International Research Journal of Pure & Applied Chemistry

21(15): 1-16, 2020; Article no.IRJPAC.60726
ISSN: 2231-3443, NLM ID: 101647669

Antimicrobial Activity and Interactions of *Toddalia asiatica* Isolated Coumarins with Two Known Drugs

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Authors' contributions

This work was carried out in collaboration among all authors. Author IO designed the study, did experiments, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BFJ, JCK and RWK managed the analyses of the study. Author IO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IRJPAC/2020/v21i1530248
Editor(s):
(1) Dr. Wolfgang Linert, Vienna University of Technology, Austria.
Reviewers:
(1) Sri Rahmaningsih, Ronggolawe University (Universitas PGRI Ronggolawe Tuban), Indonesia.
(2) Mohamed Mirghani Mohamed Ahmed, International University of Africa, Sudan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/60726

Original Research Article

Received 25 June 2020
Accepted 31 August 2020
Published 04 September 2020

ABSTRACT

Five coumarins namely, 5, 7-dimethoxy-6-(3'-hydroxy-3'-methylbutan-2-oxo) coumarin coumarin (**1**), Toddalolactone (**2**), Coumurrenol (**3**), gleinadiene (**4**) and Toddaculin (**5**) were isolated from either the stem and/or root bark of *Toddalia asiatica* , with compound **1** being reported for the first time. These were obtained using chromatographic methods and identified using spectroscopic techniques, as well as comparison of their physical data with already published results. Combinations of compound **3** and fluconazole displayed additive effect in inhibiting the growth of *Penicillium digitatum* with reduced MIC to 125 *µ*g/mL compared to that of fluconazole alone at 250 *µ*g /mL. Combination of compounds **1** and **3** also showed additive effect in inhibiting *Rhizopus stolonifer* lowering the MIC from 500 *µ*g/mL (for both molecules) to 250 µg /mL. Interaction in antibacterial activity between two isolated compounds **1** and **3** was also evident. These lowered the MIC in action against *Staphylococcus aureus* to 250 *µ*g/mL compared to individual compounds with MIC of 500 *µ*g/mL while showing additive effect. All the crude extracts apart from that of stem bark hexane and the individual isolated compounds showed considerable activity against all the organisms tested.

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Keywords: Toddalia asiatica; coumarins; interactive effects; antimicrobial.

1. INTRODUCTION

Toddalia asiatica (L) Lam. (Rutaceae) (Syn: *Paullinia asiatica* L., *Scopolia aculeata* Sm., *Toddalia aculeata* Pers.) is widely available in East Africa where it is traditionally used in management of malaria related symptoms mainly by the Maasai and Kipsigis communities of Kenya amongst other ailments [1-5]. It is a single species belonging to Toddalioideae subfamily and *Toddalia* genus [6]. It is also widely distributed in other humid tropical areas in south Asia, South East Asia and China. Extracts from the plant are known to possess a number of pharmacological activities including antipyretic, analgesic, antibacterial, vulnerary, stimulant, antiperiodic, antidiarrheal, diuretic amongst others [3,5,7-12]. Isolates from these extracts have been shown to possess biological activities similar to those observed for the crude extracts [13,14,15]. Classes of compounds isolated in the plant species belong to the groups; alkaloids, flavonoids, coumarins, limonoids and lignans with some being volatile oils [1,16,17]. One of the main interests in *T. asiatica* has been prenylated coumarins which possess broad pharmacological activities, including anti-coagulant, anti-tumor, antiviral, anti-inflammatory, antioxidant, anti-microbial and enzyme inhibition properties [18-21]. This is thought to be strongly influenced by the various substituents on the ring structure of the molecules prominent of which is the prenyl group which has diverse structural features [22]. Investigation of encounters between multiple bioactive molecules has been of great interest to scientists. Combinations involving different isolated compounds, known drugs and extracts, besides reducing the effective dose of a drug, also potentially reduce side effects of medicines [23]. WHO recommends the use of Artemisinin-based combination therapy as first line treatment protocol for malaria based on studies showing its efficacy [24]. This regimen was demonstrated to reduce malaria-associated morbidity and mortality globally. Studies have revealed *in vitro* synergistic effects between plant extracts and antibiotics with a significant reduction of minimum inhibitory concentration (MIC) in antibiotics [25-27]. A fourfold reduction in the MIC of gentamicin when combined with the phytochemicals, protocatechuic acid, quercetin, caffeic acid on one hand and by the same factor for sulfadiazine in combination with the same compounds on another, in their action against *Pseudomonas aeruginosa* have been revealed [28]. A 34-fold reduction of MIC of *Klebsiella pneumoniae* resistant in combination tests involving ethanol extract of *Punica granatum* rind with ciprofloxacin was demonstrated by Rafiq and coworkers [29]. Allicin, a phytochemical present in garlic, has been shown to work synergistically in combination with *β*-lactam antibiotics against *Staphylococcus* spp. and *P. aeruginosa* [30]. Further studies on combinations of antibiotics and phytochemicals may provide new therapeutic options for antimicrobial infections. The aim of this study was to evaluate interactive effects between the antimicrobials gentamicin and fluconazole in combination with the coumarins isolated from *T. asiatica* against the bacteria *Staphylococcus aureus* and *Escherichia coli* and the fungi *Penicillium digitatum* and *Rhizopus stolonifer*, respectfully. This was the first study of interactive effect of the prenylated coumarins against the tested microbes.

2. METHODOLOGY

2.1 General Experimental Procedures

Melting points were determined using the Gallenkamp melting point apparatus and were uncorrected. Mass spectra were obtained on Electron impact mass spectra (EI-MS) using a Finnigan GC-MS. NMR spectra were obtained using Bruker Avance 600 (^{1}H 600MHz, ^{13}C NMR 150 MHz). Solvents used were deuterated $CDCl_3$, CD_3OD and $(CD_3)_2CO$. Chemical shifts were given in (ppm) values with trimethylsilane (TMS) used as the internal standard. Homonuclear Correlation Spectroscopy (COSY), Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra were obtained using the standard Bruker software.

2.2 Plant Material Collection and Preparation

The root and stem barks of the plant were collected from Kakamega forest and identified by botanists at the Department of Biological Sciences Herbarium, Masinde Muliro University. Herbarium species was deposited in the University herbarium for reference.

2.3 Extraction and Isolation of *T. asiatica* Bark Material

Plant material was air-dried and ground to powder before sequential extraction using the solvents hexane, DCM and methanol for 24 hours in each case. Filtered extract was concentrated *in vacuo* using a rotary evaporator at 45 ^{o}C to produce semi-solid material. TLC analysis was done using various solvent systems and plates sprayed with vanillin-H_2SO4 (5%) or exposed to iodine vapour. The samples were stored in the refrigerator (-4 ^{o}C). The hexane stem bark extract (35 g) was subjected to gradient elusion chromatography (silica gel; Hex; EtOAc/*n*-Hexane) to obtain combined fractions H1 – H7 (TLC analysis). Fraction H3 (7 % EtOAc/*n*-Hexane) was further subjected to fractional crystallization (Hex:CH_2Cl_2) to give compound **4** (0.12 g) as cream crystals. Compound **2** (0.22g) was isolated from DCM stem bark by subjecting its extract (40 g) to gradient elution column chromatography (silica gel; Hex; EtOAc/*n*-Hexane) to afforded 8 combined fractions (E1 - E8). Fraction E7 (eluted with 50 % EtOAc/*n*-Hexane) was subjected to fractional crystallization (*n*-Hexane:CH_2Cl_2:MeOH) to obtain white powder. Methanol stem bark extract (25 g) was also subjected to fractionation by gradient elution column chromatography (*n*-Hexane/ EtOAc:EtoAc/MeOH) to obtain combined fractions M1 - M7. Compound **3** (0.18 g) was obtained as yellow crystals from M4 (80 % EtOAc/*n*-Hexane) on subjection to fractional crystallization (*n*-Hexane:CH_2Cl_2:MeOH). The crude hexane root bark extract (28 g) was subjected to Gradient elution column chromatography with n-hexane containing increasing amounts of EtOAc to afforded 6 combined fractions HR1 - HR6. Fractions HR3 (20 % EtOAc/*n*-Hexane) was subjected to further fractional crystallization to obtain compound **1** (0.15 g) as white powder.

2.4 Antimicrobial Activities

The minimum inhibitory concentrations (MICs) of test samples and the positive control drugs gentamicin (1.0 *µ*g/disc) and fluconazole (1.0 *µ*g/disc) were measured by the microdilution broth susceptibility assay Sanguinetti, & Posteraro, [31] against the microorganisms *S. aureus*, *E. coli*, *P. digitatum* and *R. stolonifer*, obtained from MMUST microbiology laboratory. The inocula of bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The samples were dissolved in 10 % DMSO and diluted two-fold in sterile 96-well microtiter plates in duplicate using BHI broth. Standardized inocula of test strains were added and after incubation at 37 ^{0}C for 24 h on a rotary shaker at 200 rpm, MICs were read as the lowest concentration with inhibition of the growth of the test organisms, compared to the positive control gentamicin, fluconazole and medium containing 10 % DMSO as negative control.

2.5 Molecular Interactive Effect on Antimicrobial Activity

Prior to performing the molecular interactive test, the MICs of plant extracts and antibiotics were determined using micro dilution plate method with resazurin in Mueller-Hinton broth [32]. MIC was defined as the lowest concentration showing clear zone of inhibition. Agar well-diffusion method was followed to determine the antimicrobial activity of combined compounds [33]. The test samples (equal volumes of mixtures of compounds **1/3** and equal volumes of mixtures of compound **1**, **3**/standards) were introduced into the wells. Stock solution of each combined mixtures were prepared at a concentration of 1 mg/ml in different mixtures both of ethyl acetate. Control experiment comprising the solvent ethyl acetate was set up. The plates were incubated at 37 °C for 18 to 24 h for bacterial pathogens and 28 °C for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index calculated. Triplicates were maintained and the experiment was repeated thrice for each replicate the readings were taken in three different fixed directions and the average values recorded. The zones of inhibition (mm) were recorded from measurements of clear zones around the agar wells. *In vitro* interactions between antimicrobial agents were determined and quantified by calculating the fractional inhibitory concentration index (FICI) using the formula given below [32,34]:

$$FICI = \frac{MIC_a \text{ in combination}}{MIC_a} + \frac{MIC_b \text{ in combination}}{MIC_b}$$

Where; MIC_a is MIC of **1** and MIC_b is MIC of **3**

Interpretation of the FICI:

FICI = 0.5 Synergistic - a combination has a greater effect than the added effects of each constituent.

FICI > 0.5 to 1 Additive - a blend has an effect equal to the sum of the effects of each component.
FICI > 1 to 4 Indifferent - a blend has identical effect to that of the most active constituent.
FICI > 4 Antagonistic - a combination has reduced activity relative to the effect of the most efficient individual constituent

3. RESULTS AND DISCUSSION

Chemical investigation of the root and stem barks of *T. asiatica* by extraction followed by chromatographic fractionation yielded five coumarins namely; 5, 7-dimethoxy-6- (3'-methy-3'-hydroxyl-but-2-none) coumarin (**1**), Toddalolactone (**2**), Coumurrenol (**3**), gleinadiene (**4**) and Toddaculin (**5**), with compound **1** being reported for the first time. The structures of the compounds were elucidated by comparison of their spectral data (Tables 1, 2) with those from literature [35-37]. Antimicrobial investigation was done against gram negative bacteria, *Eschirichia coli*, gram positive bacteria, *S. aureus* and fungi *P. digitatum* and *R. stolonifer* for the extracts, individual isolated compounds and their combination with gentamycin and fluconazole to study possible interaction in antimicrobial activity.

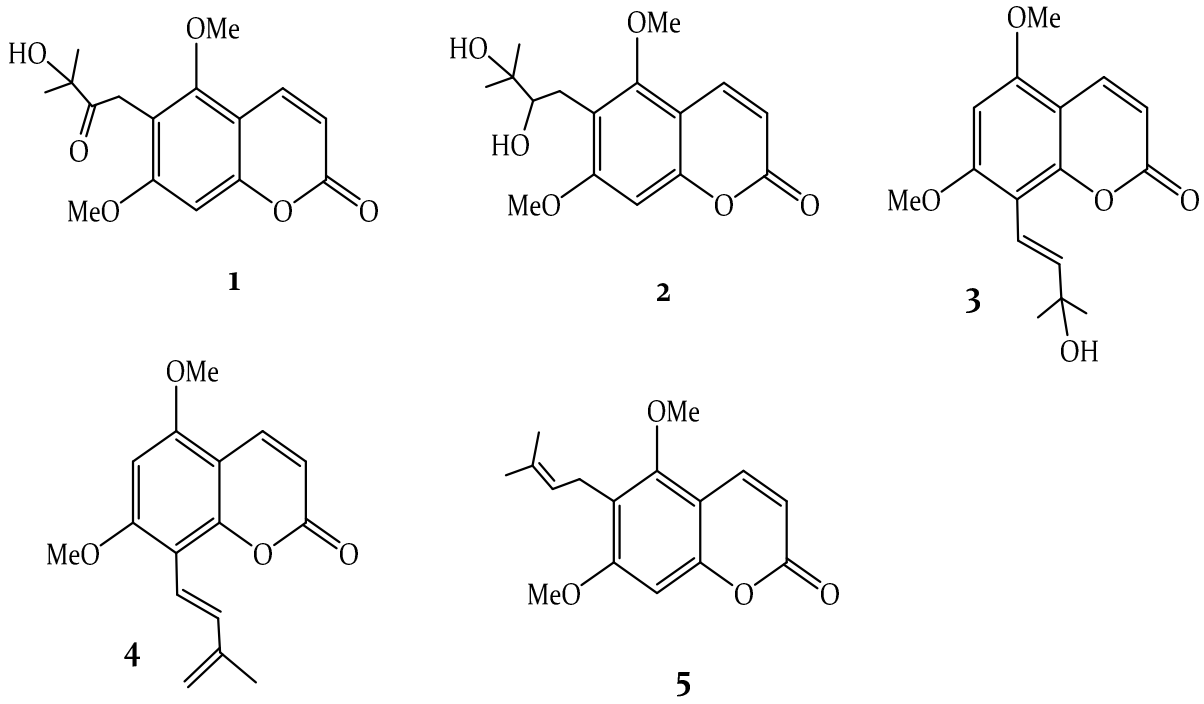

Table 1. ^{1}H NMR (600 MHz) of compounds 1–5 from *T. asiatica* in $CDCl_3$

H	1	2	3	4	5
3	6.21d(9.6)	6.24d(9.6)	6.15d(9.6)	6.20d(9.6)	6.22d(9.6)
4	7.85d(9.6)	8.04d(9.6)	8.10d(10.2)	7.91d(9.6)	7.86d(9.6)
6	-	-	6.58s	6.32s	-
8	6.66s	6.77s	-	-	6.62s
1'	3.92s	2.86	6.83s	7.27d(16.2)	3.36d(6.9)
2'	-	3.66m	6.83s	6.67d(16.2)	5.16t(6.9)
4'	1.50s	1.26s	1.39s	5.11d	1.78s
5'	1.52s	1.27s	1.39s	2.02s	1.68s
5-OCH_3	3.76s	3.99s	3.98s	3.93s	3.88(s)
7-OCH_3	3.81s	3.89s	3.99s	3.79s	3.87(s)
OH	3.47s		3.3		

"s", "d", and "m" represents singlet, doublet and multiplet

Table 2. ^{13}C NMR (100 MHz) data of compounds 1–5 from *T. asiatica* in $CDCl_3$

Carbon	1	2	3	4	5
2	161.4	163.4	163.1	161.2	161.3
3	112.9	112.6	110.9	110.9	112.3
4	138.8	141.2	140.9	138.7	137.0
4a	107.4	108.4	107.6	107.2	107.1
5	156.0	163.8	163.4	153.5	155.2
6	125.9	120.3	92.3	90.3	120.3
7	160.9	157.8	157.4	161.1	161.7
8	95.7	96.3	104.8	103.8	95.9
8a	156.5	156.2	154.5	155.6	154.7
1'	31.6	27.1	115.3	135.7	22.7
2'	212.3	78.3	142.9	117.1	122.2
3'	77.0	74.0	72.1	143.3	132.1
4'	27.0	25.5	30.1	117.0	25.7
5'	26.9	25.5	30.1	18.3	17.8
5-OCH_3	63.7	63.8	56.8	56.0	56.1
7-OCH_3	56.4	56.7	56.8	55.9	63.1

Compound **1** was isolated as yellow crystals and its structure was determined by comparison of its spectroscopic data (Tables 1 and 2) with those from literature and co-isolated compounds which indicated that it was a coumarin. Its ^{1}H NMR spectrum (Table 1) showed two proton doublets at δ_H 6.21 (J=9.6 Hz) and δ_H 7.85 (J=9.6 Hz) and a singlet occurring at δ_H 6.66 which could be attributed to *α*-benzopyrone protons at H-3, H-4 and H-8, respectively. This showed that compound **1** had a 5, 7-dimethoxy coumarin basic structure as in co-isolated compounds (Table 1). The existence of a 3-methyl,3'-hydroxy-butan-2-one side chain was indicated in the ^{1}H-NMR spectrum by the singlets at δ_H 3.92 (H-1', 2H), 1.50 (H-4', 3H), 1.52 (H-5', 3H) and an OH group at δ_H 3.47 (1H, s, OH-3'). H-1' appears downfield given that it experienced double anisotropic effect from the ring and the carbonyl □-systems. Signals for this side chain moiety were seen in the ^{13}C-NMR spectrum at δ_c 31.6, 212.3 (C=O), 78.0 (C-OH), 26.9 and 27.0 representing C-3', 2', 1', 4' and 5', respectively. A study of the HMQC and HMBC spectra revealed correlation between H-1' (δ 3.92) and the carbons at 156.0 ppm (C-5) and 160.9 ppm (C-7) placing the prenyl group at position C-6 in the ring system. This could further be confirmed by the fact that the methoxy at C-5 (δ_c 63.7) was downfield shifted compared to that at C-7 (δ_c 56.4) implying that the former was *ortho* disubstituted [38]. The structure of this compound was proposed to be 5, 7-dimethoxy-6-(3'-hydroxy-3'-methylbutan-2-oxo) coumarin.

Antimicrobial evaluation was done for the various extracts, compounds and compound combinations by determining zones of inhibition against pathogens/saprophytes. Both the methanol and dichloromethane DCM *T. asiatica* stem bark extracts were active against *S. aureus* showing inhibitions zones of 16.7 and 11.0 mm at 1,000 *μ*g/mL (Table 3). The methanol extract was more potent in this test displaying a maximum minimum inhibition concentration (MIC) of 250 *μ*g/mL. Against the gram- negative bacteria *E. coli*, DCM extract showed higher inhibition of 12.3 mm compared to the more polar methanol extract with an inhibition zone of 9 mm at 1,000 *μ*g/mL. Both extracts recorded an MIC of 500 *μ*g/mL compared to that of the standard at 125 *μ*g/mL (Table 4). Tests against the two fungi, *R. stolonifer* and *P. digitatum* showed the methanol extract to be more potent with inhibition zones of 18.3 and 21.0 mm, respectively, at 1000 µg/mL (Tables 5 and 6). In the case of *R. stolonifer* this extract had a lower MIC value (250 *μ*g/mL) than that of the standard (500 *μ*g/mL) while in the case of *P. digitatum* the same MIC value was recorded for this extract. The hexane stem bark extract showed no activity against all the organisms tested but crude of the root bark extract inhibited the growth of the two fungi tested. The highest activity was seen against *R. stolonifer* of 12.0 mm inhibition zone at 1000 *μ*g/mL and an MIC value of 250 µg/mL.

Compounds **1**, **2**, **3** and **4** all showed appreciable activities against both gram positive and negative bacteria. Toddalolactone (**2**), the most polar

compound, showed the highest potency with inhibition zones of 18 and 16 mm against *S. aureus* and *E. coli* at a concentration of 1,000 *µ*g/mL compared to that of the standard at 26 and 24 mm, respectively. This may suggest the significance of a 1, 2-diol substitution on the side chain of the coumarin skeleton in enhancing antibacterial activity. This compound had lower MIC values of 250 and 500 *µ*g/mL, respectively, for the two bacteria compared to gentamycin at 125 *µ*g/mL. Gleinadiene (**4**) recorded inhibition zones of 13 and 10 mm against *S. aureus* and *E. coli,* respectively, at MIC value of 500 *µ*g/mL for both organisms (Table 7). In tests against *P. digitatum* and *R. stolonifer,* all the isolated compounds showed significant activities up to concentrations of 500 *µ*g/mL. Compound (**2**) was the most potent recording inhibition of 18 mm and 16 mm against the two fungi, respectively, at a concentration of 1,000 *µ*g/mL. These results were comparable with those of Fluconazole though with a lower MIC (250 *µ*g/mL) (Table 8). The high activity of the methanol crude extract could be attributed to polar constituents like compound **2**. This test also seems to indicate significance of 1, 2-diol substitution of the prenyl side chain in enhancing antifungal activity. Further it seems that the position of the prenyl group may not have an effect on the bioactivity of the compounds.

Table 3. Zones of inhibition of crude extracts against *S. aureus* bacterial growth in different solvents

Plant Part	Crude extract	Zone of inhibition (mm) at various concentrations			
		1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL
Stem bark	Hex	-	-	-	-
	DCM	11.00±0.58	8.33±0.33	-	-
	MeOH	16.67±0.67	14.33±0.33	12.67±0.33	-
Root bark	Hex	-	-	-	-
Gentamycin		25.33±0.67	22±0.00	18±0.58	13.67±0.88

Table 4. Zones of inhibition of crude extracts against growth of *E.coli* bacteria

Plant Part	Crude extract	Zone of inhibition (mm) at various concentrations			
		1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL
Stem bark	Hex	-	-	-	
	DCM	12.33±0.33	9.33±0.67	-	-
	MeOH	9.00±0.58	7.67±0.33	-	-
Root bark	Hex	-	-	-	-
	Gentamycin	24±0.00	18.67±0.88	17.67±0.58	12.33±0.67

Table 5. Zones of inhibition of crude extracts against *Penicillium digitatum* fungi growth

Plant Part	Crude extract	Zone of inhibition (mm) at various concentrations			
		1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL
Stem bark	Hex	-	-	-	-
	DCM	13.00±0.00	11.00±0.58	-	-
	MeOH	21.00±1.00	19.67±0.58	15.5±0.5	-
Root bark	Hex	10.67±0.67	8.33±0.33	-	-
Fluconazole		16.33±0.88	14±0.00	12.33±0.88	-

Table 6. Zones of inhibition of crude extracts against *R. stolonifer* fungi growth

Plant part	Crude extract	Zone of inhibition (mm) at various concentrations			
		1000 µg/mL	500 µg/mL	250 µg/mL	125 µg/mL
Stem bark	Hex	-	-	-	-
	DCM	7.45±0.58	6.24±0.25	-	
	MeOH	18.33±0.88	15.67±0.33	13.67±0.33	
Root bark	Hex	12.00±0.58	10.33±0.33	8.33±0.33	-
Fluconazole		23.67±1.45	20±0.58	-	-

Table 7. Zone of inhibition of pure compounds against *S. aureus* and *E.coli*

Compound	Zone of inhibition (mm) at various concentrations							
	1000*µ*g /mL		500*µ*g /mL		250*µ*g /mL		125 *µ*g /mL	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
1	9	10	8	-	-	-	-	-
2	18	16	16	13	8	-	-	-
3	10	9	9	8	-	-	-	-
4	13	10	12	-	-	-	-	-
Gentamicin	26	24	22	20	18	18	14	12

Table 8. Zone of inhibition of pure compounds against *P. digitatum* and *R. stolonifer*

Compound	Zone of inhibition (mm) at various concentrations							
	1000*µ*g /mL		500*µ*g /mL		250*µ*g /mL		125 *µ*g /mL	
	PD	RS	PD	RS	PD	RS	PD	RS
1	10	12	8	9	-	-	-	-
2	18	16	16	14	-	-	-	-
3	14	13	10	8	-	-	-	-
4	13	14	12	13	-	-	-	-
Fluconazole	16	24	14	20	12	-	-	-

PD- P.digitatum RS -R.stolonifer

A number of phytochemicals have proven therapeutic potential as antimicrobial compounds and have also been shown to increase the susceptibility of the organism to various drugs [28,29,31]. In the current study, interaction in antimicrobial activity between two isolated compounds **1** and **3**, and in combination with antimicrobial agents, gentamycin and fluconazole was evident. Against *S. aureus*, the MIC of combined compounds **1** and **3** improved to 250 *µ*g/mL compared to individual compounds both with MIC at 500 *µ*g/mL. The FIC index for this combination was found to be 1.0 indicating additive effect (Tables 9 and 11). Combination of gentamycin with compound **3** however showed indifference effect (FIC 1.25) with the lowest MIC value of 125 *µ*g/mL. Its combination with compound **3** also showed indifferent effect in the test against *S. aureus*. Test of interaction of samples against *E. coli* showed that combination of compounds **1** and **3** and the two compounds with gentamicin produced indifferent effect (FIC 1.5, 3.0 and 2.5, respectively). Combination of compound **3** with fluconazole gave additive effect (FIC 1.0) while mixtures of compounds **1** & **3** and compound **1** with fluconazole both gave indifference effect with FIC indices of 1.5 each in the test against *P. digitatum* (Tables 10 and 12; Fig. 1). The best result against this organism was in the combination of compound **3** with fluconazole with an MIC of 125 *µ*g/mL. In the test against *R. stolonifer* combinations of compounds **1** with **3** showed improved activity when compared with individual compounds with FIC of 1.0 (additive). Combinations of both compound **1** and **3** with Fluconazole showed indifference effects with FIC indices of 3.0 and 1.5, respectively (Tables 10,12; Fig. 2).

Table 9. Zone of inhibition of pure and combined compounds against *S. aureus* and *E.coli*

Combinations	Zone of inhibition (mm) at various concentrations							
	1000*µ*g /mL		500*µ*g /mL		250*µ*g /mL		125 *µ*g /mL	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
1	9	10	8	-	-	-	-	-
3	10	9	9	8	-	-	-	-
1+3	11	10	10	9	9	-	-	-
1 +Gent.	24	22	22	20	18	-	-	-
3 +Gent.	28	24	26	21	20	-	10	
MeOH extract	12	10	11	8	10	-	-	-
Gentamycin	26	24	22	20	18	14	16	-

Table 10. Zone of inhibition of pure and combined compounds against *P. digitatum* and *R. stolonifer*

Compound	Zone of inhibition (mm) at various concentrations							
	1000µg /Ml		500µg /Ml		250µg /mL		125 µg /Ml	
	PD	RS	PD	RS	PD	RS	PD	RS
1	10	12	8	9	-	-	-	-
3	14	13	10	12	8	-	-	-
1+3	26	27	24	22	20	16	-	-
1 +Fluc	20	22	16	20	14	-	-	-
3 +Fluc	28	27	26	24	22	20	18	-
MeOH extract	22	24	18	19	16	14	-	-
Fluc	16	24	14	20	12	16	-	-

PD - P.digitatum, RS -R.stolonifer

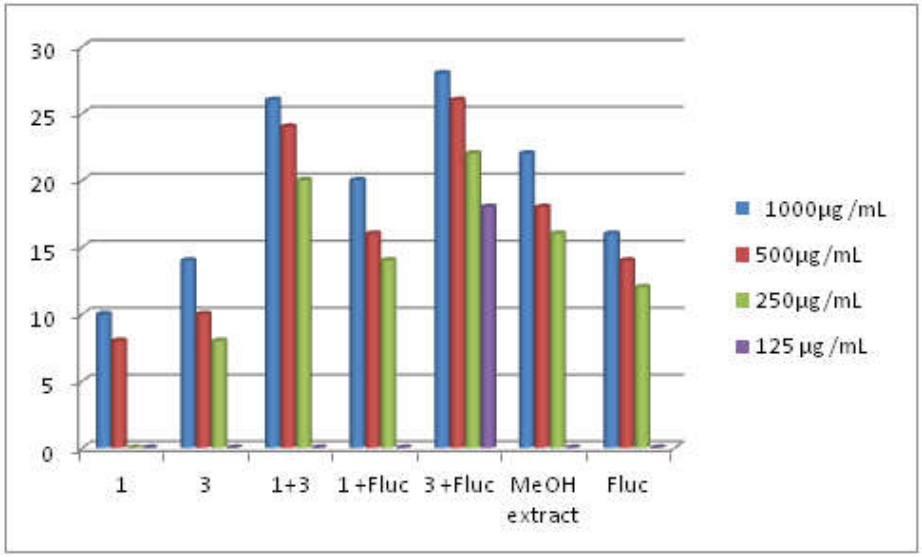

Fig. 1. A graph on zones of inhibition to growth of *P. digitatum* by various compound combinations

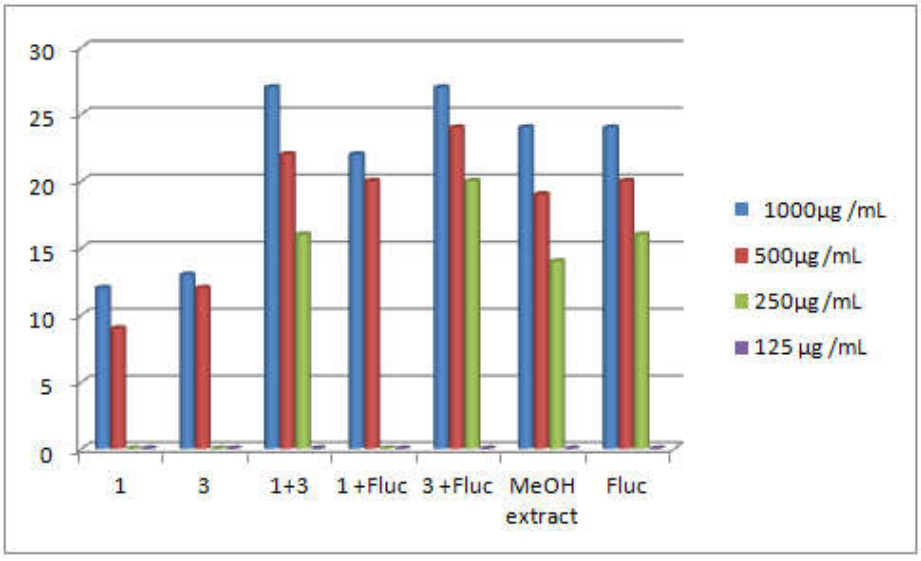

Fig. 2. A graph on zones of inhibition to growth of *R. stolonifer* by various compound combinations

Table 11. Test for interaction between combined compounds 1, 3 and Gentamicin

Test Organism	Bioactivity (FICI)		
	Compound 1 + 3	Compound 1 + G	Compound 3 + G
S. aureus	1.0	2.5	1.25
E.coli	1.5	2.5	3.0

Table 12. Test for interaction between combined compounds 1, 3 and Fluconazole

Test Organism	Bioactivity (FICI)		
	Compound 1 + 3	Compound 1 + F	Compound 3 + F
P. digitatum	1.5	1.5	1.0
R. stolonifera	1.0	3.0	1.5

4. CONCLUSION

This study has revealed the presence of five coumarins namely 5, 7-dimethoxy-6-(3'-hydroxy-3'-methylbutan-2-oxo) coumarin (**1**), being reported for the first time, and Toddalolactone (**2**), Coumurrenol (**3**), gleinadiene (**4**) and Toddaculin (**5**), which were re-isolated. Combinations of compounds **1** and **3** and compound **3** and fluconazole showed additive interaction against the fungi *R. stolonifer* and *P. digitatum*, respectively. Additive effect was also observed in the combination of compounds **1** and **3** in test against the gram-positive bacteria *S. aureus*. Other tests involving combination of the two compounds and the antimicrobials gentamycin and fluconazole showed indifference effect. Further work examining the effects of combined antibacterial agents and phytochemicals from this plant on related bacteria is being pursued.

ACKNOWLEDGEMENTS

We acknowledge Mr. Opanga for the provision of laboratory facilities and technical assistance, Mr Osogo Aggrey for the assistance in carrying out the bioassays and Dr. Lilechi Danstone assistance with NMR and MS experiments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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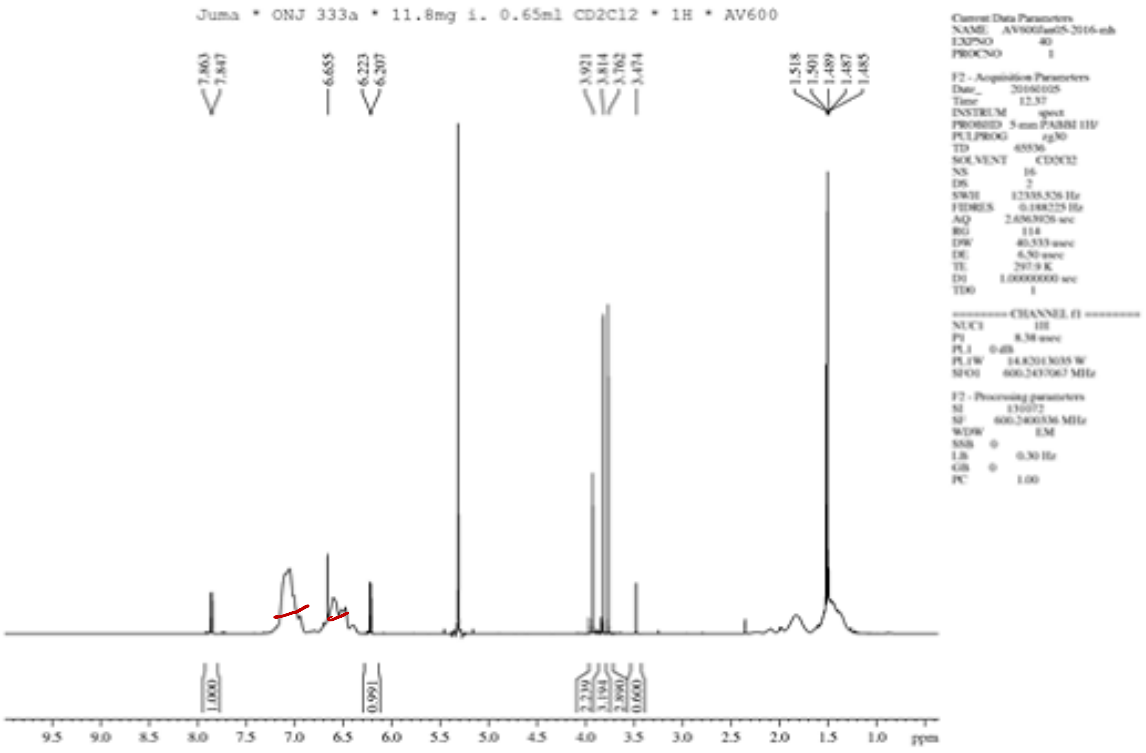
APPENDIX



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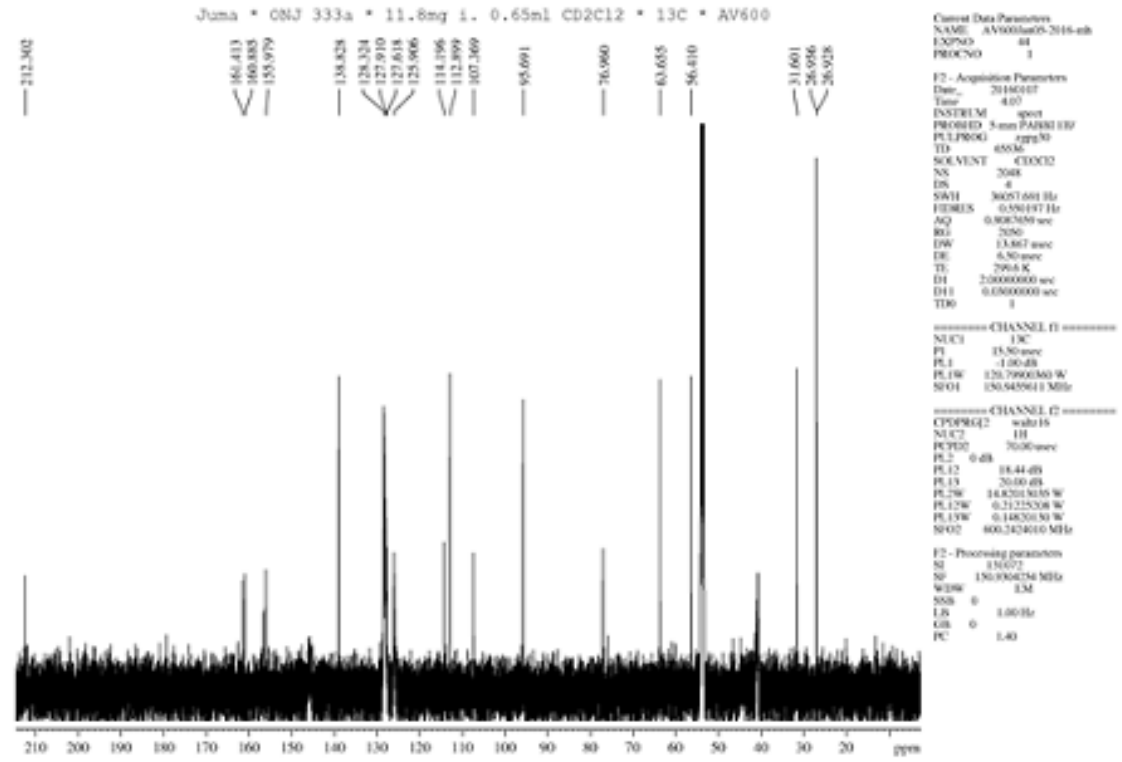

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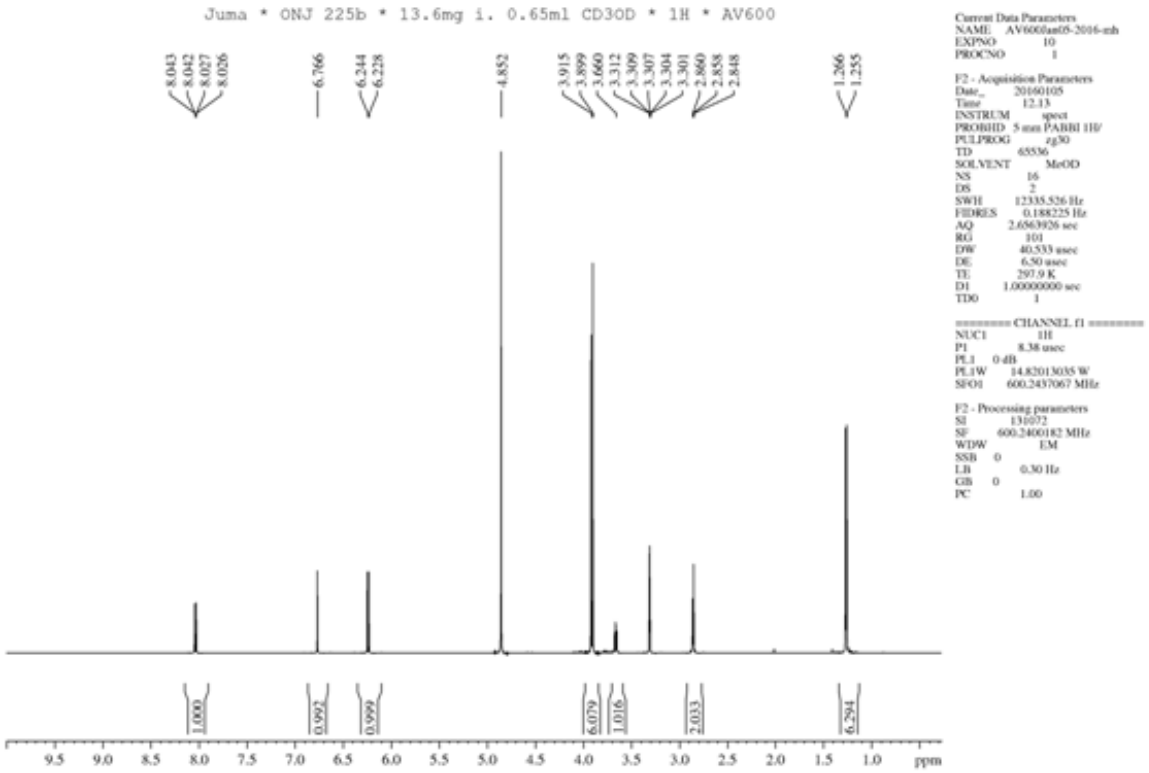

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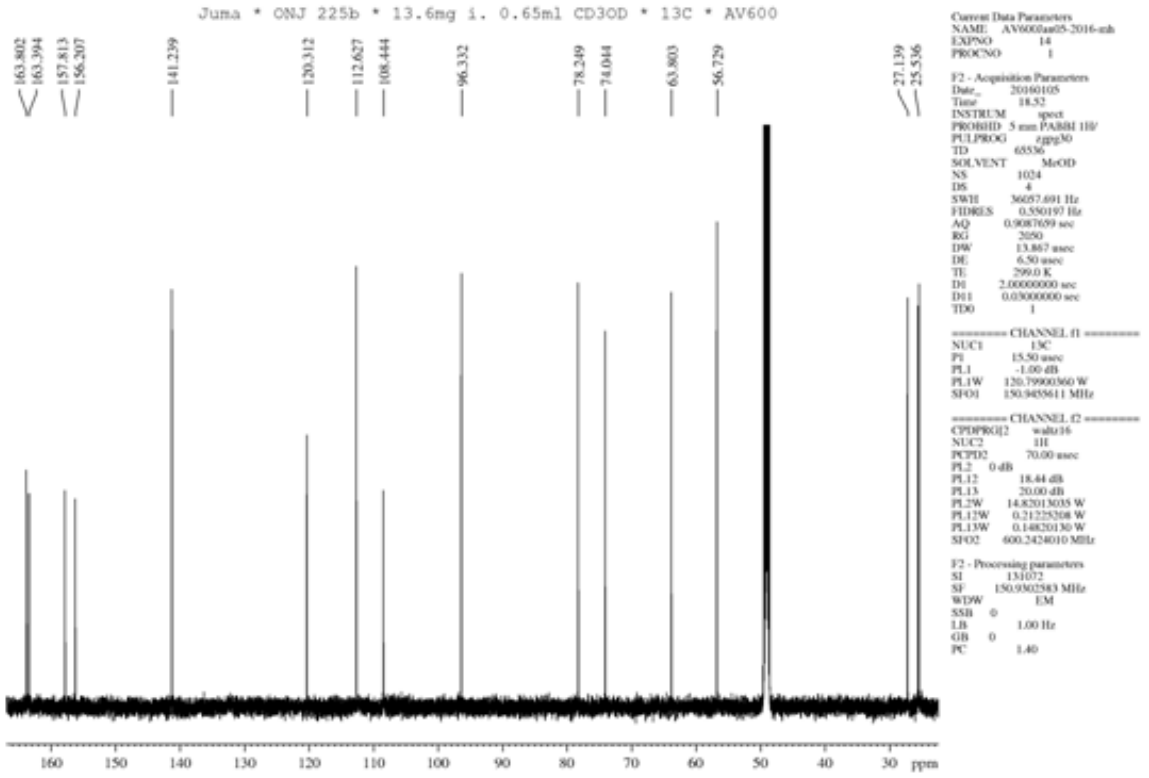

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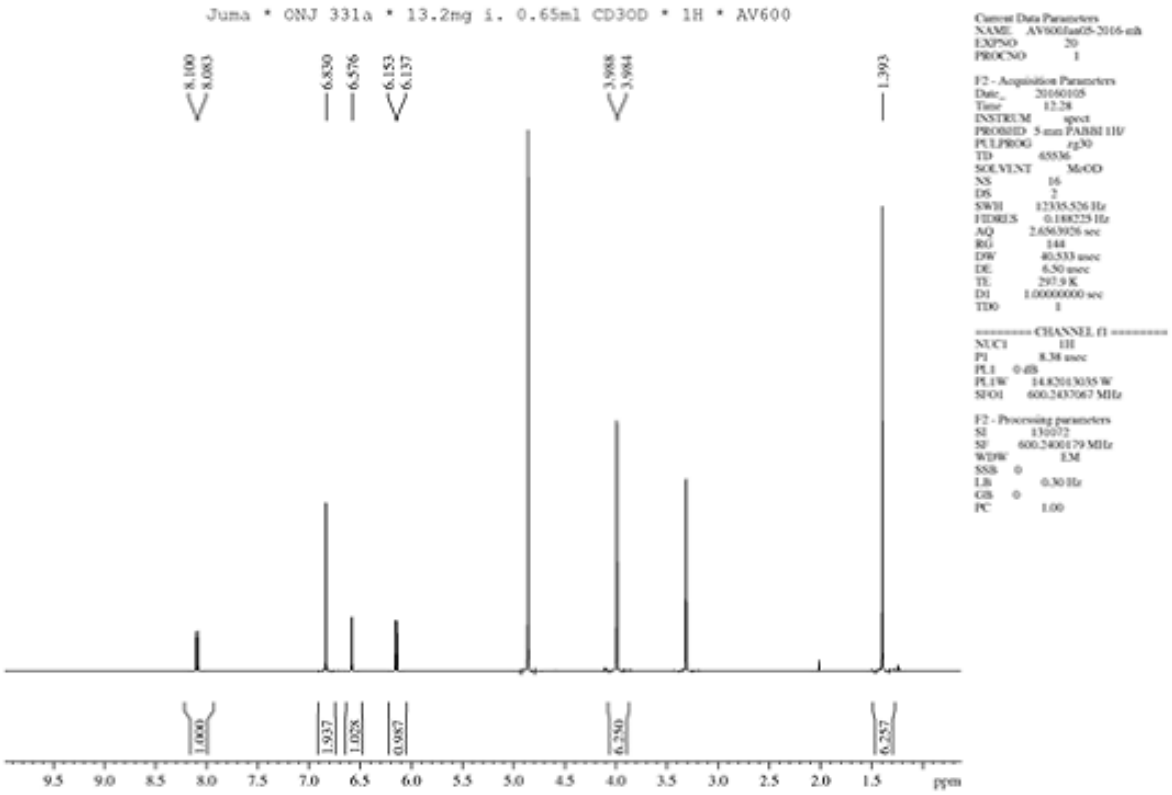

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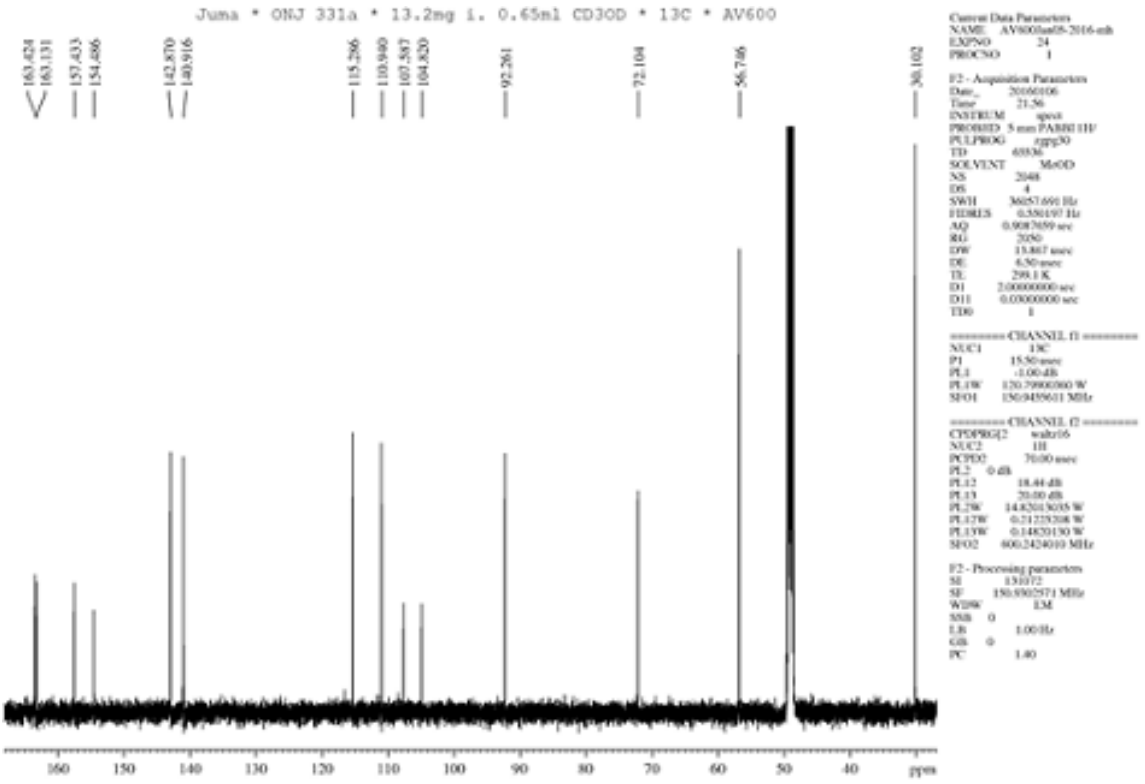

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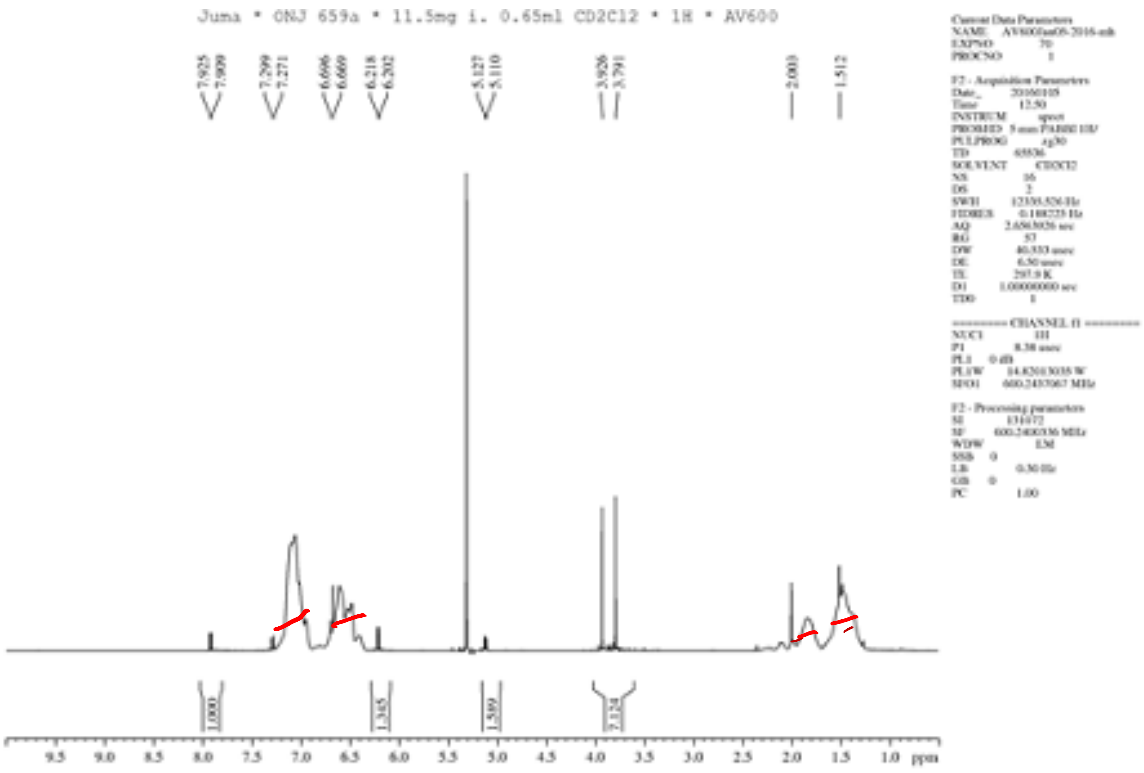

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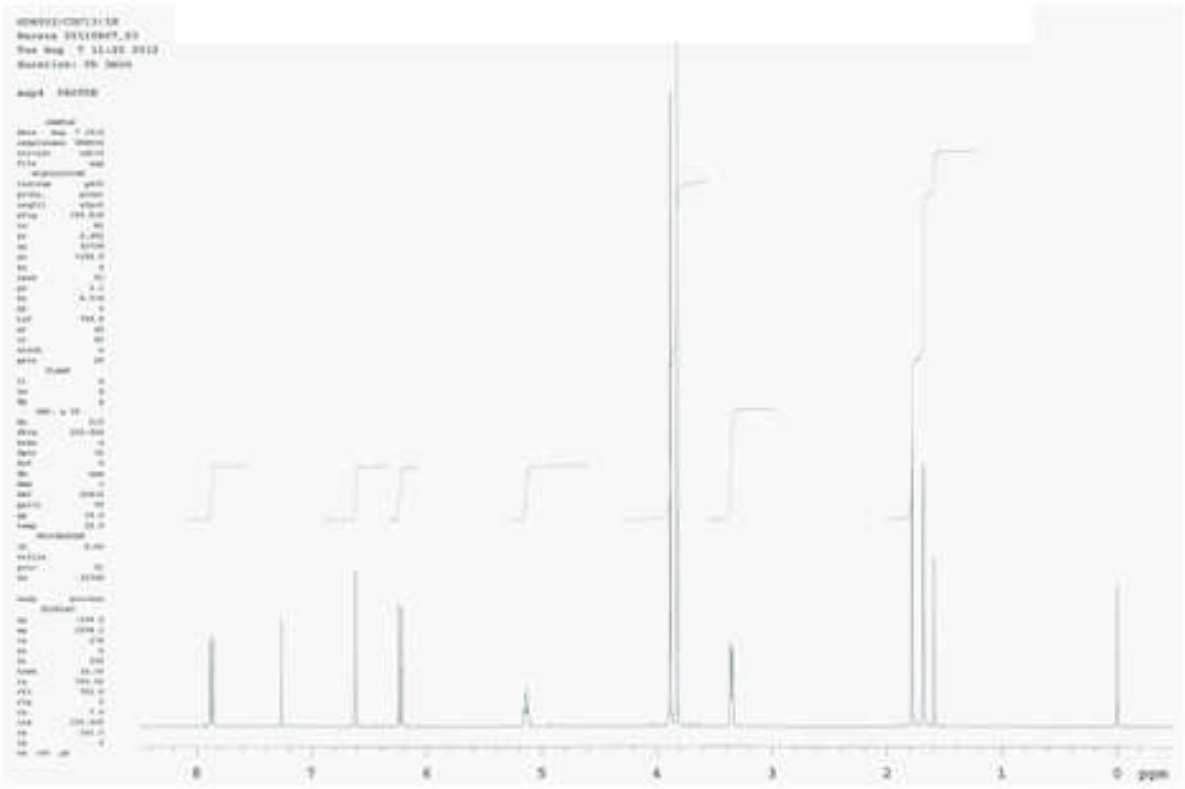

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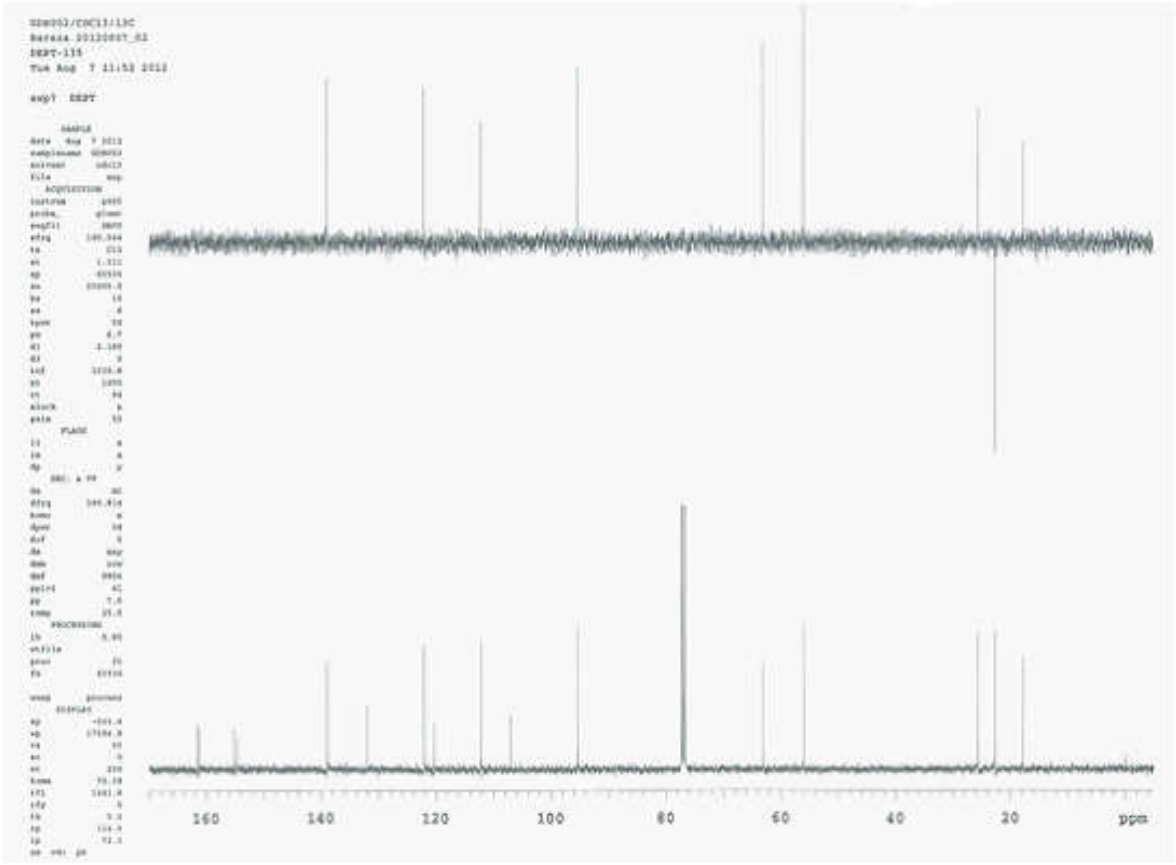

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