SMOo) was isolated as a colourless oily form (250 mg) from ethyl acetate extract by chromatographic separation methods. The chemical structure of the isolated compound was identified by using modern spectroscopic techniques such as FT IR, ^{1}H NMR, COSY, ^{13}C NMR, HMQC, DEPT, HMBC and EI-MS. In addition, the antibacterial activity of the isolated compound was investigated by the agar well diffusion method and the isolated compound showed significant inhibitory activity on *S. aureus* (MRSA).

Keywords: thermophilic bacterial strain, antagonistic activity, antibacterial activity, spectroscopic data

Introduction

Infectious disease outbreaks become a challenge of worldwide nowadays. It is also the major cause of death which leads the 25% of all causes per year. Recently, antibacterial drugs have become less effective or even ineffective, resulting in an accelerating global health security emergency that is rapidly outpacing available treatment options (WHO, 2014). Natural products isolated from microorganisms have not only been the source of most of the antibiotics currently on the market but also the largest contributors to drugs in the history of medicine. To obtain antimicrobials from microorganisms, many scientists are trying to study unusual habitats for the discovery of new bioactive compounds (Harvey *et al*., 2001).

An extremophile is an organism that thrives in physically or geographically extreme conditions that are detrimental to most life on earth (Rampelotto, 2010). Over the last year, the extremophile with different categories, thermophiles (high temperature), acidophiles (low pH), alkaliphiles (high pH), halophiles (high salinity), and psychrophiles (low temperature) have the capability to produce new bioactive compounds under extreme or unusual conditions (Tango and Islam, 2002). Thermophilic bacteria (optimum growth temperature of 50 °C or above) have attracted great attention among extremophiles because they are sources of new bioactive metabolites (Singh *et al*., 2011). Hot water springs are situated throughout the length and breadth of India and Myanmar, at places with boiling water (e.g., Manikaran, Himachal Pradesh and Helin, an Ancient city of Myanmar) (Win Min Thant, 2008).

In 2010, a detailed experimental work for determination of antibacterial activity and isolation of a pure compound (SMOo) from the thermophilic bacterial strain No. 21 was reported (Sann Myint Oo *et al*., 2010). The suggested structure was mainly determined by FT IR spectroscopy at that time. In this paper, the complete structure of isolated compound, a phthalate derivative, is reported by extensive NMR spectroscopy. Phthalate derivatives are colourless liquid chemicals that have been used as plasticizers to improve the plasticity and the flexibility of materials. They can also be synthesized by plants and bacteria or fungi, and many studies have reported different biological activities of these compounds (Ortiz & Sansinenea, 2018).

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Materials and Methods

Strain Collection

The ten isolated thermophiles which respond to antagonistic activities were provided by the Department of Biotechnology, Mandalay Technical University. They were cultured using the sample collected from the Henlin hot spring (Temp. 51°C and pH 5-6), the ancient city of Myanmar (Win Min Thant, 2008). Among them, the effective strain No. 21 showing the antagonistic activity on *S. aureus* (MRSA) was selected for this research work.

Fermentation of the Effective Strain

280 mL of inoculum was cultured from the selected strain at 90 rpm and room temperature for two days. For the production of a bioactive compound, the inoculum (280 mL) was transferred to the fermenter (5 L) containing 2.8 L of the prepared broth (antibiotic producing peptone medium). Running condition as impeller rotation, OD and temperature fixed at 220 rpm, 12.5 and 30 ºC was performed. The fermentation process was derived for 96 hours (Crueger *et al*., 1989).

Isolation and Purification of a Bioactive Compound

The fermentation broth was extracted three times with ethyl acetate (1:1, v/v). The ethyl acetate extract was separated from an aqueous phase by separating funnel. It was then concentrated by using a rotary evaporator and evaporated to dryness at room temperature to obtain the crude extract. The crude extract (750 mg) was fractionated using column chromatographic separation packed with silica gel and eluted with *n*-hexane gradually enriched with ethyl acetate to afford 42 fractions. Then, these fractions were confirmed for purity and homogeneity by analytical TLC. The fractions with the same R_f values were combined to give four fractions (I-IV). Among these combined fractions, the combined fraction (I) was rinsed by re-dissolving in *n*-hexane for further purification. It was checked by TLC for purity [*n*-Hexane: EtOAc, 9:1 (v/v)] (Sann Myint Oo *et al*., 2010).

Structural Elucidation of a Bioactive Compound

FTIR spectrum of an isolated compound was measured at the Department of Chemistry, University of Mandalay. ^{1}H NMR, COSY, ^{13}C NMR, HMQC, DEPT, HMBC, and EI-MS spectra were also determined at Meijo University, Nagoya, Japan. The isolated compound was elucidated by using the above spectroscopic techniques.

Screening of Antibacterial Activity of Isolated Compound

The antibacterial activity of isolated compound was determined on tested organism, *S. aureus* (MRSA), by agar well diffusion and agar drop diffusion methods. *n*-Hexane solvent was used as control. 1.05 g of Muller-Hinton and 1.5 g agar were dissolved in the sterilized beaker containing 50 mL of distilled water. It was then stirred with magnetic stirrer and sterilized by autoclaving at 121 °C for 15 minutes. After sterilization of medium, 20 mL of this medium was poured on the sterilized petridishes. The medium was then allowed to harden and dried in microwave oven at 40 °C for ten minutes. 0.1 mL of tested organism was inoculated into 20 mL of medium at 40 °C for four hours and swabbed onto the Muller-Hinton agar pate. Using a 5 mm punch, a well was made on the agar plate. An isolated compound (SMOo) solution (25 μL) that are dissolved in *n*-hexane was introduced into the well and dropped on the agar plate. It was incubated in an incubator at 27 °C for 24 hours. After incubation, the clear zone of agar well diffusion was measured and recorded (Collin, 1965).

Results and Discussion

Isolated Organic Compound from the Bacterial Fermentation Broth

The combined fraction (I) separated from the ethyl acetate extract (1.67 g) by column chromatographic method gave one spot on TLC with R_f value of 0.66 [*n*-Hexane: EtOAc, 9:1 (v/v)] and UV active. A pure colourless oily form (250 mg, 31.2% based on theyl acetate exract) was obtained.

Identification of Isolated Compound

FT IR spectrum (Figure 1) of the isolated compound showed the presence of the sp^2 =C-H stretching of the alkenic group at 3070 cm^{-1}. The absorption bands appeared at 2958, 2931 and 2873 cm^{-1} were responsible for the C-H stretching vibrations of sp^3 hydrocarbon. The sharp peak at 1728 cm^{-1} indicated the carbonyl stretching of the ester carbonyl group (-C=O). The conjugated C=C stretching of the aromatic ring was associated with the bands of 1600 and 1581 cm^{-1}. The intense peaks at 1272, 1122 and 1072 cm^{-1} attributed to the –C-(C=O)-O- stretching vibration of the ester group whereas those at 767, 705 and 651 cm^{-1} were corresponding to the =C-H bending vibration of an olefinic group.

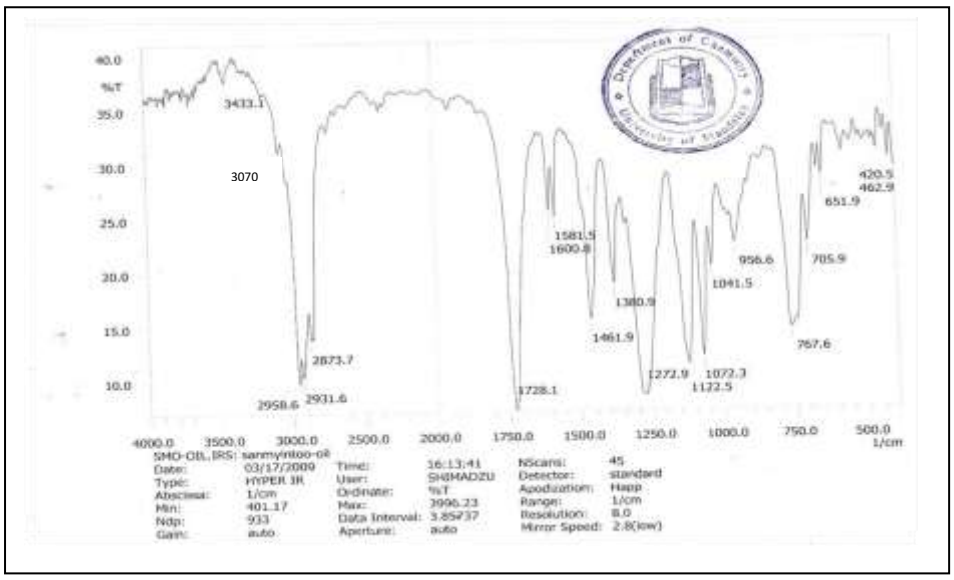


Figure 1. FT IR spectrum of the isolated compound

The ^{1}H NMR spectrum (Figure 2) displayed the characteristic aromatic proton signals of the *o*-disubstituted benzene ring [7.70 (2H, dd, *J* = 8.8 and 3.2 Hz, H-3 and H-6)] and [7.52 (2H, dd, *J* = 8.8 and 3.2 Hz, H-4 and H-5]. Two similar ester moieties were evidenced at 4.21 (4H, *m*, H-1'), 1.68 (2H, *m*, H-2'), 1.34-1.43 (16H, *m*, H-3',4',5' and 7') and 0.91 (12H, *t*, H-6' and H-8'). The ^{13}C NMR (Figure 3), HMQC (Figure 4) and DEPT (Fiture 5) spectra exhibited a total of 12 carbon signals, including characteristic signals due to an ester carbonyl at δ_C 167.7 (COO-), aromatic quaternary carbon at δ_C 132.4 (C-1 and C-2), two aromatic methine carbons at δ_C 130.8 (C-4 and C-5), 128.8 (C-3 and C-6), ester oxygen bearing methylene carbon at δ_C 68.1 (C-1'), one sp^3 methine carbon at δ_C 38.7 (C-2'), four methylene carbons at δ_C 30.3 (C-3'), 28.9 (C-4'), 22.9 (C-5'), 23.7 (C-7') and two methyl carbons at δ_C 14.0 (C-6') and 10.9 (C-8').

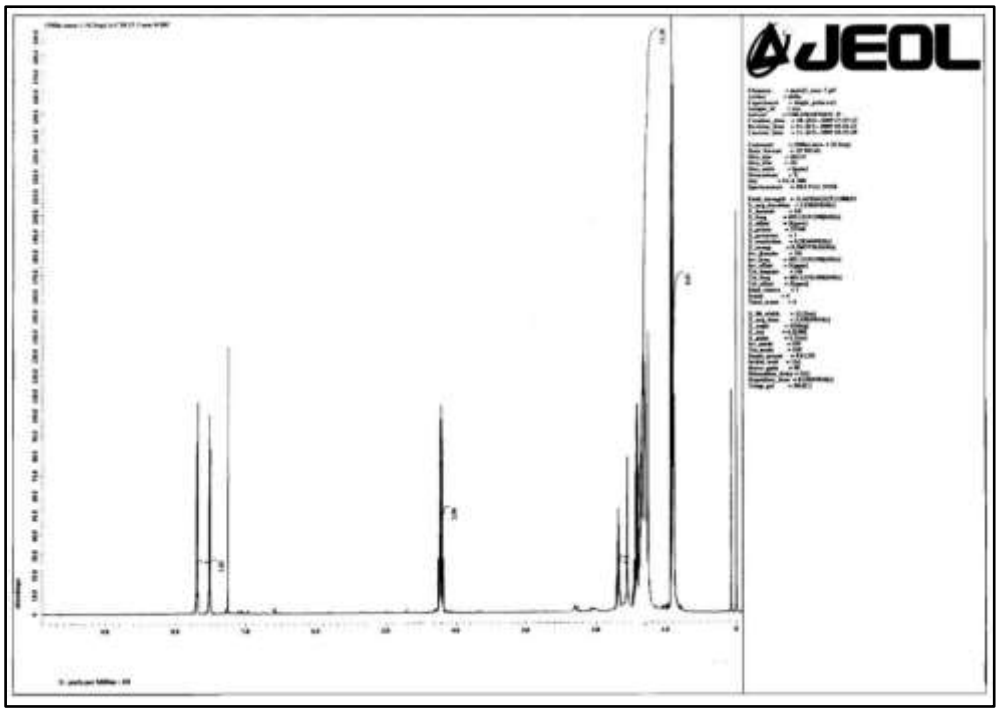


Figure 2. ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of the isolated compound

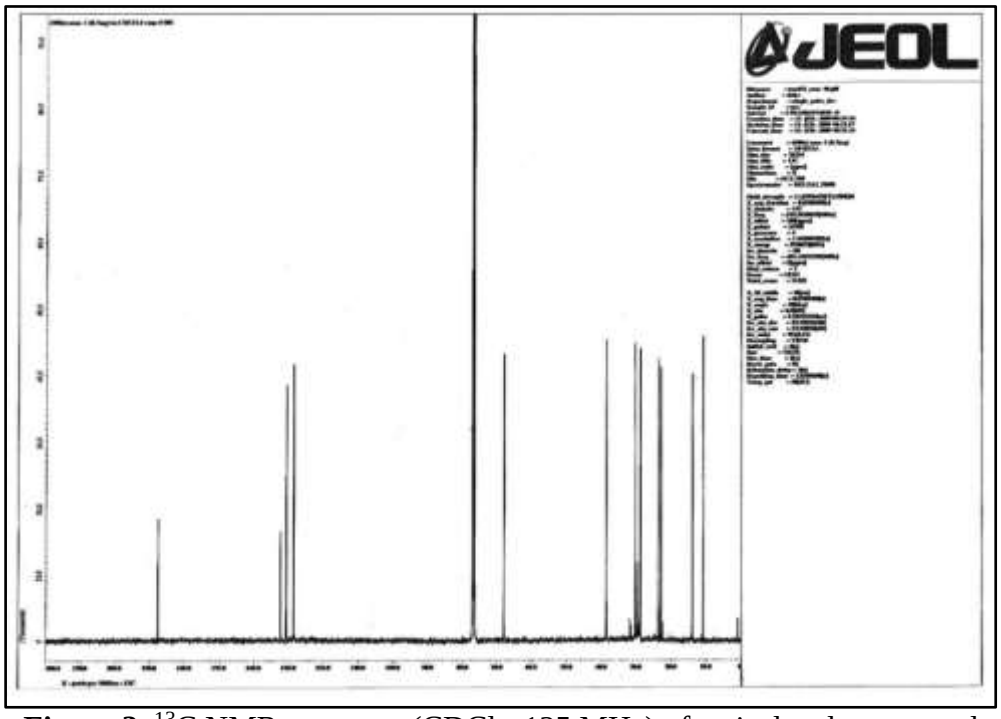


Figure 3. ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of the isolated compound

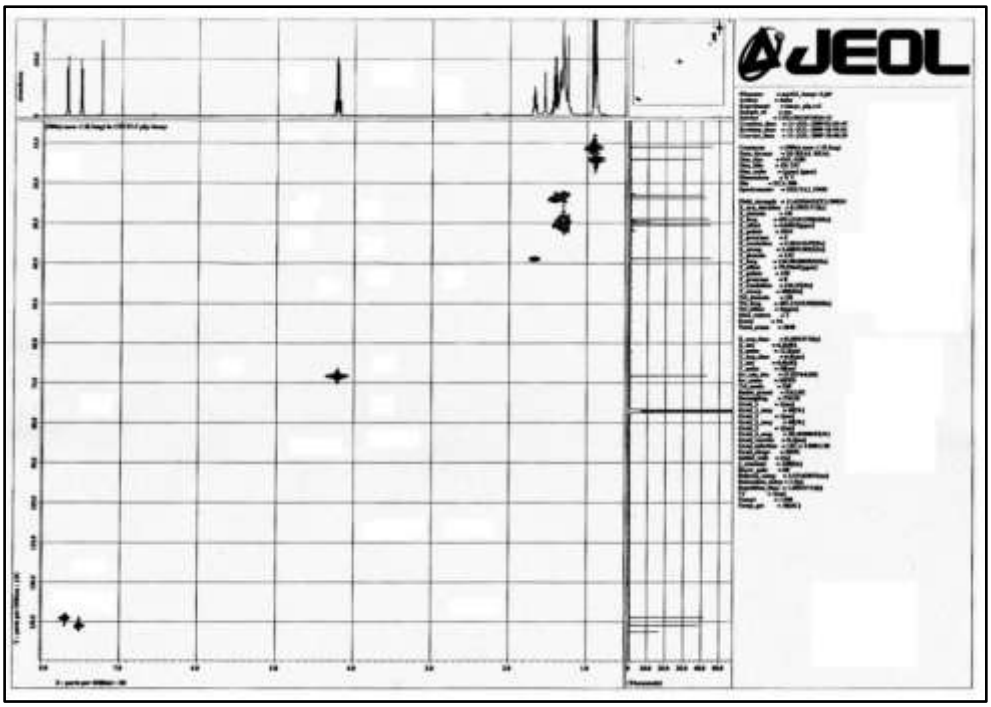


Figure 4. HMQC spectrum of the isolated compound

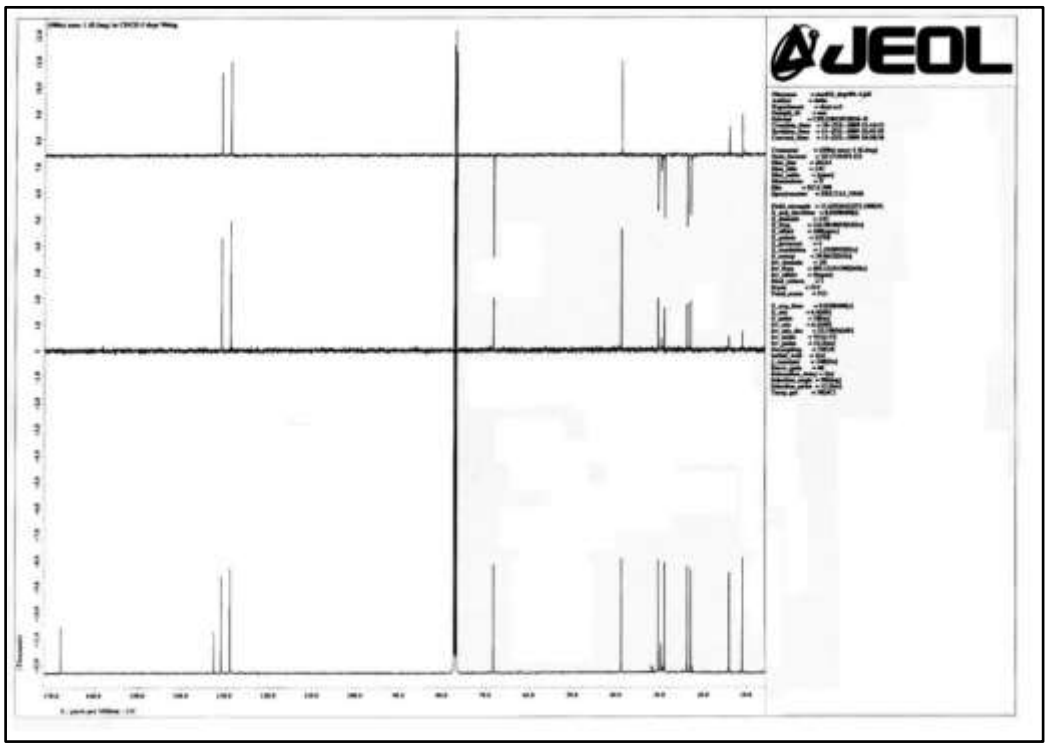


Figure 5. DEPT spectrum of the isolated compound

Moreover, the $^{1}H–^{1}H$ COSY spectrum (Figure 6) on the isolated compound indicates the presence of two partial structures which are ortho-disubstituted benzene ring and ester oxygen-bearing 2-ethyl hexoxy group. In the ^{1}H and ^{13}C NMR, and HMBC (Figure 7) spectra, the ester oxygen-bearing methylene protons [4.21 (4H, *m*, H-1')] in 2-ethyl hexoxy group respond long range coupling with the ester carbonyl carbon at 167.7 (COO-) extending the ester moiety in the

isolated compound. Two similar ester moieties are achieved due to the presence of double proton integration numbers and carbon intensity lines. The three fragments, one ortho-disubstituted benzene ring and two ester groups, are connected by HMBC correlation between the aromatic protons at 7.70 and ester carbonyl carbon at 167.7. Based on the above evidence, the structure of the isolated compound was identified to be bis (2-ethyl hexyl) phthalate as shown in Figure 8.

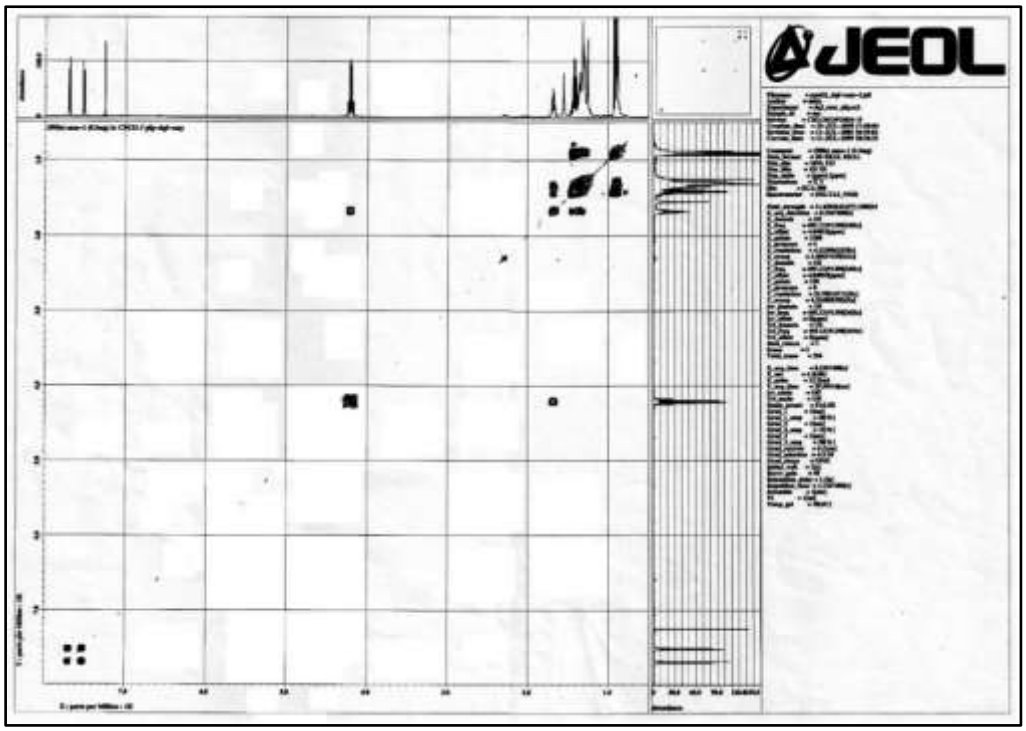


Figure 6. $^{1}H–^{1}H$ COSY spectrum of the isolated compound

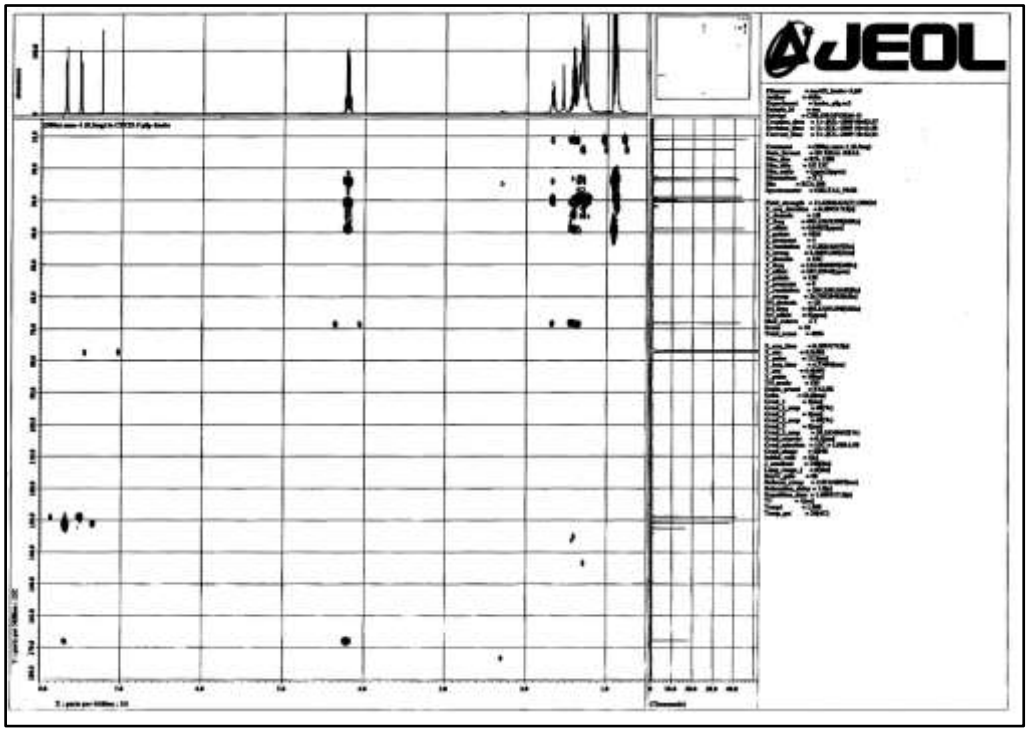


Figure 7. HMBC spectrum of the isolated compound

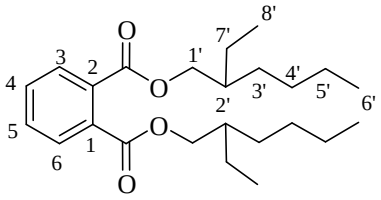


Figure 8. Chemical structure of the isolated compound

In the EI-MS of compound (Figure 9), the ions at *m/z* 149, 167 and 279 may correspond to the following fragments ***a, b*** **and** ***c***, respectively as seen in Figure 10. The molecular ion peak is present at *m/z* 390.

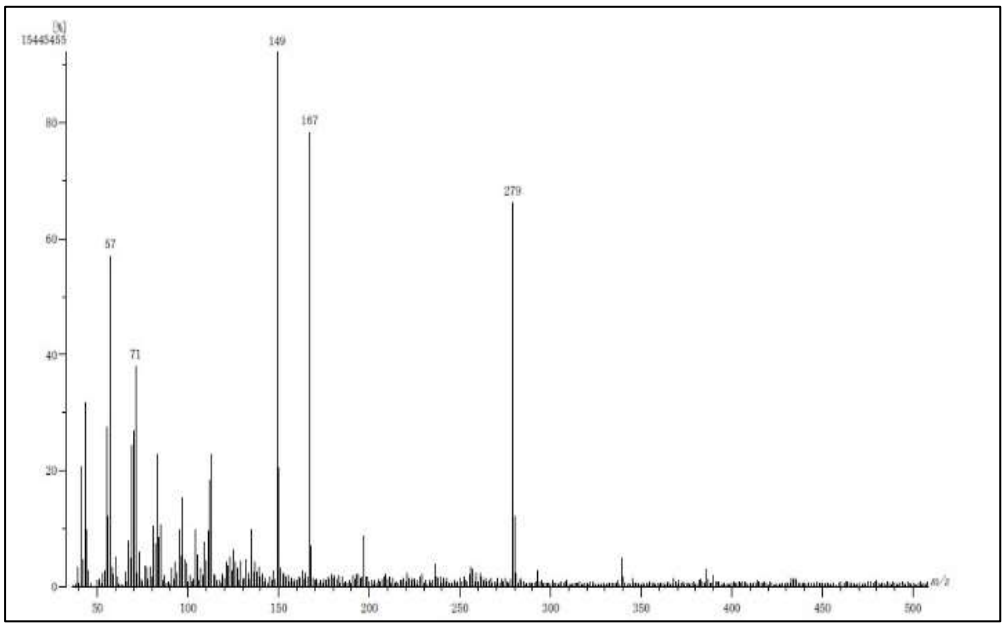
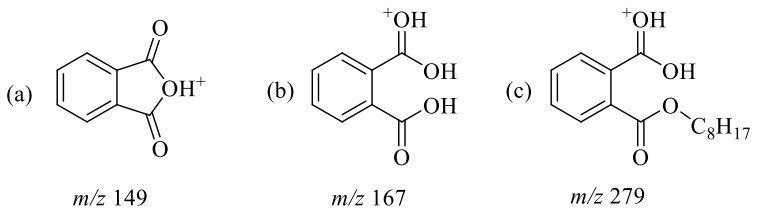


Figure 9. EI-MS spectrum of the isolated compound



m/z 149 *m/z* 167 *m/z* 279

Figure 10. EI-MS analysis of the isolated compound

Based on NMR, including observation of HMBC correlation, mass spectral data and comparison of the spectral data with those reported in the literature (Rao *et al.*, 2000), the isolated compound (SMOo) could be confirmed as bis (2-ethyl hexyl) phthalate.

It was found that the reported spectral data of di- 2-ethyl hexyl phthalate isolated from *Cassia auriculata* leaves are expressed in Table 1 (Rao *et al.*, 2000).

Table 1. The ^{1}H (500 MHz), ^{13}C (125 MHz) NMR and DEPT Spectral Data of the Isolated Compound (SMOo) in $CDCl_3$

Position	^{1}H (J in Hz)		^{13}C (δ)		DEPT
	Isolated compound	Literature data[R]	Isolated compound	Literature data[R]	
1,2	-	-	132.4	132.4	C
3,6	7.70 (2H, *dd*, *J* = 8.8 and 3.2Hz)	7.75 (2H, *dd*, *J* = 7.5 and 1.5Hz)	128.8	128.7	CH
4,5	7.52 (2H, *dd*, *J* = 8.8 and 3.2Hz)	7.55 (2H, *dd*, *J* = 7.5 and 1.5Hz)	130.8	130.8	CH
1'	4.21 (4H, *m*)	4.25 (4H, *d*)	68.1	68.1	CH_2
2'	1.68 (2H, *m*)	1.65 (2H, *m*)	38.7	38.7	CH
3'	1.34-1.43 (4H, *m*)[a]	1.3 (4H, *m*)	30.3	30.3	CH_2
4'	1.34-1.43 (4H, *m*)[a]	1.3 (4H, *m*)	28.9	28.9	CH_2
5'	1.34-1.43 (4H, *m*)[a]	1.3 (4H, *m*)	22.9	22.9	CH_2
6'	0.91 (6H, *t*)[a]	0.91 (6H, *t*)	14.0	14.0	CH_3
7'	1.34-1.43 (4H, *m*)[a]	1.3 (4H, *m*)	23.7	23.7	CH_2
8'	0.91 (6H, *t*)[a]	0.91 (6H, *t*)	10.9	10.9	CH_3
COO-	-	-	167.7	167.7	C

[R]Rao *et al.*, 2000
[a]Overlapped

Antibacterial Activity of the Isolated Compound

The antibacterial activity of the isolated compound (SMOo) was investigated by the agar well diffusion method and found to possess high inhibitory activity (18 mm, +++) on *S. aureus* (Figure 11). *n*-Hexane as control did not show activity (-) on the tested organism.

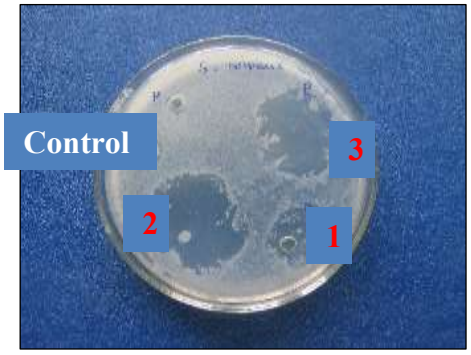


Figure 11. Antibacterial Screening of the isolated compound

Agar well = 5 mm
No activity = 5 mm (-)
Low activity = 5-10 mm (+)
Medium activity = 10-15 mm (++)
High activity = Above 15 mm (+++)

(1): Agar well diffusion method
(2) and (3): Agar-drop diffusion method
Control: *n*-Hexane

Conclusion

In the research work, the selected thermophilic bacterial strain No. 21 was found to produce a potent bioactive compound. The isolated compound (SMOo) with the molecular formula of $C_{24}H_{38}O_4$, showed the antibacterial activity. The bioactive compound (SMOo) was able to be elucidated as a well-known organic compound, di- (ethyl hexyl) phthalate (DEHP), by using spectroscopic data.

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