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Full Length Research Paper

Novel polyene from *Vernonia urticifolia* (Asteraceae)

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Phytochemical investigation of *Vernonia urticifolia* resulted in isolation of urticifolene, a new polyene metabolite together with lutein and sitosterol, which were fully characterized using NMR spectroscopy with the application of correlation spectroscopy (COSY), Nuclear overhauser enhancement spectroscopy (NOESY), heteronuclear multiple bond spectroscopy (HMBC) and heteronuclear single quantum coherence (HSQC). The biological assay determination showed that Lutein inhibited the growth of *Enterococcus faecium* at low concentration (MIC) of 8 µgmL^{-1}, while urticifolene inhibited the growth of *Enterococcus faecium* and *Pseudomonas aeruginosa* at low concentration (MIC) of 16 and 32 µgmL^{-1}, respectively. In contrast to the related polyenes, nystatin and amphotericin B, which exhibit no antibacterial activity, urticifolene exhibited inhibitory property against all the bacteria investigated.

Key words: Urticifolene, lutein, antibacterial activity, *Vernonia urticifolia*, Asteraceae.

INTRODUCTION

Ethnomedicinally, *Vernonia* species are employed in the treatment of diverse range of ailments, including measles, skin rashes, backache, malaria (Anoka et al., 2008), asthma, bronchitis, dysentery and worms (guinea, round and thread) (Misra et al., 1984). The root of *V. cinerea* is used as an anthelmintic and diuretic (Misra et al., 1984) and to treat coughs intestinal colics and chronic skin diseases (Dastur, 1977). *V. calvoana*, a leafy vegetable, is found to be a rich source of provitamin A, particularly *cis*-β-carotene (Ejoh et al., 2010).

Several *Vernonia* species have previously been investigated, of which *V. amagdalina* is the most extensively investigated for its pharmacological properties (Kupchan et al., 1963; Ohigashi et al., 1994). *V. brasiliana* and *V. brachycalyx* have shown potential antiprotozoal activity (De Almeida et al., 1997; Oketch-Rabah et al., 1997). *V. urticifolia,* the subject of the present study, is known as Motoiyokwo by the Kalenjin tribe of Kenya, who use it to treat sinuses, allergy and skin rashes (Kokwaro, 1976).

There are no previous phytochemical or pharmacological studies carried out on this plant, hence the current study was undertaken primarily to investigate the phytochemistry of *V. urticifolia* and to test the isolated compounds for antibacterial activity since extracts of *Vernonia* species have been cited to possess antimicrobial activities in traditional medicine (Kokwaro, 1976). We present herein a detailed isolation, characterization and antibacterial evaluation of the isolated compounds.

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MATERIALS AND METHODS

Plant material

The leaves of *V. urticifolia* were collected from Nakuru District, Kenya in June, 2010. The plant was identified by Mr Ezekiel Cheboi of the Department of Natural Resource, Egerton University, Kenya. A voucher specimen (Kiplimo 03) was retained at the Ward Herbarium University of KwaZulu-Natal Westville, Durban, South Africa.

Extraction and Isolation of compounds

The air-dried and ground leaves (763 g) were extracted sequentially using soxhlet apparatus with solvents of varying polarity viz: hexane, dichloromethane, ethyl acetate and methanol. The dichloromethane extract was concentrated to yield a crude extract (6.94 g), which was separated using column chromatography over silica gel (Merck 9385). A glass column (diameter 4.5 cm) was plugged with cotton wool and packed with silica gel slurry to a height of 30 cm and thinly layered with sea sand washed with HCl. The dry mixture of silica-extract was carefully layered on the silica-gel bed and eluted with hexane/ethyl acetate step gradient starting with 100% hexane to hexane: ethylacetate (1:4). Twenty fractions of 100 mL were each collected in each step and the fractions were

monitored with Thin Layer Chromatography (TLC). Fractions with similar TLC profile were combined. Six fractions were obtained after combination (A-F). Fraction D was purified in a 2 cm column using dichloromethane: ethylacetate (4:1) to yield three subfractions D_1-D_3, compound 1 (32 mg) was obtained in fractions D_2 and compound 2 (43 mg) was obtained in fraction E.

General experimental procedure

Melting points were determined on a kofler micro-hot stage melting point apparatus and were uncorrected. NMR spectra were recorded at room temperature on 400MHz varian UNITY–INOVA spectrometer. ^{1}H NMR spectra were referenced against the $CDCl_3$ signal at δ_H 7.24 and ^{13}C NMR spectra against the corresponding signal at δ_C 77.0, although coupling constants are given in Hz. The GC-MS analyses of the samples were carried out on an Agilent GC–MSD apparatus equipped with DB-5SIL MS (30 m x 0.25 mm i.d., 0.25 µm film thickness) fused-silica capillary column. The MS was operated in the EI mode at 70 eV, in *m/z* range of 42 to 350. The infrared spectra were recorded on a Nicolet impact 400D Fourier Transform Infrared (FT-IR) spectrometer, using NaCl windows with chloroform as solvent against an air background. UV spectra were obtained on a Varian DMS 300 UV-visible spectrometer.

Biological studies

Test organism

Three strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35032, *Klebsiella pneumoniae* ATCC 700603) and three Gram-positive bacteria (*Staphylococcus aureus* ATCC 29212, *Enterococcus faecium* ATCC 19434, *Staphylococcus saprophyticus* ATCC 35552) were selected for the determination of the antibacterial activities of the isolated compounds.

Determination of minimum inhibitory concentration (MIC)

The antibacterial activities of the compounds were determined using the broth microdilution method as described by Andrews (2001). Bacterial strains were cultured for 18 h at 37°C in Tryptone Soy Broth (TSB) and standardized to a final cell density of 1.5×10^8 cfu/mL equivalent to 0.5 McFarland standard. The 96-well plates were prepared by dispensing into each well, 90 µL Muller-Hinton (MH) broth and 10 µL of the bacterial inoculum. Test compounds were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL, while tetracycline (a positive control) was dissolved in ethanol. Serial two-fold dilutions were made in a concentration range of 0.002 to 1 mg/mL. Wells containing MH broth only were used as medium control and wells containing medium and cultures without the test compound were used as the growth control. Plates were covered (to avoid contamination and evaporation) and then incubated for 24 h at 37°C. The minimum inhibitory concentration (MIC) was described as the lowest concentration of the test compounds completely inhibiting the growth of microorganisms. The tests were carried out in triplicate, the results is as shown in Table 2.

RESULTS AND DISCUSSION

The dichloromethane extract of the powdered leaves of *V. urticifolia* afforded a new polyene, (urticifolene, 1), a known carotenoid (lutein, 2) (Figure 1) and a known triterpenoid, (sitosterol) that appear to be ubiquitous in the *Vernonia* family. This is the first time compound 1; a yellow oily solid is being assigned a trivial name urticifolene. The molecular formula was established to be $C_{31}H_{52}O_4$ (Figure1) with a molecular mass 488 (Appendix 1), which implied six degrees of unsaturation. The IR spectrum (Appendix 2) revealed the presence of hydroxyl (3396 cm^{-1}) and carbonyl (1713 cm^{-1}) functional groups. The UV spectrum (Appendix 3) of urticifolene (1), showed maxima with three shoulders at 237, 245 and 322 nm consistent with the conjugated double bonds present in the compound. The ^{13}C NMR spectrum (Table 1, Appendices 5 and 6) of urticifolene (1) showed ten carbon resonances in the olefinic region (123 to 135 ppm) indicating five double bonds, a carbonyl at δ_C 179.23 (accounting for the six degrees of unsaturation) and two oxygenated methine carbon resonances at δ_C 72.95 and 72.19. The ^{1}H NMR data (Table 1, Appendix 4) displayed two methyl groups bound to aliphatic carbons at δ_H 0.97 (3H, s, H-31) and δ_H 0.89 (3H, s, H-16) and an overcrowded olefinic signals, which appeared between 5 and 7 ppm, characteristic of polyenes. Also present in the ^{1}H NMR spectrum were two overlapping signals at δ_H 4.17 (d, J = 6.60 Hz) and δ_H 4.22 (d, J = 6.32 Hz), which correlated to δ_C 72.18 and δ_C 72.95 respectively, in the HSQC spectrum (Appendix 9) confirming the presence of two hydroxyl groups at positions H-3 and H-14 as indicated by the IR spectrum. The COSY spectrum (Appendix 7) showed a correlation between H-3 and H-2 and the HMBC experiment (Appendix 8) showed a correlation between C-3 and H-2 in the aliphatic region, and H-5 in the olefinic region. The geometry of the double bond at C_4, C_6, C_8, C_{10} and C_{12} were determined from the proton coupling constants of $J_{6,7}$ = 15.0 Hz, and $J_{10,11}$ = 15.5 Hz. The other three double bonds were established to be *cis* double bonds as a result of their coupling constants, which were observed to be below 8 Hz. The triplet at 5.97 ppm (J = 11.0 Hz, H-8), supported a double bond at C-9 with a *cis* geometry. The protons H-12 and H-13 also displayed downfield chemical shift values of δ_H 5.57 and δ_H 5.66 that are close to those observed for the olefinic protons at H-8 and H-9, suggesting the presence of a *cis* double bond at Δ^{12}. Urticifolene (1) displayed high structural similarity to laetiporic acid isolated from *Laetiporus sulphureus* with the exception of the aliphatic methylene chain containing 16 carbons in urticifolene (1) that is absent in laetiporic acid (Davoli et al., 2005). Compound 2 was identified as Lutein due to characteristic peaks observed in the spectroscopic data. The UV spectrum showed absorption maxima at 454, 480 and 430 nm. This was consistent with the UV data of (9Z, 9'Z, 3R, 3'R, 6'R)-lutein isolated from *Brassica napus* (Rape) (Kull and Pfander, 1997).

The results of the antibacterial activity (Table 2) showed that urticifolene, 1 and lutein, 2 have broad spectrum of activity. The minimum inhibitory concentration (MIC) determination showed that lutein at low

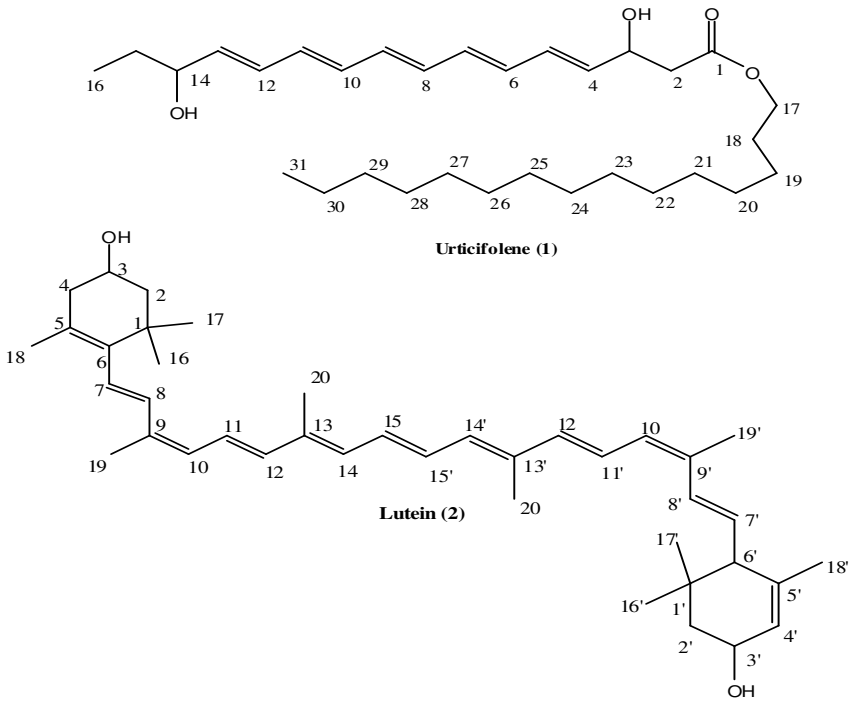

Figure 1. Structures of compounds (1 & 2) isolated from *Vernonia urticifolia*

Table 1. ^{1}H and ^{13}C NMR spectral data of compound 1 and 2 (400 or 100 MHz).

Position	δ_C (1)	δ_H (1)	Position	δ_C (2)	δ_H (2)
1	179.23	-	1	37.13	-
2	35.22	2.33	2	48.45	178, 150
3	72.95	4.22	3	65.10	4.03
4	123.72	5.36	4	42.56	2.05, 2.42
5	125.82	6.50	5	126.17	-
6	125.91	6.50	6	138.00	-
7	127.84	5.97	7	128.73	6.12
8	127.78	5.97	8	130.81	6.65
9	132.81	5.42	9	135.07	-
10	132.90	5.43	10	130.04	6.07
11	134.87	5.65	11	124.49	6.73
12	135.20	5.57	12	137.57	6.28
13	135.71	5.66	13	136.42	-
14	72.18	4.17	14	132.58	6.23
15	33.93	2.33	15	130.09	6.63
16	14.19	0.89	16	30.26	1.08
17	37.25	1.60	17	28.73	1.09
18	31.76	1.28	18	21.62	1.78
19	29.69	1.28	19	29.70	1.97
20	29.69	1.29	20	12.81	1.97
21	29.36	1.33	1’	34.04	-
22	29.34	1.33	2’	44.64	1.37, 1.85
23	28.89	1.33	3’	65.93	4.25
24	28.87	1.33	4’	128.81	5.50
25	27.61	2.19, 2.06	5’	137.77	-

Table 1. Contd.

26	27.58	2.19, 2.06	6’	54.97	2.47
27	25.09	1.60	7’	131.30	5.47
28	24.61	1.34	8’	130.09	6.65
29	22.59	1.29	9’	135.70	-
30	20.74	2.06	10’	125.60	6.05
31	14.04	0.97	11’	124.94	6.74
			12’	138.50	6.28
			13’	136.49	-
			14’	137.73	6.23
			15’	130.81	6.63
			16’	29.50	0.85
			17’	24.29	1.03
			18’	22.86	1.64
			19’	12.76	1.90
			20’	13.11	1.95

Table 2. Minimum inhibitory concentration (MIC) of urticifolene and lutein.

Test organism	Concentration (µg/mL)	
	Urticifolene	Lutein
E. faecium, (ATCC 19434)	16	8
S. aureus, (ATCC 29212)	256	32
S. saprophyticus, (ATCC 35552)	128	256
E. coli, (ATCC 25922)	256	32
K. pneumonia, (ATCC 700603)	256	32
P. aeruginosa, (ATCC 35032)	32	256

concentration of 8 µg/mL completely inhibited the growth of *E. faecium* (ATCC 19434) and at 32 µg/mL, it inhibited the growth of *S. aureus* (ATCC 29212), *E. coli* (ATCC 25922) and *K. pneumonia* (ATCC 700603). Urticifolene inhibited the growth of *P. aeruginosa* (ATCC 35032) and *E. faecium* (ATCC 19434) at low concentration of 16 µg/mL, but the MIC for *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 700603) and *S. aureus* (ATCC 29212,) were recorded as 256 µg/mL. These findings indicated that urticifolene (1) and lutein (2) possessed inhibitory activity against bacteria. Other investigations showed that the carotenoid methanol extract of citrus peel of *Shatian pummel* exhibited inhibitory properties within a range of 18.75 to 140.00 µg/mL against *E. coli*, *S. aureus* and *B. subtilis* (Tao et al., 2010).

The constituents of citrus carotenoids cover a wide range of compounds, such as β-cryptoxanthin, violaxanthin isomers, lycopene and β-carotene (Tao et al., 2010) and therefore, the synergistic effect of these compounds could have possibly enhanced the activity of the methanol carotenoid extract. It has also been reported that some polyenes (of which urticifolene, 1 belongs), such as amphotericin B and nystatin have no antibacterial activity whilst others, such as faeriefungin inhibit the growth of a variety of bacterial isolates. The mode of action of carotenoids is not clearly understood, but Cucco et al. (2007) suggest that β-carotene could lead to accumulation of lysozyme (a bacterial immune enzyme that digests bacterial cell walls). But the mode of action of polyene against bacteria is unknown, though the fungicidal activity involves interaction of the lipophilic polyene structure with cytoplasmic membrane sterol found in fungi and mammalian cells resulting in alteration of the cell membrane, leakage of cell constituents and cell death (Mulks et al., 1990).

Conclusion

The phytochemical study of this plant resulted in the isolation of two compounds, a polyene and a carotenoid. These compounds exhibited moderate antibacterial activity.

In contrast to the related polyenes antibiotics nyastin and amphotericin B, which are inactive against bacteria,

urticifolene exhibited inhibitory activity against all the bacteria investigated. These results provide scientific validity and credence to the ethnomedicinal use of this plant in the treatment of ailments caused by some of the bacteria used in this study and highlights the usefulness of *V. urticifolia* in the treatment of bacterial infections.

ACKNOWLEDGEMENTS

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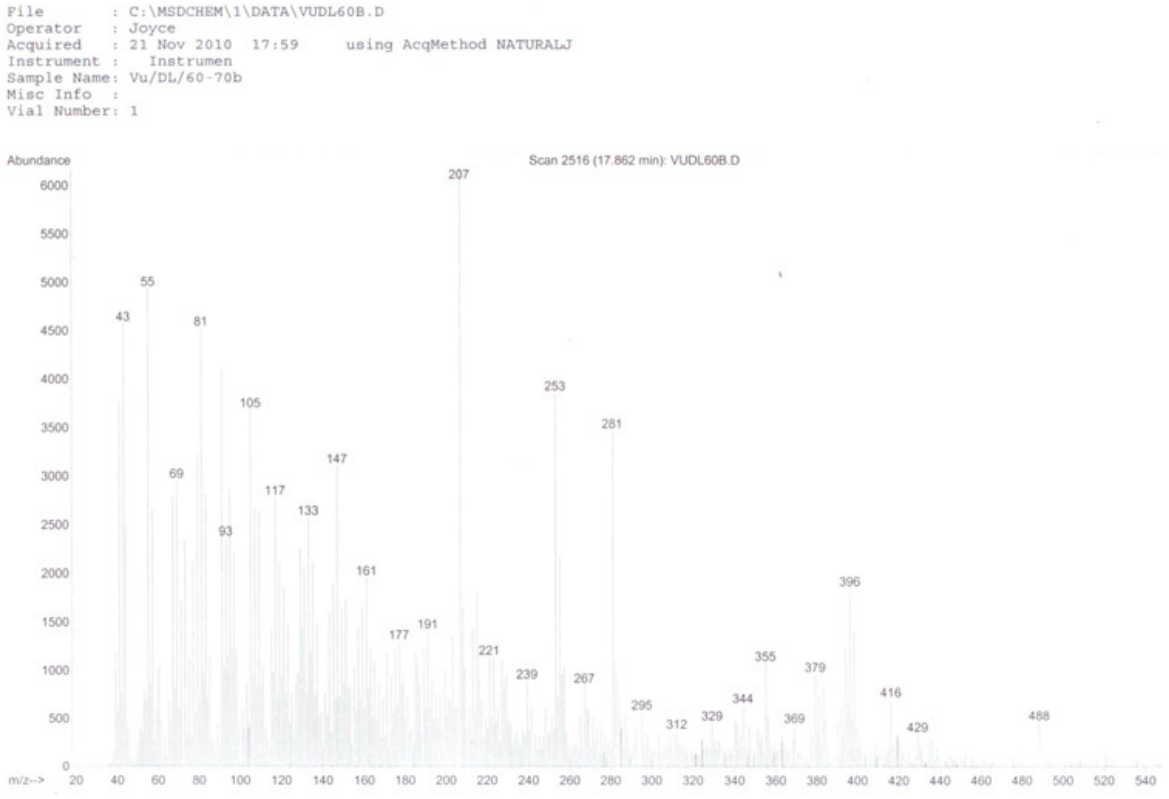
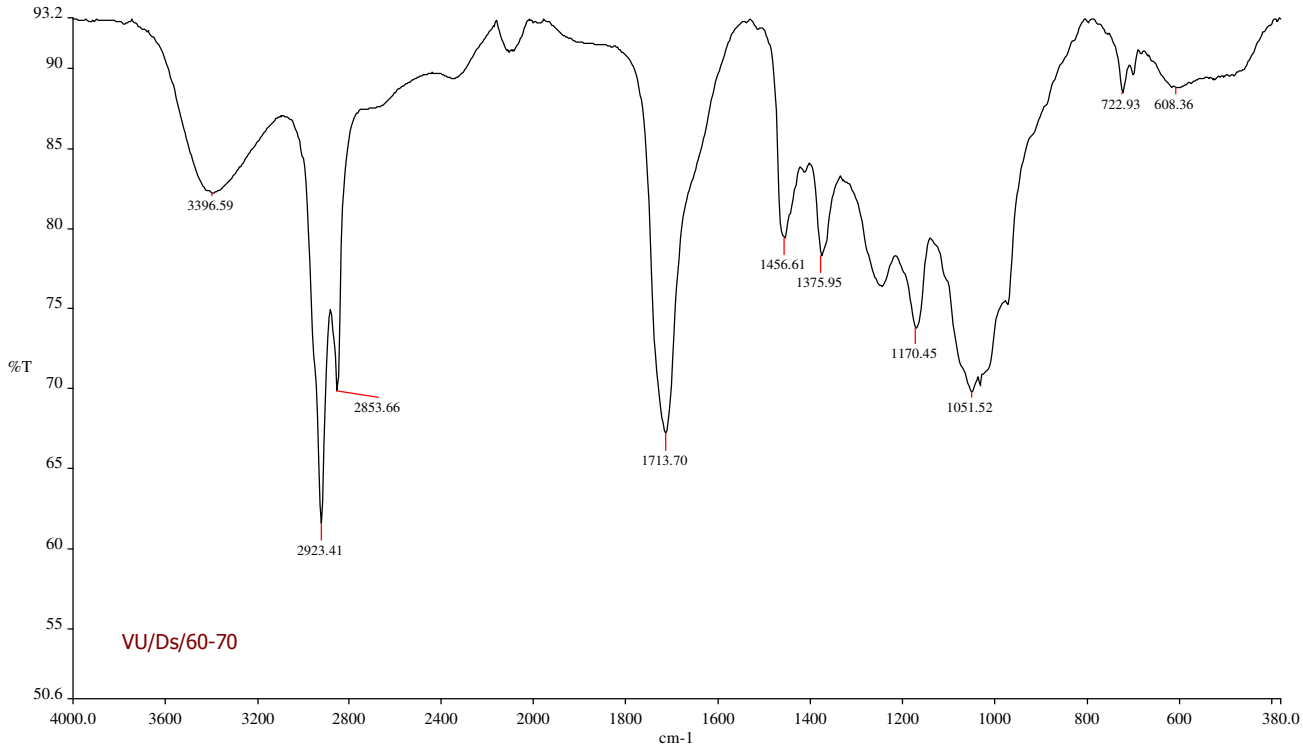
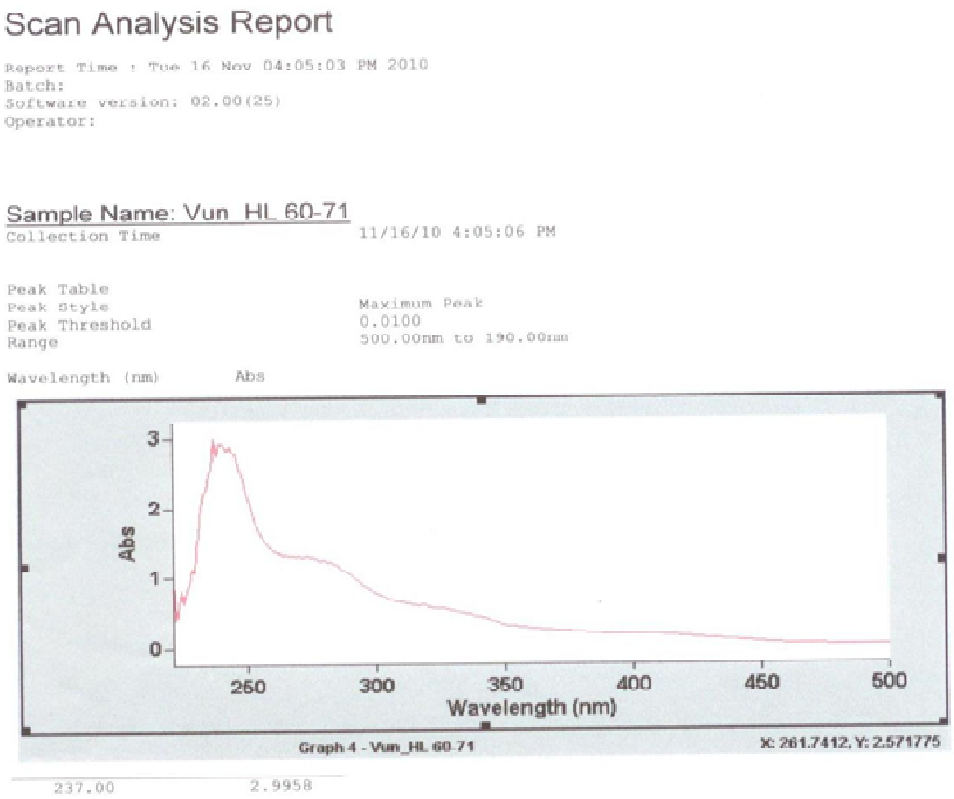
APPENDIX



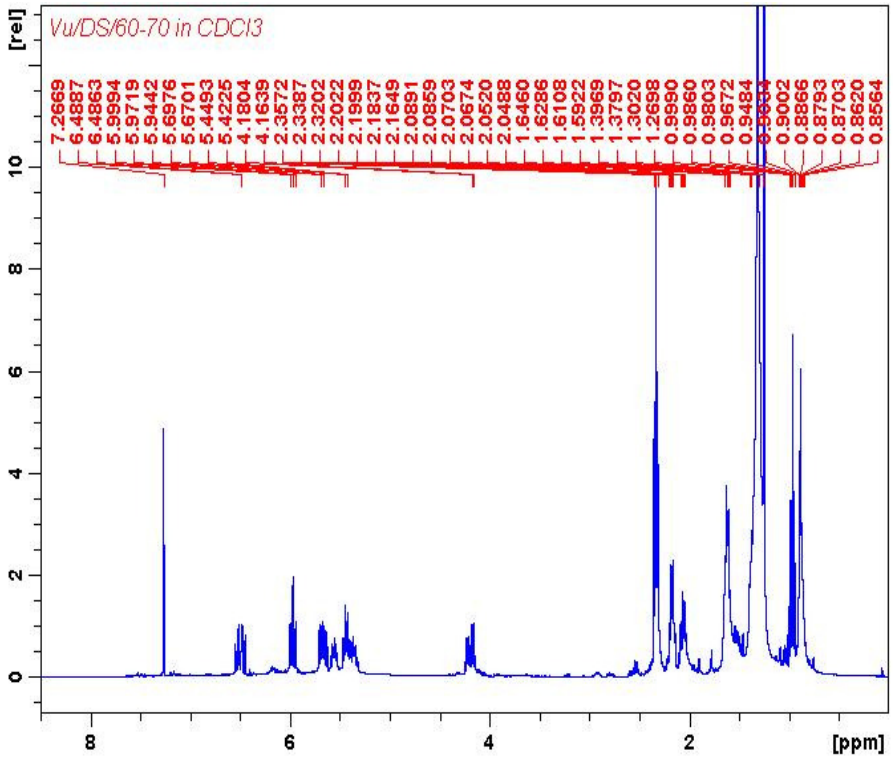
Appendix 1. Mass spectrum of compound 1.



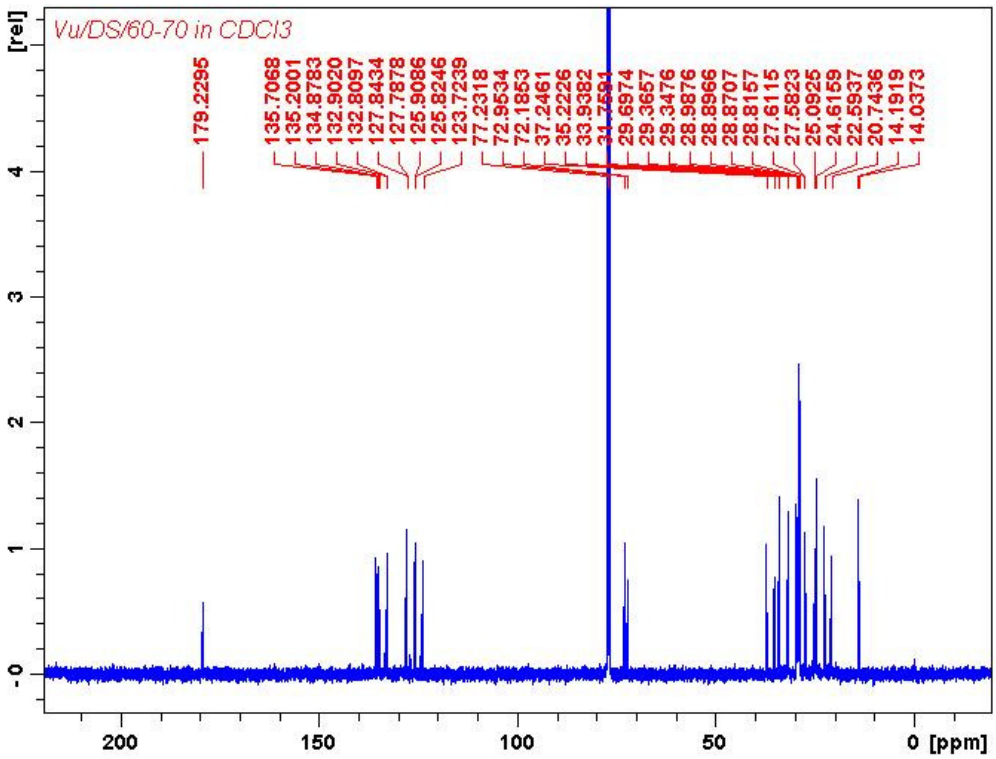
Appendix 2. IR spectrum of compound 1.



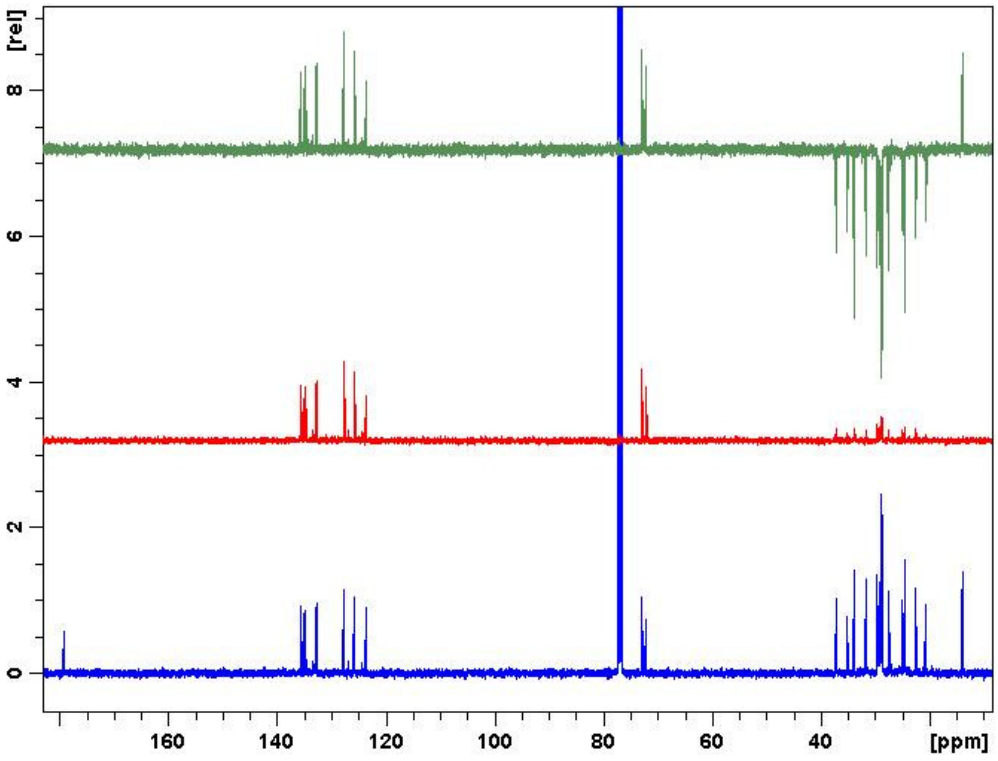
Appendix 3. UV spectrum of compound 1.



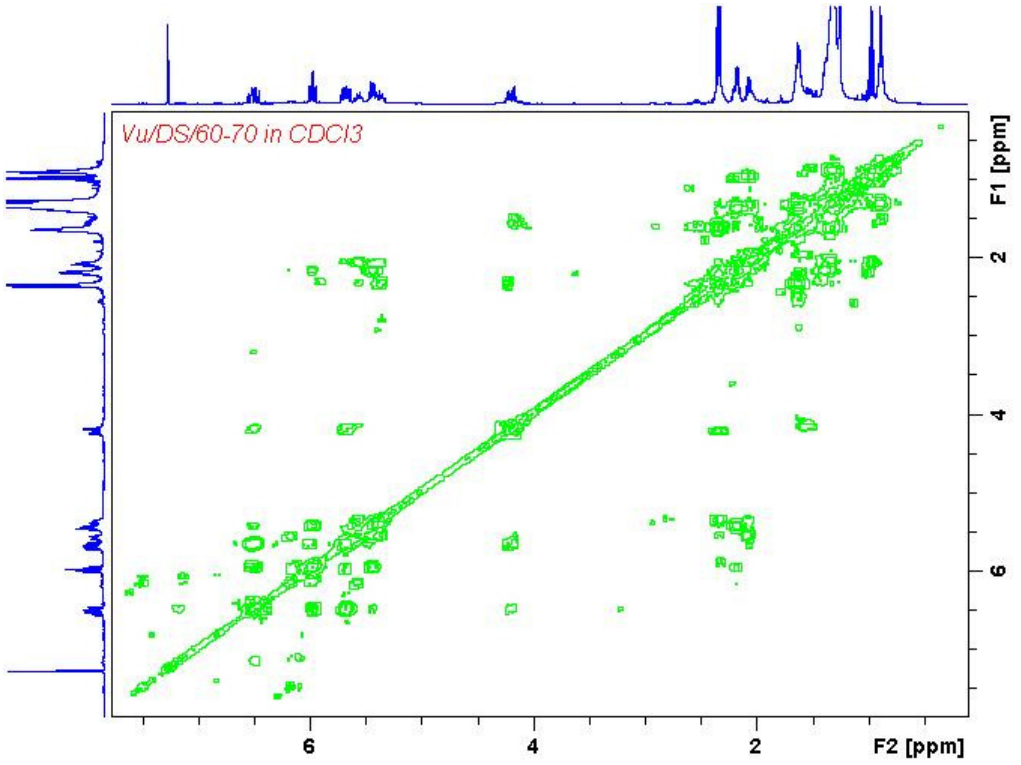
Appendix 4. ^{1}H NMR spectrum of compound 1.



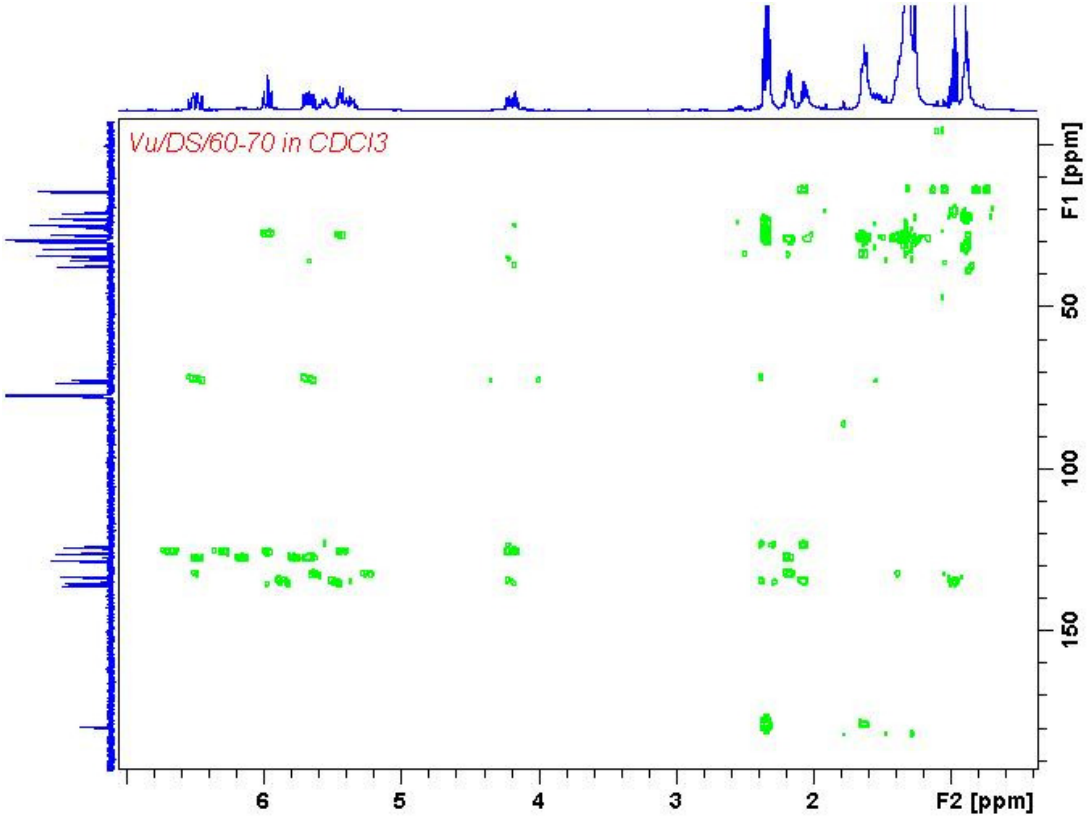
Appendix 5. ^{13}C NMR spectrum of compound 1**.**



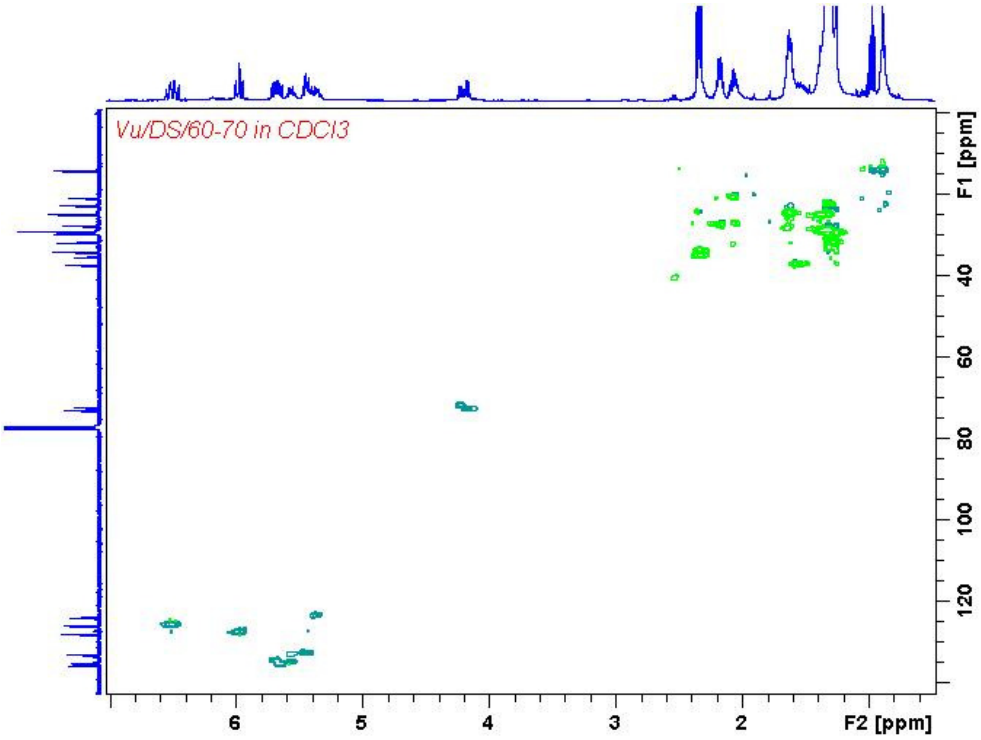
Appendix 6. DEPT 90 and 135 NMR spectrum of compound **1**.



Appendix 7. COSY spectrum of compound **1.**



Appendix 8. HMBC spectrum of compound **1**.



Appendix 9. HSQC spectrum of compound 1.