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RESEARCH ARTICLE

ISOLATION OF FOUR FUROQUINOLINE ALKALOIDS FROM *TECLEA NOBILIS* AND THEIR ACTIVITY AGAINST *SCHISTOSOMA MANSONI* MIRACIDIA

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ABSTRACT

Schistosomiasis is one of the neglected diseases affecting approximately 200 million people worldwide. In this research, we isolated and evaluated the antimiracidial activity of secondary metabolites from *Teclea nobilis* (Rutaceae) which has been used ethnomedicinally against helminthes. Ethyl acetate extract was fractionated over silica gel column chromatography yielding four furoquinoline alkaloids which were identified through analysis of their 1D and 2D NMR, mass spectrometry data and comparison with literature data as; tecleoxine **A**, methylnkolbisine **B**, kokusaginine **C** and nkolbisine **D**. Serial dilutions of these compounds were exposed to *Schistosoma mansoni* miracidia. Compounds **A, B** and **C** were tested for bioassay and analyzed as a mixture because they were not resolved individually into purified forms due to their minimal amounts and close Rf values. Miracidial mortality was determined after 30 minutes under a dissecting microscope. Log probit regression analysis at 95% confidence level was used to calculate LC_{50} and LC_{90} using IBM SPSS version 21.0 software. Compounds **A**, **B** and **C** mixture were the most potent with LC_{50} and LC_{90} values of 270.2 and 690.9 ppm, respectively. Synergism could have been the reason for the high potency. Compound **B** was the least potent with mortality recorded LC_{50} and LC_{90} values of 287.9 and 631.7 ppm, respectively. These findings indicate that these compounds could be useful as lead compounds for the development of schistosomicide. Also, considering that a large number of human populations, mostly from poor backgrounds, suffer chronic schistosomiasis, the discovery of plant compounds that may control schistosomiasis could be of great value.

Key words: alkaloids, miracidial activity, *Schistosoma mansoni* and *Teclea nobilis*

INTRODUCTION:

The plant *Teclea nobilis* is an evergreen shrub or tree of the Rutaceae family with a smooth grey bark native to Ethiopia, Kenya, Tanzania, and Uganda rainforests. It has simple leaves on glabrous branchlets. It is reported to have been used ethnomedicinally in many African countries and Saudi Arabia [1]. The leaves and stem bark has been used as a remedy for gonorrhea as well as reported to have anti-inflammatory, analgesic and antipyretic effect as well as antihelmintic activity [2-3]. Schistosomiasis is one of the neglected tropical diseases and just like other helminthosis is of both public health and economic importance [4]. The helminthosis burden, in terms of prevalence, is equivalent to 50% of that of malaria and 25% of that of HIV/AIDS with approximately 2.9 billion people being infected [5]. Africa is the worst hit with this menace with estimated 90% of the 200 million people with schistosomiasis alone living in Africa [5-6].

Currently, Praziquantel is the drug of choice in the treatment of Schistosomiasis. However, with the recent reports of the development of resistance to praziquantel, [7-8] there is the need to research on new alternative drugs. One of the strategies in combating Schistosomiasis is through interruption of the parasite life cycle, by control of snails, miracidia, cercaria and adult worms. There is increasing need to find alternative cercaricide, miracidiacides and schistosomicides which are cheaper, safer, offer complete cure and are environmentally benign from plants [9].

Interest in the control of *S. mansoni* lies in the fact that it is one of the major contributors of the neglected tropical diseases especially in sub-Saharan Africa. We describe here the isolation, structure elucidation and miracidicidal activity of four alkaloids tecleoxine **A**, methylnkolbisisne **B**, kokusaginine **C** and nkolbisine **D**. These alkaloids are

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for the first time being reported to have anti-helmintic activity against *S. mansoni* miracida.

MATERIALS AND METHODS:

Collection of plant material:

Fresh leaves of *T. nobilis* were collected from the Kakamega rainforest (0°10'-0°21' N, 34°44'-34°58' E) in Kenya. It was identified at the Department of Biological Sciences, Egerton University, where a voucher specimen was deposited. The materials were then air-dried under shade to constant weight.

Extraction and isolation:

The air-dried powdered *T. nobilis* leaves were soaked in methanol, exhaustively extracted at room temperature and concentrated in vacuo at 40^0C. The methanol crude extract obtained was suspended in water and subjected to hexane and ethyl acetate sequential liquid-liquid partitioning. The ethyl acetate fraction was then chromatographed over silica gel column, eluted with 6:4 ethyl acetate-hexane solvent mixtures to yield four fractions F_1, F_2, F_3, and F_4. Fractions F_3 and F_4 were then subjected to silica gel preparative TLC plates using ethyl acetate-hexane (6:4) as eluent. Fraction F_3 gave a sub-fraction (213.1 mg) which upon analysis of its NMR and MS spectra were found to be a mixture of, compounds **A, B** and **C.** These compounds **A-C** were not resolved individually into purified forms due to their minimal amounts and close R_f values. They were thus tested for bioassay and analyzed as a mixture. Fraction F_4 yielded compound **D** (192.6 mg). The NMR spectra were recorded on a Bruker Advance 500 MHz NMR spectrometer. All the readings were done in Deuterated chloroform and chemical shifts assigned by comparison with the residue proton and carbon resonance of the solvent. Tetramethylsilane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). The mass spectra of the compounds were recorded on a Brucker Exactive Mass Spectrometer with electrospray ionization (ESI) Method. The Thermo Xcalibur Qual computer software was used in the analysis of the mass chromatograms.

These compounds **A-D** were isolated as dark, oily substances which showed strong fluorescence under 254 and 365 nm UV light on silica gel TLC plates. The structures of these four known compounds were determined based on the analysis of their 1D and 2D experiments coupled with comparison of their spectral data with that in literature. The ^{1}H and ^{13}C NMR spectral data are listed in table 1 below. However, the coupling constants and the proton multiplicities of the compounds were not determined as the H spectra had distorted baseline with presence of broad peaks.

Miracidicidal assay:

S. mansoni eggs were recovered from stool of infected sand harvesters from Lake Victoria in Usoma village Kisumu County, Kenya. The eggs were kept at 4^0C overnight in the refrigerator. The eggs were then transferred into a 1000 ml conical flask, filled to the brim with dechlorinated water and exposed to bright light for one hour. The flask was then covered with a dark cloth for 10 minutes leaving the top part. The top part of the water was poured into a petri-dish and observed for miracidia under a 10X objective dissecting microscope. The bioassays were performed at the Kenya Medical Research Institute (KEMRI), Centre for Disease Control (CDC), Kisumu, Kenya. 24 wells multiwell plates were used to test the effects of the compounds and observe the mortality of the miracidia under a dissecting microscope. Twenty miracidia were picked using a micropipette and transferred into 1ml dechlorinated water. The compounds were solubilized in DMSO and diluted with dechlorinated tap water. The concentration of DMSO was kept below 1%. Serial dilution concentrations of 1000, 500, 450, 400, 350, 300, 250, 200, 150, 100, 50 ppm were added to each experimental well. Two negative controls were also set up; one with dechlorinated water and another one with 1% DMSO in dechlorinated water. 1000 ppm solution of praziquantel was used as a positive control. All experiments were carried out in three replicates and mortality observed after 30 minutes. The miracidia were considered dead if they showed no signs of movement. The dead miracidia in the three replicates was combined and expressed as the percentage mortality of each concentration. Probit analysis of concentration mortality data was conducted to estimate the LC_{50} and LC_{90} values using the statistical package for social science (SPSS) version 21.0 software.

RESULTS AND DISCUSSION:

Compound **A** was found to have the molecular formula $C_{18}H_{19}NO_2$. The positive high-resolution electron impact mass spectrometry (HREIMS) of this compound showed a molecular ion peak at *m/z* 330.13 ($[M+H]^+$) (calculated for [$C_{18}H_{19}NO_5$ + H]; *m/z* 330.14). The APT and HSQC NMR spectra showed the presence of four methyls, one methylene, five methines and eight quarternary carbons. The carbon signals at 67.8, 61.2, 56.6, 24.6 and 19.0 ppm were attributed to the epoxyprenyl group. The ^{1}H NMR spectrum of this compound showed the presence of four aromatic protons absorbing at 7.47, 6.96, 7.43 and 7.28 ppm whose positions were confirmed by HMBC experiments. The position of the two methoxy groups and the epoxyprenyl substituent to their respective positions were confirmed by HMBC experiments. By comparison of these spectral data and that in literature

[10] led to the conclusion that the compound **A** is tecleoxine.

Compound **B** was found to have the molecular formula $C_{19}H_{23}NO_6$.It was identified as with molecular ion peak *m/z* 362.2 ($[M+H]^+$) (calculated for [$C_{19}H_{23}NO_6$+ H] (*m/z* 362.15) using positive high-resolution electron impact mass spectrometry (HREIMS). This compound was found to have NMR spectral data which were closely related to those of compound **A** except the presence of a methoxy group at C-3'and, a hydroxyl group at C-2' instead of an epoxide ring. The APT and HSQC NMR spectra showed the presence of an additional methoxy group at δ52.5 and its position established by HMBC experiments where the methoxy proton at δ3.62 correlated with C-3'. The position of the substituent, 2'-hydroxy-3'-methoxyisoprenyl, was confirmed to be at C-6 through HMBC experiments. These data and that in literature [10] confirmed the compound to be methylnkolbisine.

Compound **C** was found to have the molecular formula $C_{14}H_{13}NO_4$. It was identified as with molecular ion peak *m/z* 260.1 ($[M+H]^+$) (calculated for [$C_{14}H_{13}NO_4$ + H] (*m/z* 260.3) using positive high resolution electron impact mass spectrometry (HREIMS). The compound differed from compound **A** by the presence of a methoxy group at C-6 instead of the epoxyprenyl group. The position of the methoxy group, whose carbon and protons were absorbing at 56.3 and 3.73 ppm respectively, was confirmed by the use of HMBC experiments where the proton at 3.73 ppm showed correlations with C-6. This compound was identified as kokusaginine.

Compound **D** was found to have the molecular formula $C_{18}H_{21}NO_6$. It was identified as with molecular ion peak *m/z* 348.1($[M+H]^+$) (calculated for [$C_{18}H_{22}NO_6$ + H] (*m/z* 348.4) using positive high resolution electron impact mass spectrometry (HREIMS). This compound had NMR spectral data that were in close resemblance with those of compound **A**. However, there were two hydroxyl groups at C-2' and C-3' instead of an epoxide ring. The position of the 2', 3'-dihydroxyisoprenyl side chain was confirmed by the use of HMBC experiments. The NMR and HREIMS spectral data of this compound and those in the literature [11] confirmed that the compound was nkolbisine. This compound, unlike compounds **A-C** that were earlier isolated from *T. nobilis*, is for the first time being reported in *T. nobilis*. The structures of compounds **A-D** are shown in figure 1 and their NMR data summarized in table 1.

The compounds **A-C** were confirmed to be alkaloids tecleoxine, methylnkolbisine and kokusaginine earlier isolated from *T. nobilis* [10]. Kokusagine has also been isolated from the plants *Melicope bonickii, Haplophylum species* and *Esenbeckia leiocarpa* [12-14]. These compounds have also been isolated from other plants of the family Rutaceae including the genus *Teclea* which is well endowed with alkaloids [15-19]. Compound **D** which was identified as nkolbisine has also been isolated from other plants [11, 13, 20] but it is the first time it is being reported in *T. nobilis* species.

To the best of our knowledge, there is no reported study on the evaluation of miracidicidal activity of the furoquinoline alkaloids **A-D**. In the present paper, these alkaloids isolated from the leaves of *T. nobilis* presented lethal effects against *S. mansoni* miracidia. The percentage mortality, LC_{50} and LC_{90} values after 30 minutes of exposure for the alkaloids are summarized in table 2**.** The results of the miracidicidal tests demonstrated that the compounds **A, B** and **C** mixture was the most potent with registered LC_{50} and LC_{90} values of 270.2 and 690.9 ppm respectively. Compound **D** was the least potent with mortality recorded LC_{50} and LC_{90} values of 287.9 and 631.7 ppm, respectively. Synergism of the compounds **A-C** may have been the cause of the observed activity where they were more active than compound **D**. However, the activity of all the furoquinoline alkaloids compared favourably well with those of commercial Praziquantel, which was used as the reference standard.

To the best of our knowledge, no reported study on the evaluation of antihelmintic activity was performed against *S. mansoni* miracidia with alkaloids. However, the comparisons of LC values of the four alkaloids **A, B, C** and **D** showed excellent toxicities against *S. mansoni* miracidia. The miracidia mortality of the compounds had dose-dependent effect that is, the activity was proportional to the concentration. There are few reported studies of miracidicidal activity of isolated pure compounds on *S. mansoni* miracida. However, alkaloids, both synthetic and from natural sources, have been shown to be excellent antihelmintics. For instance, Glycoalkaloids from *Solanum* spp have been reported to have *in vitro* antischistosomal effects. Similarly, epiisopiloturine and Imidazole alkaloids from *Pilocarpus microphyllus* (Rutaceae) leaves have been reported to have in vitro effect on the survival time of *S.mansoni* Schistosomulae and adult worm stages [21]. Moreover, the commercial drugs Praziquantel and Triclabendazole are alkaloids which are known to cause tegumental damage and paralysis of the trematodes [22]. According to Satou *et al.*, [23] the isoquinoline alkaloids; Allocyptopine, Dehydrocaryodaline and Papaverine have been shown to cause larval mobility inhibition against the helminth *Toxocara canis.* The alkaloids Protopine, d-Corydoline and l-Stylopine have also been reported to have anthelmintic activity against *Strongyloides ratti* and

Strongyloides venezuelensis [24]. Satou *et al.,* [25] also reported that β-Carboline alkaloids isolated from the plants *Picrasma quassoides* and *Ailanthus altissima* exhibit larval toxicity against *Toxocara canis*.

Table 1: ^{1}H and ^{13}C NMR spectral data for compounds A-D ($CDCl_3$)

No.	^{1}H (δ ppm)				No.	^{13}C (δ ppm)			
	A	**B**	**C**	**D**		**A**	**B**	**C**	**D**
2	7.47	7.47	7.47	7.52	2	142.5	142.5	142.5	142.5
3	6.96	6.96	6.96	7.01	3	104.7	104.7	104.7	104.8
3a					3a	102.2	102.2	102.2	102.3
4					4	155.9	155.9	155.9	155.9
4a					4a	112.7	112.7	112.7	112.9
5	7.43	7.43	7.43	7.42	5	102.5	102.5	102.5	102.1
6					6	146.7	146.7	146.7	146.6
7					7	152.9	152.9	152.9	152.6
8	7.24	7.24	7.24	7.28	8	106.4	106.4	106.4	106.4
8a					8a	142.3	142.3	142.3	142.3
9a					9a	162.9	162.9	162.9	163.0
1’	4.12	3.42, 3.37		4.31, 4.14	1’	67.8	71.0		70.9
2’	3.17	3.63		3.83	2’	61.2	68.3		75.2
3’					3’	58.6	71.8		72.1
4’	1.30	1.34		1.29	4’	24.5	26.4		26.4
5’	1.28	1.34		1.27	5’	19.0	26.8		25.9
3’- OMe		3.62					52.5		
4-OMe	4.32	4.32	4.32	4.38	4-OMe	58.9	58.9	58.9	58.9
6-Ome			3.73					56.3	
7-Ome	3.90	3.90	3.90	3.93	7-Ome	55.9	55.0	55.0	56.0

Table 2: **Miracidicidal activity of compounds A-D**

Concentrations and LC (ppm)	**Mean % mortality**	
	Compounds A-C	**Compound D**
1000	98.3±2.4	100.0±0.0
500	86.7±2.4	88.3±2.4
450	78.3±2.4	76.7±2.4
400	70.0±0.0	66.7±4.7
350	61.7±2.4	56.7±2.4
300	48.3±2.4	46.7±4.7
250	41.7±2.4	41.7±2.4
200	30.0±0.0	28.3±2.4
150	20.0±4.1	16.7±6.2
100	8.3±4.1	3.3±2.4
50	3.3±2.4	1.7±2.4
1% DMSO[a]	0.0±0.0	0.0±0.0
Praziquantel[b]	100.0±0.0	100.0±0.0
LC_{50}	270.2(232.6- 312.1)	287.9 (253.0-326.6)
LC_{90}	690.9 (552.6-979.5)	631.7 (521.4-853.5)

a- Negative control, b – Positive control

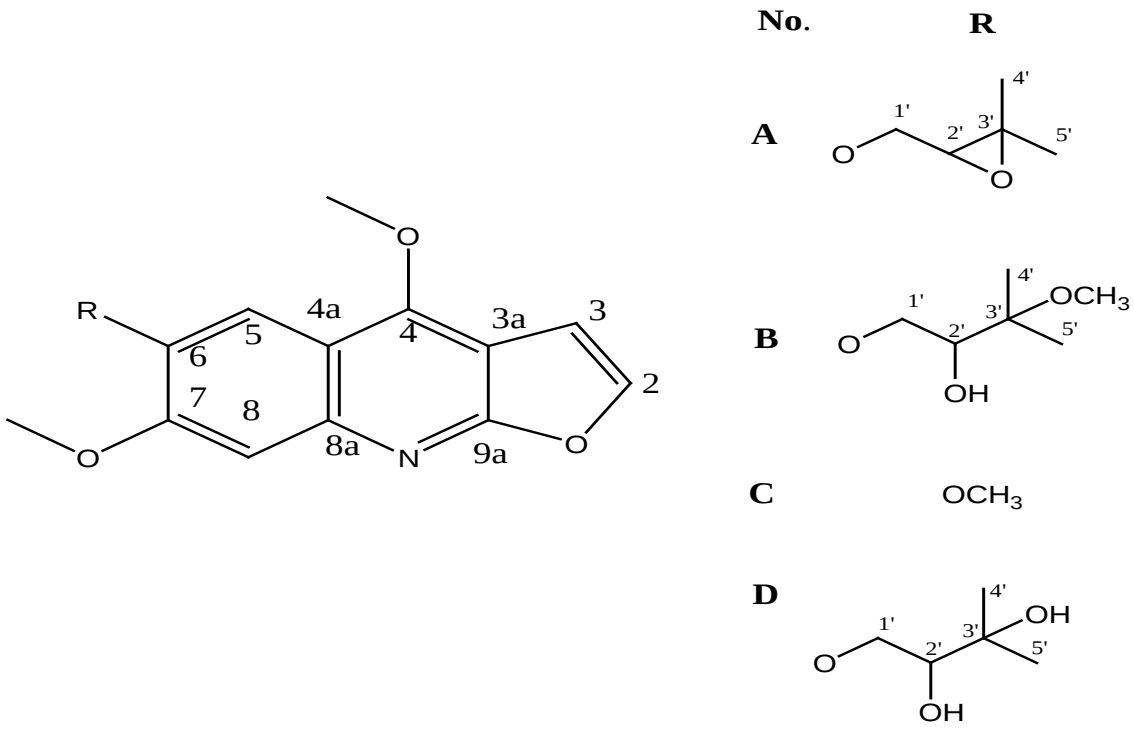

Figure 1: Structures of isolated compounds A-D

CONCLUSION:

This study suggests that the furoquinoline alkaloids **A-D** can be potential schistosomicide. Furthermore, these findings could be useful in the research for new alternative cercaricides, miracidiacides and schistomicides of plant origin which could be safer, cheaper and environmentally benign. These compounds can also be used as lead compounds for the development of wormicides.

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