STRUCTURE ELUCIDATION AND SOME BIOACTIVITIES OF PURE ORGANIC COMPOUND FROM THE STEM BARK OF *DIOSPYROS EHRETIOIDES* WALL.(AUK-CHINSA)

Khaing Khaing Win[1], Htay Htay Shwe[2], Thida Win[3]

Abstract

The stem bark of *Diospyros ehretioides* Wall. (Auk-chinsa) was selected for the phytochemical screening, the determination of the cytoxicity of the crude extract, the isolation of pure organic compound and the identification of the structure of the isolated compound. The cytotoxic activity of the crude extract was determined in HeLa cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and it showed high activity with IC_{50} value of 0.85μg/mL. The pure organic compound (**KKW-1**) was isolated by thin layer and column chromatographic separation techniques. The molecular formula of the isolated compound could be observed according to the HR-MS spectral data. The molecular formula of compound (**KKW-1**) is found as $C_{22}H_{14}O_6$. In addition, the complete structure of naphthoquinone compound (**KKW-1**) was elucidated by applying 1D and 2D NMR spectroscopy.

Keywords: *Diospyros ehretioides,* naphthoquinone, cytotoxic activity, HeLa cells, MTT Assay

Introduction

Herbal medicine is the oldest form of health care known to mankind. Herbs had been used by all cultures throughout history. These historic formulations were created over a period of hundreds of years before the advent of modern medicine.

Most of the people use the traditional indigenous medicinal plants and their usage in therapy play a very important role in Myanmar. A plant can provide thousands of molecules with different biological activities. The persistent use of chemical termiticides is at present of environmental concern

1. Dr.,Assistant Lecturer, Department of Chemistry, Monywa University
2. Dr,Lecturer, Department of Chemistry, University of Mandalay
3. Dr,Rector, University of Mandalay

and has resulted in the need to search for plant-derived compounds as alternatives in termite control (Carter, 1978).

Reports published decades ago had revealed that several wood species possess natural resistance to termite infestation but only a limited number of them had been examined (Sandermann *et al*., 1958).Termite resistant woods are said to contain allelochemicals such as quinones, flavonoids and terpenoids that possess natural repellent and toxic properties (Scheffrahn, 1991).

The family Ebenaceae consists of only three genera, of these, the genus *Diospyros*is by far the largest, with 500 species (Willis, 1966).This genus is widespread in the tropics and the warm temperate regions of the world, of which 24 species are native to India. *Diospyros sylvatica,* also known locally as gatha is a moderate sized tree distributed in the hills of Vizianagram and neighbouring Orissa state (Gamble, 1997).

Chemical examination of Ebenaceae has been generally confined to the genus *Diospyros.* Anumber of *Diospyros* species are used in herbal medicine for the treatment of whooping cough, leprosy, dysentery, menstrual troubles, and abdominal pains and as antibiotics (Watt and Breyer-Brandwijk, 1962). The wood of this genus has considerable economic importance as a source of hard wood timbers and also as edible-fruits (Irvine, 1961).

Chemical studies on a number of species have revealed that the stems and leaves of this genus have been reported to contain triterpenoids (Bhakuni *et al*., 1971), while the roots are well known to contain naphthols and naphthoquinones. The antibacterial, antifungal and termite resistant properties of *Diospyros* have all been attributed to the presence of naphthoquinones (Waterman and Mbi, 1979). In a brief study conducted on the resistance of various timbers of *Diospyros* species, the wood of *D.celebica* was found to be highly resistant to the subterranean termites; *Reticulitermes lucifugus* and *Reticulitermes flavipes* (Sandermann *et. al*., 1957).

The naphthoquinones, 7-methyljuglone and its dimer isodiospyrin isolated from the wood of *D. Virginiana* were reported to possess termiticidal activity against *Reticulitermes flavipes*(Carter *et al*., 1978). Naphthoquinones and naphthalene derivatives have been isolated previously from this genus,

but a survey of the literature revealed that triterpenes namely α– amyrin, lupeol and betulin were the only constituents reported from the bark of this plant.

In this research paper, the pure organic compound (KKW-1) from the stem bark of Auk-chinsa (Figure 1) was isolated by applying column and thin layer chromatographic methods. The molecular formula of the compound (KKW-1) was determined by HR-ESI-MS spectrometry. The complete structure was identified by using modern sophisticated techniques such as ^{1}H NMR, ^{13}C NMR, DQF-COSY, HSQC and HMBC respectively.

Botanical Description

Family name	-	Ebenaceae
Botanical name	-	*Diospyros ehretioides* Wall.
Local name	-	Auk-chinsa
Parts used	-	Stem bark
Medicinal uses	-	anticancer activity, anti HIV activity, antibacterial activity, antimalarial activity, and anti-inflammatory activity

Figure 1: Fruit, leaf and flower of Auk-chinsa

Materials and Methods

General

For column chromatography silica gel 60 (Merck) with a particle size of 63-200 μm was used. Silica gel 60 F_{254} precoated aluminium sheets (0.2 mm; Merck) were used for TLC controls. Melting point was measured by the electric melting point apparatus.

All NMR experiments were recorded on a Bruker Avance 600 (operating at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C) at 300 K. The

spectra were recorded in chloroform-d_3 and referenced against residual non deuterated solvent. ESI-MS (LR and HR mode) spectra were measured on a TSQ 7000.

Plant Material

The stem bark of Auk-chinsa was collected from Yezin, Pyinmanar Township, Mandalay Region. The collected sample was washed, dried in air and crushed into small pieces. Then, the dried sample which was stored in a well stoppered bottle was used throughout the experiment.

Extraction and Isolation

The air dried sample of the stem bark of Auk-chinsa (500g) was extracted with pet ether (2000 mL). It was concentrated under reduced pressure. And then it was re-extracted again with ethyl acetate and concentrated by rotatory evaporator. The pet-ether crude extract (1.41 g) and ethyl acetate crude (1.87) g were obtained. The pet-ether portion (1.4g) was fractionated with column chromatography on silica gel as adsorbent and stepwise eluted with n-hexane : EtOAc (4:1) to give pure compound (25.2 mg).

Results and Discussion

Preliminary Phytochemical Test for the Stem Bark of Auk-chinsa

The results of phytochemical tests of crude extract of stem bark of Auk-chinsa are tabulated in Table 1.

Table 1: Phytochemical Constituents of Stem bark of Auk-chinsa

No.	Tests	Reagents	Observation	Results
1.	Alkaloid	1 % HCl, Dragendorff's reagent	Orange ppt	+
2.	Flavonoid	EtOH, Conc. HCl, Mg turnings	Pink color solution	+
3.	Terpenoid	Pet ether, Acetic anhydride, Conc. H_2SO_4	Pink color solution	+
4.	Steroid	$CHCl_3$, Acetic anhydride, Conc. H_2SO_4	Blue color solution	+
5.	Glycoside	H_2O, 10 % Lead acetate	White ppt	+
6.	Reducing Sugar	H_2O, Benedict solution	Brick red ppt	+
7.	Polyphenol	EtOH, 10 % $FeCl_3$, 1 % $K_3Fe(CN)_6$	Greenish blue color solution	+
8.	Saponin	H_2O, shake	Forthing	+
9.	Tannin	H_2O,10 % $FeCl_3$ soluiton, H_2SO_4	Yellowish brown color solution	+

(+) = Presence of constituents (-) = Absence of constituents

According to this Table 1, the stem bark of Auk-chinsa contains many phytochemical constituents.

Antimicrobial Activities of Various Crude Extracts from the Stem Bark of Auk-chinsa

The crude extracts in three solvents (n-hexane, EtOAc, EtOH) were sent to Pharmaceutical Research Department (PRD) for antimicrobial activity measurements by Agar well diffusion method. The resulting data are shown in Table 2.

Table 2: Antimicrobial Activities of Various Crude Extracts from the Stem Bark of Auk-chinsa

Crude Extract	**Diameter of Inhibition Zone (mm)**					
Solvent	I	II	III	IV	V	VI
n- hexane	-	-	-	-	-	-
EtOAc	18 (++)	31 (+++)	25 (+++)	25 (+++)	11 (+)	20 (+++)
EtOH	-	-	11 (+)	-	-	-

Microorganisms

I = *Bacillus subtilis* II = *Staphylococcus aureus*
III= *Pseudomonas aeruginosa* IV = *Bacillus pumilus*
V = *Candida albicans* VI = *Escherichia coli*
Agar well - 10 mm
10 mm~ 14 mm (+); 15 mm ~ 19 mm (++); 20 mm above (+++)

The ethyl acetate extract of stem bark responds high activities on all selected organisms except *Bacillus subtilis* and *Candida albicans*. The n-hexane extract of stem bark has no activity on all organisms. The ethanol extract shows low activity on *Pseudomonas aeruginosa* and no activity on other microorganisms (Figure 2).

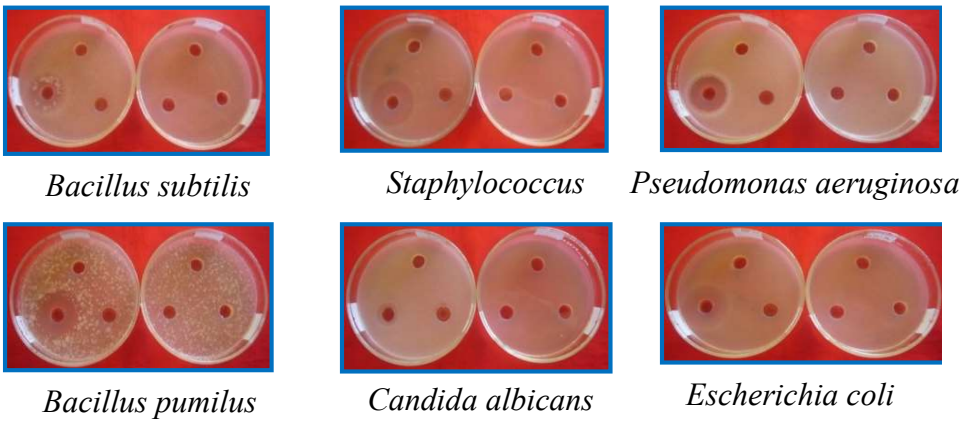
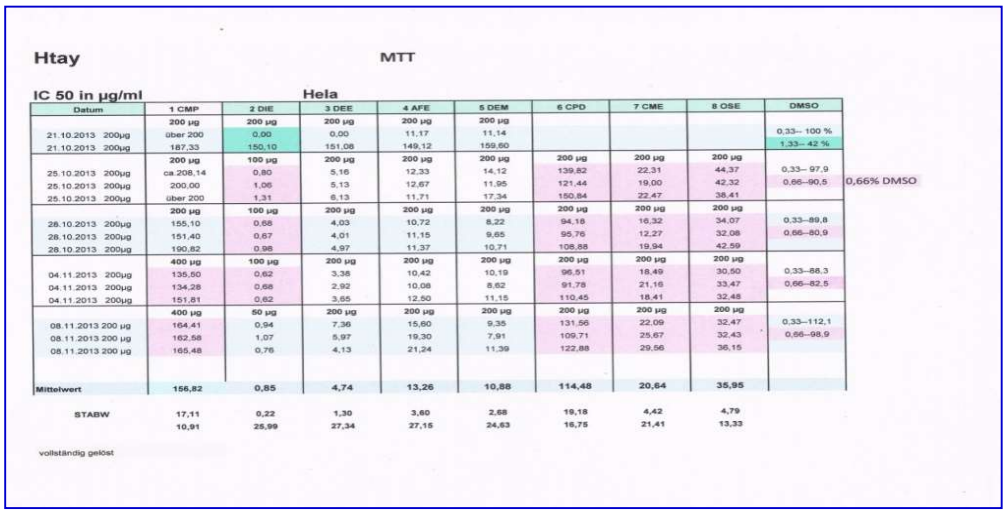


Figure 2: Antimicrobial activities of the crude extracts from the stem bark of Auk-chinsa against six selected microorganisms

Cytotoxic Activity of the Stem Bark of Auk-chinsa

The ethyl acetate crude extract was sent to Pharmaceutical Biology Department, University of Regensburg, Germany for cytotoxicity measurement on HeLa cell by MTT assay. The high IC_{50} value of 0.85 µg/mL shows that this plant has high anticancer activity on HeLa cell (cervical cancer). The resulting data are shown in Figure 3.



Htay

MTT

IC 50 in µg/ml

Hela

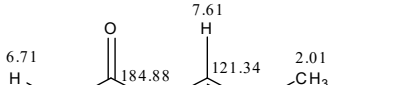
Datum	1 CMP	2 DIE	3 DEE	4 AFE	5 DEM	6 CPD	7 CME	8 OSE	DMSO	
	200 µg	200 µg	200 µg	200 µg	200 µg					
21.10.2013 200µg	über 200	0,00	0,00	11,17	11,14				0,33-- 100 %	
21.10.2013 200µg	187,33	150,10	151,08	149,12	159,60				1,33-- 42 %	
	200 µg	100 µg	200 µg	200 µg	200 µg	200 µg	200 µg	200 µg		
25.10.2013 200µg	ca.208,14	0,80	5,16	12,33	14,12	139,82	22,31	44,37	0,33-- 97,9	
25.10.2013 200µg	200,00	1,06	5,13	12,67	11,95	121,44	19,00	42,32	0,66--90,5	0,66% DMSO
25.10.2013 200µg	über 200	1,31	6,13	11,71	17,34	150,84	22,47	38,41		
	200 µg	100 µg	200 µg	200 µg	200 µg	200 µg	200 µg	200 µg		
28.10.2013 200µg	155,10	0,68	4,03	10,72	8,22	94,18	16,32	34,07	0,33--89,8	
28.10.2013 200µg	151,40	0,67	4,01	11,15	9,65	95,76	12,27	32,08	0,66--80,9	
28.10.2013 200µg	190,82	0,98	4,97	11,37	10,71	108,88	19,94	42,59		
	400 µg	100 µg	200 µg	200 µg	200 µg	200 µg	200 µg	200 µg		
04.11.2013 200µg	136,50	0,62	3,36	10,42	10,19	96,51	18,49	30,50	0,33--88,3	
04.11.2013 200µg	134,28	0,68	2,92	10,08	8,62	91,78	21,16	33,47	0,66--82,5	
04.11.2013 200µg	151,81	0,62	3,65	12,50	11,15	110,45	18,41	32,48		
	400 µg	50 µg	200 µg	200 µg	200 µg	200 µg	200 µg	200 µg		
08.11.2013 200 µg	164,41	0,94	7,36	15,60	9,35	131,56	22,09	32,47	0,33--112,1	
08.11.2013 200 µg	162,58	1,07	5,97	19,30	7,91	109,71	25,67	32,43	0,66--98,9	
08.11.2013 200 µg	165,48	0,76	4,13	21,24	11,39	122,88	29,56	36,15		
Mittelwert	156,82	0,85	4,74	13,26	10,88	114,48	20,64	35,95		
STABW	17,11	0,22	1,30	3,60	2,68	19,18	4,42	4,79		
	10,91	25,99	27,34	27,15	24,63	16,75	21,41	13,33		

vollständig gelöst

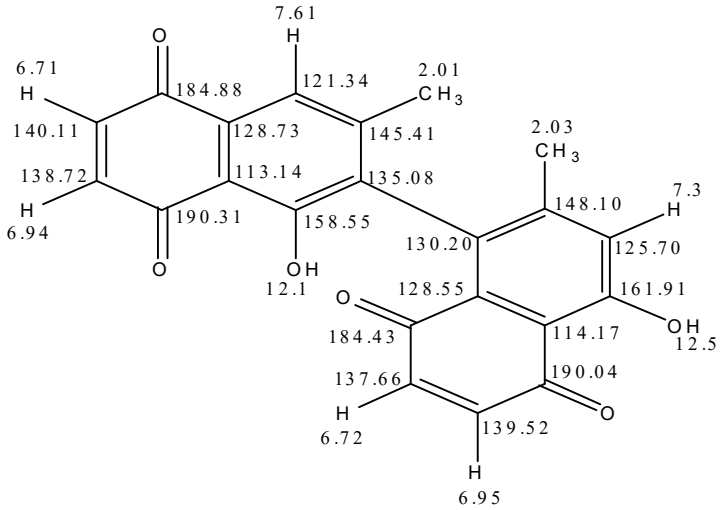
Figure 3: Cytotoxic activity result from the stem bark of Auk-chinsa testing with HeLa cells by MTT assay

Structure Elucidation of Isolated Compound

The concentrated pet- ether extract of the stem bark of *Diospyros ehretioides* was separated by column chromatography on silica gel resulted in the isolation of the naphthoquinone compound (KKW-1)with yield % of 1.787% .It was isolated as bright orange amorphous compound with melting point of 296-298°C .The analysis of the ^{1}H- NMR spectral data proton signals at 6.71, 6.72, 6.94, 6.95, 7.3, 7.61ppm revealed that the dimer structure of the aromatic compound(Table 3 and Figure 4). In addition, the four carbonyl carbon signals at 184.43, 184.88, 190.04, 190.31 and other aromatic carbon signals at 113.15, 114.18, 121.34, 125.70, 128.55, 128.75, 130.22, 135.09, 137.65, 138.72, 139.53, 140.11, 145.42, 148.10, 158.55, 161.91 ppm showed the presence of quinone compound (Table 3 and Figure 5). Furthermore , the



two methyl signals at 2.01, 2.03 and the two phenolic OH protons at 12.1, 12.5ppm described that the two 7- methyl juglone compounds were connected to form dimer type quinone compound. HSQC, HMBC and DQF-COSY experiments were utilized extensively to complete the ^{1}H- and ^{13}C-assignments and the connectivity between the dimer (Figures 6,7 and 8). HR-ESI-MS of the compound displayed a molecular ion $[M + H]^{+}$ at 375.0862 (calculated for $C_{22}H_{15}O_6$, m/z 375.0863) indicating a molecular formula of $C_{22}H_{14}O_6$ (Figure 9). Thus the compound (KKW-1) was determined to be 1',4-dihydroxy-2,3'-dimethyl-1,2'-binaphthyl-5,5',8,8'-tetraone.



Complete Structure of Compound (KKW-1)

Table 3: NMR data (δ ppm, J in Hz, ^{1}H 600 MHz, ^{13}C 150 MHz 298 K) of the isolated compound (KKW-1)

No. of Proton & Carbon	Chemical shift δ(ppm)	
	H	**C**
C(1)	–	184.88
H–C(2)	6.71 (*d*, *J* = 8.7Hz)	140.11
H–C(3)	6.94 (*d*, *J* = 8.7Hz)	138.72
C(4)	–	190.31
C(5)	–	158.55
C(6)	–	135.08
C (7)	–	145.41
H–C(8)	7.61 (*s*)	121.34
C(9)	–	128.73
C(10)	–	113.14
CH_3(11)	2.01 (3H, *s*)	20.41
C (1')	–	184.43
H–C(2')	6.72 (*d*, *J* = 8.9Hz)	137.66
H–C(3')	6.95 (*d*, *J* = 8.9Hz)	139.52
C(4')	–	190.04
C(5')	–	161.91
C(6')	7.3 (*s*)	125.70
C (7')	–	148.10
H–C(8')	7.61 (*s*)	130.20
C(9')	–	128.55
C(10')	–	114.17
CH_3(11')	2.03 (3H, *s*)	20.60

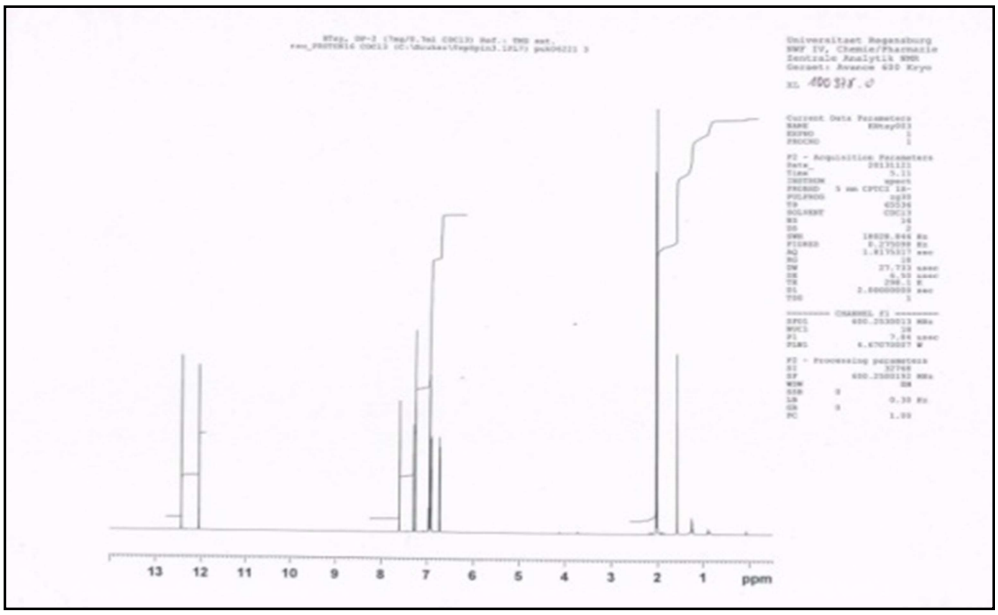


Figure 4: ^{1}HNMR spectrum of isolated compound (KKW-1)

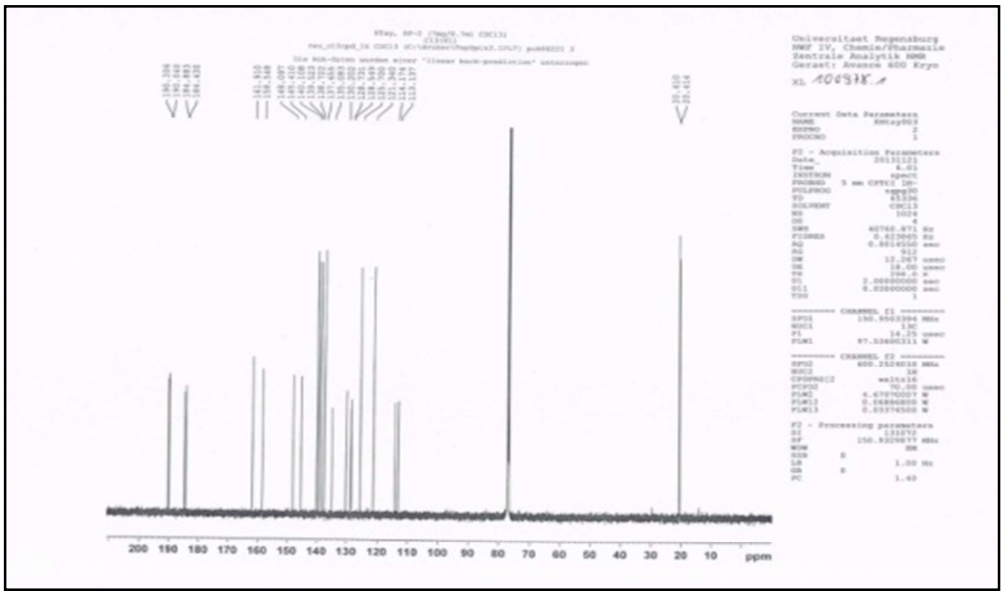


Figure 5: ^{13}CNMR spectrum of isolated compound (KKW-1)

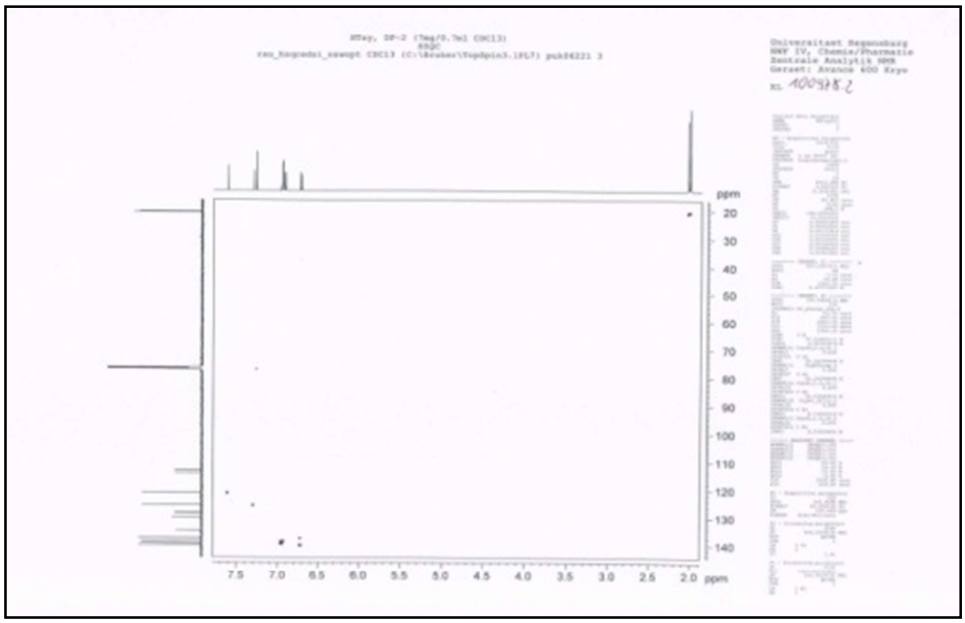


Figure 6: HSQC spectrum of isolated compound (KKW-1)

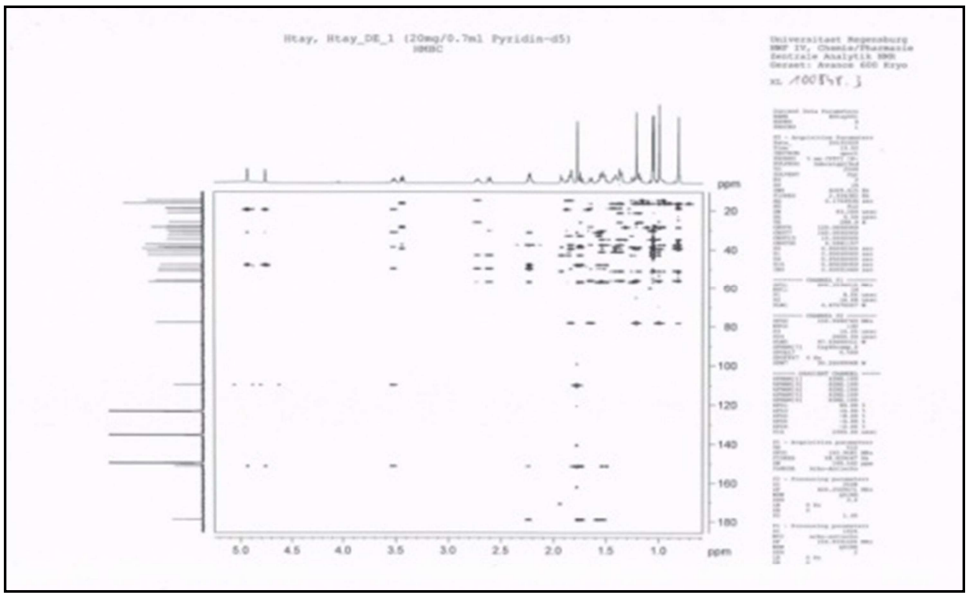


Figure 7: HMBC spectrum of isolated compound (KKW-1)

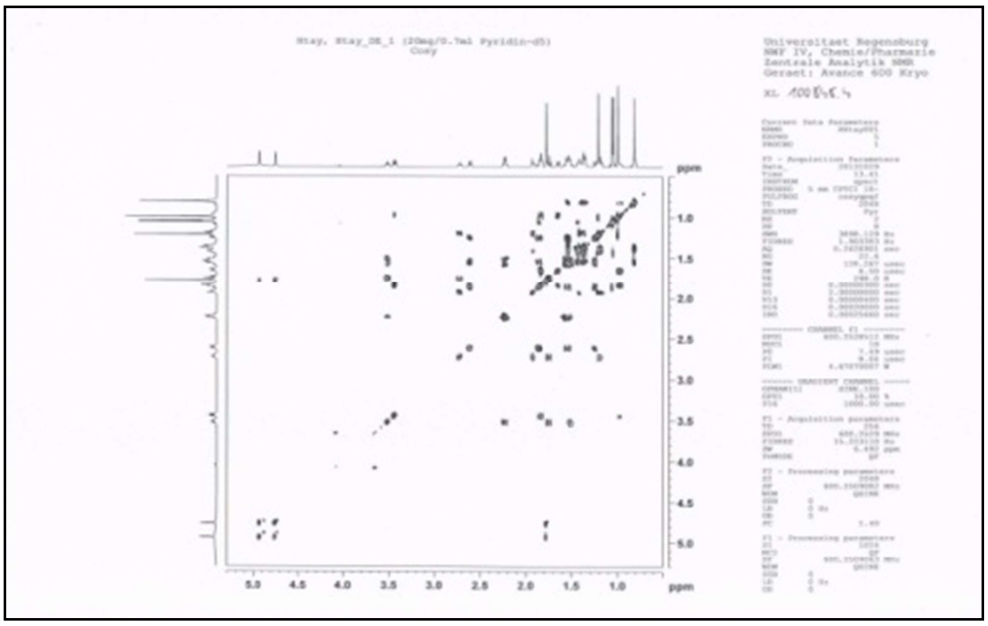


Figure 8: DQF-COSY Spectrum of isolated compound (KKW-1)

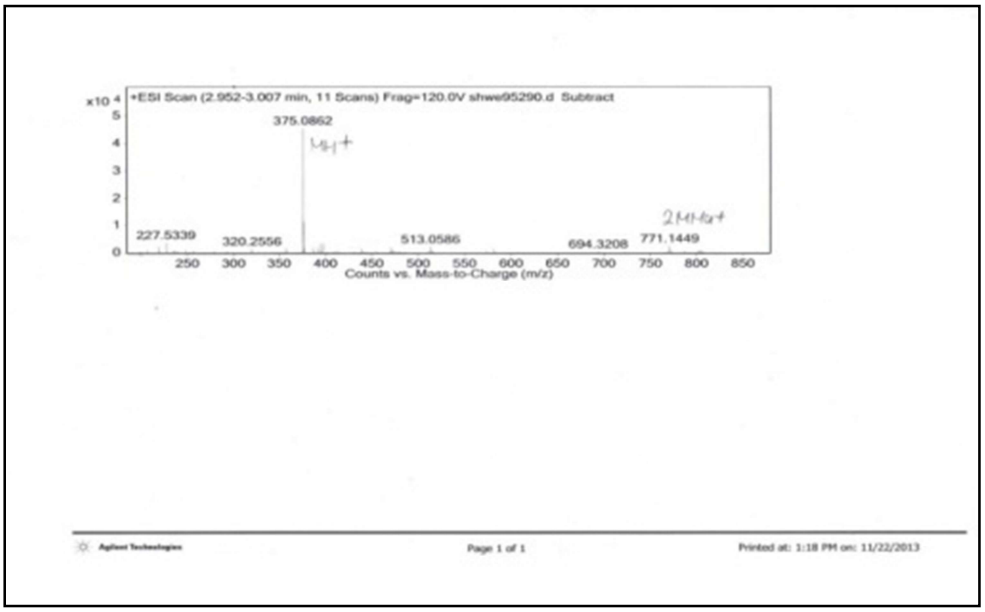


Figure 9: HR-ESI-MS Spectrum of isolated compound (KKW-1)

Conclusion

The stem bark of *Diospyros ehretioides* Wall., (Auk-chinsa) was used to determine the phytochemical constituents, to isolate the pure organic compound and to test the bioactivity and cytotoxicity of the crude extracts. The stem bark of this plant contains alkaloid, flavonoid, terpene, steroid, glycoside, polyphenol, sugar, saponin, and tannin. The high IC_{50} value of 0.85 μg/mL showed that this plant has high anticancer activity on HeLa cell (cervical cancer). The pure organic compound (KKW-1) so was isolated and by thin layer and column chromatographic separation techniques. The melting point of compound (KKW-1) was found to be 296- 298ºC. The yield percent of the isolated pure compound (KKW-1) was 1.787% (25.2 mg) based upon the pet- ether crude extract. The MS of compound (KKW-1) displayed (M^+) at m/z 374 (corresponding to $C_{22}H_{14}O_6$). The ^{1}H-NMR spectrum reflected two methyl groups (δ_H2.01 and 2.03 ppm) corresponding to δ_C 20.41 and 20.60 ppm. The existence of quinone type compound could be confirmed by the ^{13}C- NMR spectral data at four carbonyl carbons at δ_C 184.43, 184.88, 190.04 and 190.31 ppm. The ^{13}C-NMR spectrum reflected the presence of di phenol group at δ_C 158.55 and 161. 91 ppm corresponding phenolic protons at (δ_H12.1 and 12.5 ppm).Thus the compound (KKW-1) was determined to be 1',4-dihydroxy-2,3'-dimethyl-1,2'-binaphthyl-5,5',8,8'-tetraone.

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