Nigerian Research Journal of Chemical Sciences **(ISSN: 2682-6054)**
Vol. 7, Issue 2, 2019

ANTIBACTERIAL, PHYTOCHEMICAL AND SOME SPECTRAL STUDIES OF THE CRUDE METHANOL AND CHLOROFORM EXTRACTS FROM THE CORMS OF *GLADIOLUS KLATTIANUS* (HUTCH)

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ABSTRACT

The corm of *Gladiolus klattianus* (Hutch) which is used for traditional medicine was investigated. The pulverized corm was extracted exhaustively with methanol by hot continuous percolation method in soxhlet apparatus for 48 hours. The corm methanol extract was partitioned with chloroform. Phytochemical analysis of the extracts was carried out which revealed the presence of carbohydrates (monosaccharide, reducing sugars, pentose, ketose and soluble starch), glycoside (cardiac glycoside, saponins, and terpenoids), triterpenes, steroids, flavonoids and tannins in the methanol corm extract while flavonoid and tannins were absent for the chloroform corm extract; anthraquinones and alkaloids were absent in both extracts. The extracts were subjected to antimicrobial analysis using the Agar Well-diffusion method and the result revealed the methanol and chloroform corm extracts to be active against ten and eight out of the twelve human pathogens respectively. This includes *Staphylococcus aureus*, *Streptococcus faecalis*, *Corynebacterium ulcerans*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, *Neisseria gonorrhoeae*, *Shigella dysenteriae*, *Candida albicans* and *Candida krusei*. The chloroform corm extract gave a yellowish substance after chromatographic separation labeled UWA which was subjected to spectra analysis using NMR techniques, Mass spectroscopy, and Infra-Red (IR) Spectroscopy. Based on the Mass spectra and ^{13}C NMR results, the isolate was suggested to be Uzarigenin + 2-D-glucose (German). These gave credence to the traditional application of this plant for the treatment of typhoid fever, malaria fever, diarrhea, scabies, rash and urinary tract infections. The result of this study justified the use of this plant in ethno medicinal treatment of ailments.

Key words: Antimicrobial analysis, corm, *Gladiolus klattianus*, isolate.

INTRODUCTION

Gladiolus, whose name comes from the Latin word *gladius* meaning "a sword", is a genus of flowering plants from the iris family (*Iridaceae*) [1]. It is sometimes called the sword lily.The most widely-used English common name for this plant is simply gladiolus (the plural is gladioli or gladioluses) [2]**.** Other common names are: *Corn Flag*, *Afrikaners and Bluebell* [3]. Gladioli are perennial herbs. They are semi hardy in temperate climates, growing from round, symmetrical corms that are enveloped in several layers of brownish, fibrous tunics. It is one of the largest genera in *Iridaceae* family, with possibly as many as 300 species [4]. The current number of species in the genus is 255 [5]. This genus is distributed in Mediterranean Europe, Asia, Madagascar, tropical Africa and South Africa, with 160 species from southern Africa and 76 species from tropical Africa. About 10 species are native to Eurasia.

In Northern Nigeria small corms of Gladiolus are sold in markets, used in soup, taken both as a food and as a medicine. In Benue district and Basa, Nigeria, larger *Gladiolus* corms called “okpendu” or “okredu” corms are used with Guinea-corn flour (Sorghum, Gramineae) in making a sweet pap or beverage. In Lagos, corms called baka are sold as a purgative medicine to clear the system of gonorrhoeaand other conditions. Compounded with watermelon (*Citrullus*, *Cucurbitaceae*) and onion (*Allium*, *Alliaceae*); mixtures of food and medicine thus prepared, are called agunmu. In Hausa, a large *Gladiolus* corm called “rumanan doki” is used to treat dysentery in humans and Horses and is also used as a medicine for mucous diarrhea, applied by rectal injection. In Ghana, corm is prepared in enema with ginger (*Zingiber*, *Zingiberaceae*) as a potent evacuant in constipation, also used for dysentery. Bunting says it is also a valued remedy for snake-bite [6]. For persons in a faint, finely pulverized corm is put in the nostrils; if sneezing results there is hope of recovery. Fluid extract alone, or powder mixed with castor oil (*Ricinus*, *Euphorbiaceae*) and lime juice (*Citrus*, *Rutaceae*), acts promptly as a purge. Several species are used as food in Southand South tropical Africa [7-9].

MATERIAL AND METHOD

Plant collection and preparation

The plant *Gladiolus klattianus* was collected from Miligoma Shika road, Zaria, Kaduna State in the month of August, 2016. They were properly identified and confirmed at the Herbarium, Department of Botany, Faculty of Life Science, Ahmadu Bello University, Zaria, Nigeria with

voucher specimen number 6892. Thereafter, the corms were separated from the plant and air-dried for two weeks under shade in readiness for the laboratory work. The dried cormswere pounded into coarse powder using wooden mortar and pestle.

Extraction Procedure

The pulverized corm (700 g) was weighed and extracted exhaustively with redistilled and methanol (2.5 liters) for 48 hours in a soxhlet extractor. Concentration of the extract was done *in vacuo* at 40 ^{o}C using rotary evaporator (*Rota vapor*) to give the crude extract. The crude methanol extract was later subjected to solvent partitioning using chloroform to obtain the chloroform crude extract.

Phytochemical analysis of the plant material

The corms were screened for plant metabolites using the pulverized materials. Standard techniques of Brain and Turner [10] and [11] were employed inthe phytochemical screening. These metabolites include carbohydrates, glycosides, steroidal aglycone, tannins, saponins, alkaloids, flavonoids and anthracene derivatives.

Isolation

The Yhin-Layer chromatographic (TLC) and Preparative Thin-Layer chromatographic (PTLC) techniques were adopted for the isolation and purification process. Pre-coated silica gel plates were used for the trial runs to determine the appropriate solvent combinations required to separate the components of the extracts fairly well. For developing the chromatogram on the TLC plates, various solvent mixtures were tried. For the preparative TLC, larger glass plates (20 X 20 cm) of 0.5 mm thickness were used. Silica gel (Merck kieselgel 60G) for TLC and distilled water were mixed in the ratio 1:2. Silica gel (45 g) activated in the oven for a period of 30 minutes was mixed with 90 ml of water until smooth slurry was obtained.The coated plates were allowed to set for a period of five hours, and were thereafter activated before use, by heating them further in a hot-airoven at110^{o}C.

The chloroform extract was purified using PTLC technique. Five grams of the extract was dissolved in chloroformand spotted on 120 glass plates coated with silica gel (Merck Kieselgel 60G). Petroleum ether andethyl acetate solvent at a ratio of 8:2 was used for the development of the plates. The eluting solvent system used was that in which the desired component has a

Retention factor (Rf) value of 0.6 by TLC analysis. The plates were visualized under visible and UV-light (366 and 254 nm). The plates were sprayed with 10% sulphuric acid in methanol and heated at 100^{o}C for 3 minutes [12]. The compound which gave a green spot in UV light was further purified and isolated to give UWA (4 mg).The final product named UWA was analyzed using Mass spectroscopy and NMR-spectrophotometer: - Bruker Advance FT-NMR 400 MHz was used for the characterization in $CDCl_3$.

Antimicrobial screening test

Pure clinical isolates of *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Streptococcus pyogenes, Streptococcus feacalis, Corynebacterium ulcerans, Bacillus subtilis, Salmonella typhi, Klebsiella pneumonia, Neisseria gonorrhoeae, Candida albicans, Candida krusei and Shigella dysenteriae* obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, were grown on a nutrient agar slant in bijoubottles in an incubator at 37 ^{o}C for 24 h. Stock solutions of therespective plant extracts were prepared by initially dissolving 1 g of each methanol extract in 1 ml of DMSO to obtain stock solutions of each. From the stock solution, concentrations of 100, 50, 25, 12.5 and 6.250 mg/ml were prepared by serial dilution. The cork and bore diffusion method of Bauer *et al.* [13] and Barry and Thornsberry [14] were used in the anti-microbial screening. Inoculation of the prepared plates with the organism was done using a wire loop to transfer a strand of the organism into the plate followed by cross-streaking with the same wire loop to achieve uniform spread on the plate. A control was set up alongside using pure DMSO for each strain of organism. The plates were incubated at 37 ^{o}C for 24 h after which they were examined for zones of inhibition of growth.

Determination of the minimum inhibitory concentration (MIC)

The determination of the minimum inhibitory concentration was carried out on the methanol and chloroform extracts because they showed sensitivity against the growths of the test organisms. The medium was nutrient agar solution which was prepared according to the manufacturers' standard of 28 g/1000 ml. In this case double strength was prepared by dissolving 28 g in 500 ml of distilled water which was swirled and mixed thoroughly by heating to allow uniform dissolution after which 5 ml of it was dispensed into 30 sets of universal bottles and sterilized in an autoclave at 121^{o}C for 15 min. The agar was allowed to cool to 45 ^{o}C and each graded solution was then mixed gently with molten double strength nutrient agar in a Petri-dish and

allowed to solidify for one hour. Extracts'concentrations of 100, 50, 25, 12.5 and 6.250 mg/ml were prepared by serial dilution. Each plate was divided into equal sections and labeled accordingly to correspond to the test organisms. Two 5 mm diameter paper discs (Whatman No.1) were placed aseptically into each labeled section of the plate using sterilized forceps. With an automatic micropipette, 0.1 ml of each bacterial suspension was taken and transferred aseptically and carefully into each appropriate pre-labeled paper disc on the agar plates. The plates were incubated for 24 h at 37 ^{o}C after which they were observed for growths or death of the test organisms. The lowest concentration inhibiting growth was taken as the minimum inhibitory concentration.

Determination of the minimum bactericidal concentration (MBC)

This was carried out to know if the organisms could be killed completely or their growths could only be inhibited. Another 30 sets of plates of nutrient agar were prepared according to the manufacturers' standard and sterilized in an autoclave as earlier described. The paper discs in all the plates from the MIC tests were reactivated. Emphasis was paid to the MIC plates and the preceding plates. The re-activation was done in a mixture of 0.5% egg lecithin and 3% Tween 80 solution in a test tube. The reactivated organisms were sub-cultured into appropriately labeled quadrants of the sterilized nutrient agar plates using wire loop into each test tube and streaking uniformly on the labeled quadrants. This was then incubated for 24 h at 37 ^{o}C after which they were observed for growths. The MBC was the quadrant with the lowest concentration of the extract without growth.

RESULTS AND DISCUSSION

The phytochemical screening of the methanol extract of the corm of *Gladiolus klattianus* revealed the presence of carbohydrates, tannins, flavonoid, saponins glycosides, steroids and terpenes, steroidal glycosides while alkaloids and anthraquinones were absent. In the chloroform extract, alkaloids, anthraquinones, flavonoid and tannins were not detected as shown in Table 1. Phytochemicals like tannins, saponins and flavonoid have potentially significant applications against bacteria [15]. Different phytochemicals have been found to possess varied medicinal properties. For example, saponins, terpenoids, flavonoids, tannins and steroids have been reported to have anti-inflammatory effects [16-18]. Some glycosides, flavonoids, and tannins have been reported to have had hypoglycemic activities [19, 20]. Rupasingh *et al*. [21] reported

that some saponins possess hypochloesterolemic and antidiabetic properties. Some terpenoids have also been shown to decrease blood sugar levels in animal studies [22]. Steroids and saponins are important for their cardiotonic activities and anti-microbial properties. They are also used in nutrition herbal medicines and cosmetics [23]**.** Tannins have antifungal, antibacterial [24, 25] and antioxidant properties. Several authors have linked the presence of these compounds to the antimicrobial properties of plant extracts. Many triterpene saponins and their aglycones have been reported by Hostettmann and Martson [26] and Ndukwe *et al.* [27] to have varied uses as antiulcerogenic, anti-inflamatory, fibrinolytic, antipyretic, analgesic and anti-edematous in action.

Spectroscopic characterization of UWA

Spectroscopic methods were used to elucidate the structure of isolated compound UWA. The techniques were Infrared (IR) Spectroscopy (Fig.3), ^{1}H-NMR (Fig.1), ^{13}C-NMR (Fig.2) and GC-MS (Fig.4). The infrared spectrum was recorded neat, ^{1}H-NMR and ^{13}C-NMR spectra were recorded using $CDCl_3$ as solvent and the GC-MS spectra was recorded at high resolution on a mass spectrometer.

The positive tests for steroids given by extract shows that the isolated substance is likely a steroid and this was evidenced in the phytochemical tests. UWA is a yellowish substance. On subjection to IR spectroscopic analysis, the observed absorption bands were 3741.06 cm^{-1} that is characteristic of O-H stretching. Absorption at 1542.14 cm^{-1} was as a result of C=C stretching however this band is weak. Other absorption frequencies include 778.30 cm^{-1} for out of plane C-H vibration of unsaturated part. The proton NMR signal at δ 3.57 suggests a proton attached to oxygen and suggest the existence of a methyl carbon δ 2.21. Proton signal at 0.69(s), 0.80(s) and 1.02(s) is typical of angular methyl proton [28, 29]. The signals between δ 0.69 and 1.94 suggest the presence of a steroidal skeleton.

The ^{13}C-NMR (400MHz) of UWA: ^{13}C-NMR showed 35 signals. The peak at δ 121.7 might be due to a double bond which is assignable to C=C double bond as assigned for spirostene. The peaks at δ 78.98 could be due to hydroxyl functional group. Those at δ 40.7, 40.1, 39.69 and 40.2 are likely due to methine (CH) carbon atoms, while signals at δ 32.49, 32.66, 33.92, 34.82, 32.66 and 29.44 are typical of methylene (CH_2) groups. Peaks at δ 18.38, 15.61, 14.12, and 23.54 are typical of methyl (CH_3) carbon atoms. The alkenes carbons are supposed to appear at δ145.2, 121.7, [27, 28], but the NMR machine could not absorb at 145.2 due to possible malfunctioning.

The Mass Spectroscopy showed a molecular ion peak at m/z 698.8, [M^+], which might be due to the formula mass $C_{35}H_{54}O_{14}$. Other peaks such as δ 55.2, 97.2 [C_7H_{12} + H], 140.2, C_7H_{13}, 182.2 $C_6H_{14}O_6$ [M^+-507.6], 252.3, 291.2, 326.9 $C_{12}H_{22}O_{10}$ [M^+-363.9], 366.7 $C_{27}H_{42}$, 401.7 $C_{28}H_{49}O$, 532.7 $C_{27}H_{48}O_{10}$, 653.2 can be obtained from fragmentations.

The antimicrobial screening of the extract of the corm of this plant showed that the methanol extract was more active than the chloroform extract as is seen in Table 2. This could be due to the presence of some very active bioactive substances in this fraction. The MIC results are presented in Tables 3. The crude methanol extracts of the corm inhibited the growth of *S. aureus, E. coli, C. ulcerans, B. subtilis, N. gonorrhoeae, C. albicans, S. dysenteriae and S. typhi at* a concentration of 25 mg/ml and *S. feacalis, B. cereus,* at a concentration of 12.5 mg/ml as recorded in Tables 3, while the chloroform extract is minimal at 25 mg/ml against *S. aureus, E. coli, C. ulcerans, B. subtilis, S. feacalis, B. cereus N. gonorrhoeae and C. albicans* as recorded in Table 3. The MBC (Tables 4) generally showed slightly higher concentrations than that of the MIC and the methanol extracts could completely exterminate the test organisms from growing. The MBC was lowest in the methanol extract which indicates that it contains the highest concentrations of the bioactive constituents. The isolated compound, UWA, also showed great antimicrobial activity in line with the extract from which it was isolated with MIC at a concentration of 12.5 mg/ml against five of the organisms as recorded in Tables 3. In conclusion the findings in this study justified the use of this plant in ethno medicinal treatment of gonorrhea and other conditions, mucous diarrhea, as a potent evacuant in constipation, dysentery and remedy for snake bite, which are caused by some of theseorganisms used.

Table 1: Phytochemical constituents of the cormsof *Gladiolus klattianus*

CONSTITUENTS	TEST	INFERENCE	
		Chloroform Extract	Methanol Extract
Carbohydrate	a. Molisch's	+	+
	b. Fehling	+	+
Cardiac Glycosides	a. Keller-kiliani	+	+
	b. Kedde's	+	+
	c. Salkowski	+	+
Saponins glycoside	Frothing	+	+
Steroid and Triterpenes	Liebermann-Burchard's	+	+
Tannins	a. Iron (III) chloride	-	+
	b. Lead Subacetate	-	+
	c. Bromine water	-	+
Alkaloids	a. Wagner	-	-
	b. Mayer	-	-
	c. Dragendorff's	-	-
Anthraquinones	a. Borntrager's	-	-
	b. Combine Anthracene	-	-
Flavonoid	a. Shinoida	-	+
	b. 10% NaOH & HCl	-	+
Steroidal Glycosides	Liebermann-Burchard's	+	+

KEY: (+) = Present; (-) = Absent.

Table 2: Antimicrobial Activities and Zones of Inhibition of chloroform extract (CE), methanol extract (ME) and the isolate UWA of the corm of *Gladiolus klattianus* with control

Test Organisms	ACTIVITY					Zone of Inhibition (mm)				
	CE	ME	UWA	CONTROL		CE	ME	UWA	CONTROL	
				SP	FL				SP	FL
S. aureus	S	S	S	S	R	24	27	20	44	ND
S. pyogenes	R	R	R	S	R	ND	ND	ND	42	ND
S. feacalis	S	S	S	S	R	22	30	22	43	ND
C. ulcerans	S	S	R	S	R	24	27	ND	44	ND
B. subtilis	S	S	S	S	R	20	20	29	40	ND
B. cereus	S	S	S	S	R	27	34	27	47	ND
E. coli	S	S	S	S	R	20	24	23	41	ND
S. typhi	R	S	S	S	R	ND	19	21	36	ND
N. gonorrhoeae	S	S	S	R	R	27	30	28	ND	ND
K. pneumonia	ND	ND	S	S	R	ND	ND	27	40	ND
S. dysenteriae	R	S	R	S	R	ND	20	ND	40	ND
C. albicans	S	S	S	R	S	22	25	25	ND	35
C. krusei	R	R	R	R	S	ND	ND	ND	ND	32

KEY: R= Resistance. S= Sensitive. mm= Millimetre(s), SP = Sparfloxacin; FL= Fluconazole, ND = Not determined.

Table 3: Minimum Inhibition Concentrations of Extracts and isolate against Microorganisms.

Test Organisms	Concentration(mg/cm^3) $x10^1$														
	CE					ME					UWA				
	10	5	2.5	1.25	0.625	10	5	2.5	1.25	0.625	10	5	2.5	1.25	0.625
S. aureus	-	-	o*	+	++	-	-	o*	+	++	-	-	o*	+	++
S. pyogenes															
S. feacalis	-	-	o*	+	++	-	-	-	o*	+	-	-	o*	+	++
C. ulcerans	-	-	o*	+	++	-	-	o*	+	++					
B. subtilis	-	-	o*	+	++	-	-	o*	+	++	-	-	-	o*	++
B. cereus	-	-	o*	+	++	-	-	-	o*	+	-	-	-	o*	++
E. coli	-	-	o*	+	++	-	-	o*	+	++	-	-	o*	+	++
S. typhi						-	-	o*	+	++	-	-	o*	+	++
N. gonorrhoeae	-	-	o*	+	+ +	-	-	o*	+	+ +	-	-	-	o*	+
K. pneumoniae											-	-	-	o*	+
S. dysenteriae						-	-	o*	+	++					
C. albicans	-	-	o*	+	++	-	-	o*	+	++	-	-	-	o*	+
C. krusei															

KEY: no growth (no turbidity), o*= MIC, + = light growth (Turbid), ++ = Dense growth (Moderate turbidity), mg = micro-gram.

Table 4: Minimum Bactericidal (MB)/ Minimum Fungicidal (MF) Concentration of extractsand isolate against Microbes

Test Organisms	Concentration(mg/cm^3) x10^1														
	CE					ME					UWA				
	10	5	2.5	1.25	0.625	10	5	2.5	1.25	0.625	10	5	2.5	1.25	0.625
S. aureus	o*	+	++	+++	++++	-	o*	+	++	+ ++	o*	+	++	+++	++++
S. pyogenes															
S. feacalis	o*	+	++	+++	++++	-	o*	+	++	+ ++	-	-	o*	+	++
C. ulcerans	o*	+	++	+++	++++	-	o*	+	++	+ ++					
B. subtilis	o*	+	++	+++	++++	o*	+	++	+++	++++	-	-	o*	+	++
B. cereus	-	o*	+	++	+++	-	-	o*	+	++	-	-	o*	+	++
E. coli	o*	+	++	+++	++++	o*	+	++	+++	++++	-	o*	+	++	+++
S. typhi						o*	+	++	+++	++++	o*	+	++	+++	++++
N. gonorrhoeae	o*	+	++	+++	++++	o*	+	++	+++	++++	-	-	o*	+	++
K. pneumoniae											-	o*	+	++	+++
S. dysenteriae						o*	+	++	+++	++++					
C. albicans	o*	+	++	+++	++++	-	o*	+	++	+++	-	o*	+	++	+++
C. krusei															

KEY: - = no colony growth, + = Scanty colony growth, o*= MBC/MFC,++ = moderate colony growth, +++ = Heavy colony growth, ++++ = Heavy colony growth.

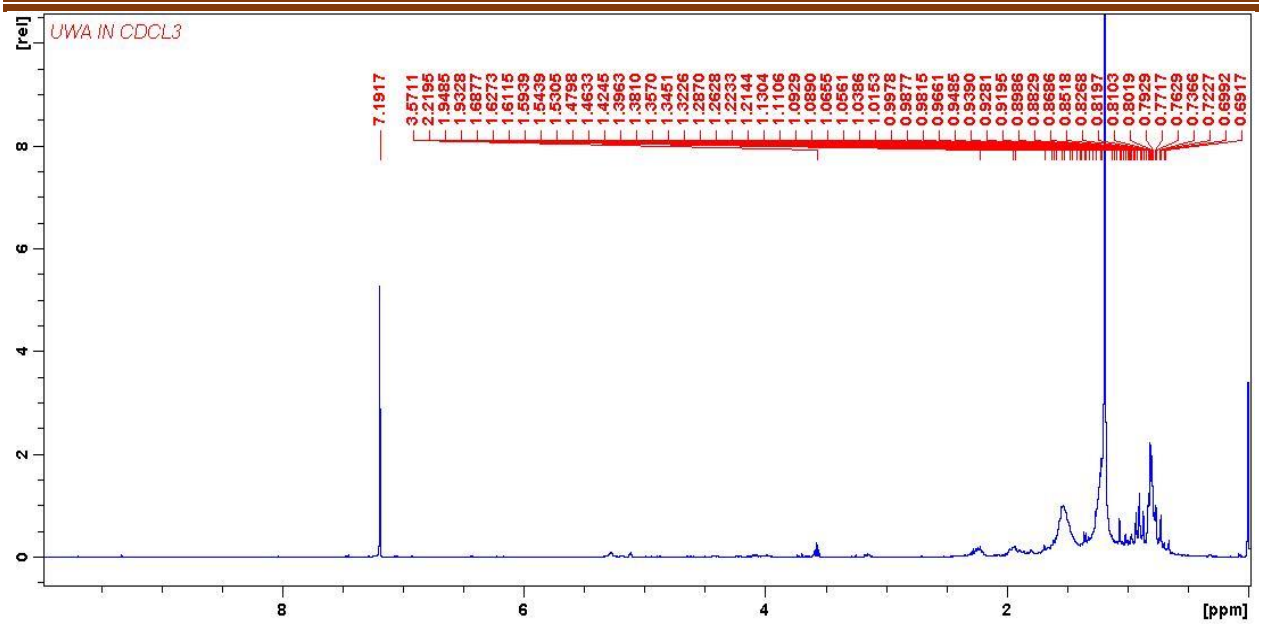


Figure 1: Proton-NMR spectra of UWA in $CDCl_3$

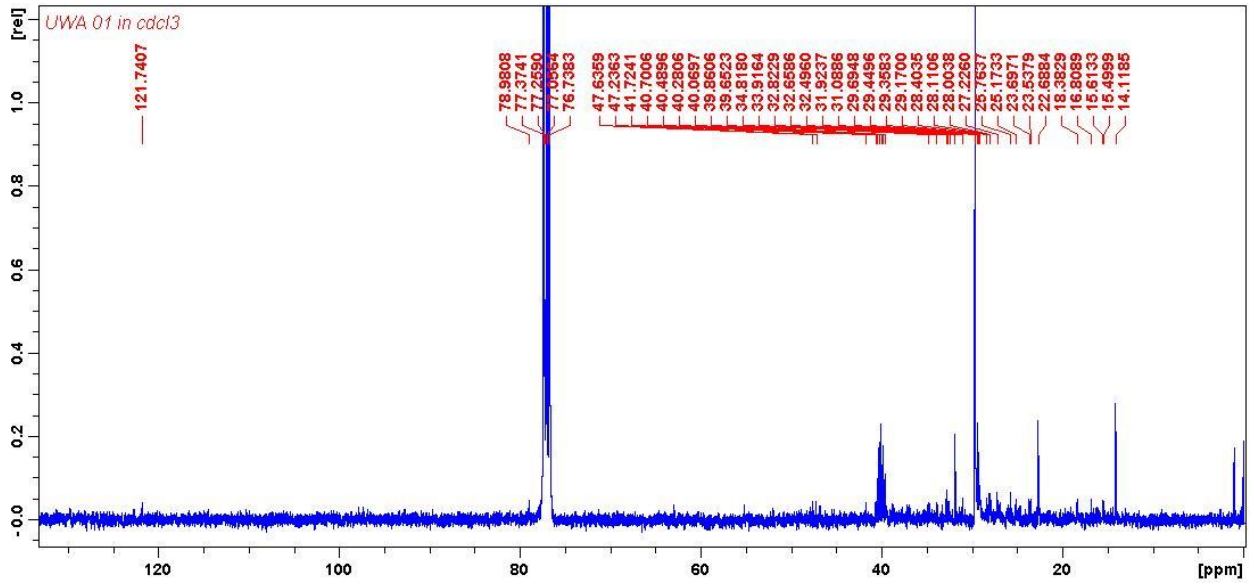
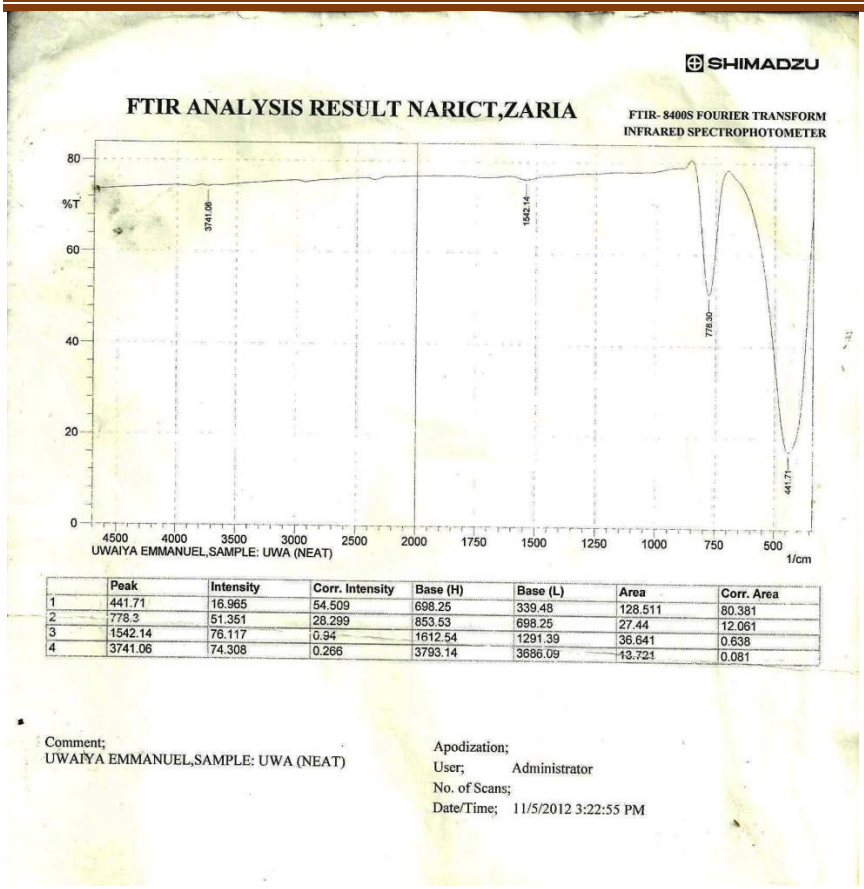


Figure 2: ^{13}C- NMR Spectra of UWA in $CDCl_3$



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	441.71	16.965	54.509	698.25	339.48	128.511	80.381
2	778.3	51.351	28.299	853.53	698.25	27.44	12.061
3	1542.14	76.117	0.94	1612.54	1291.39	36.641	0.638
4	3741.06	74.308	0.266	3793.14	3686.09	13.721	0.081

Figure 3: IR Spectral of UWA

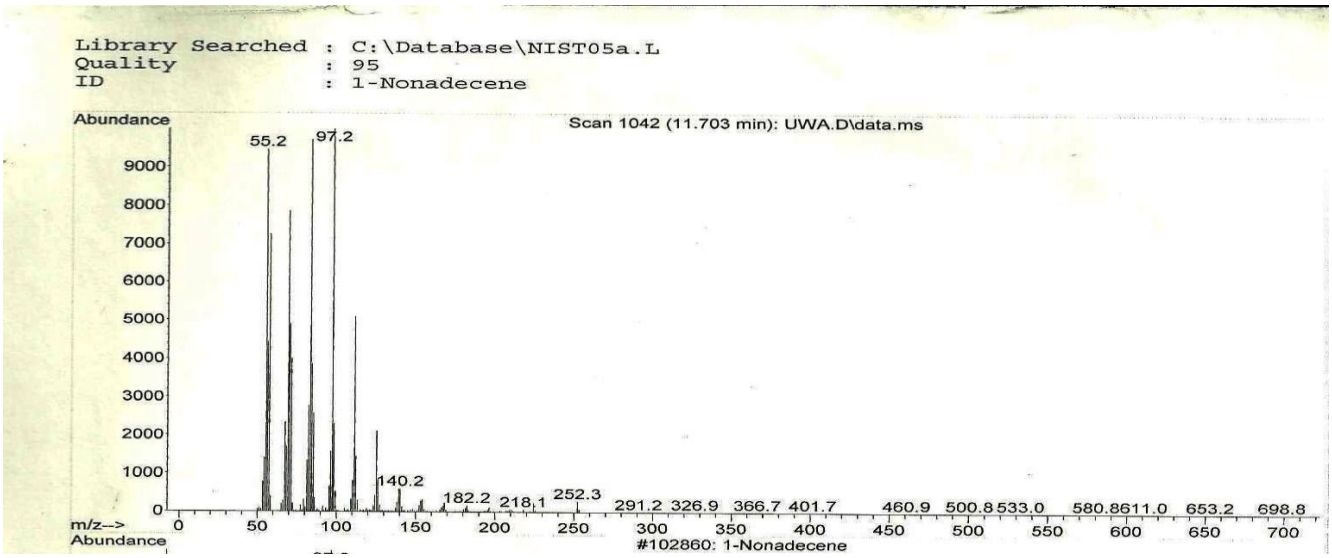


Figure 4: GC-MS data of UWA

The suggested structure for UWA (UZARIN)

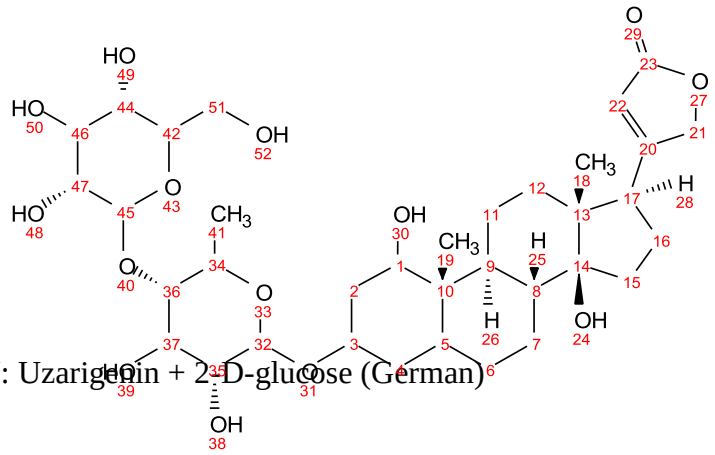


Figure 5: UZARIN: Uzarigenin + 2-D-glucose (German)

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