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Original Research

Cytotoxic and antimicrobial activities of substituted phenanthrenes from the roots of *Combretum adenogonium* Steud Ex A. Rich (Combretaceae)

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ABSTRACT

Aim: The aim of this study was to isolate the bioactive compounds from the roots of *Combretum adenogonium* and assess for its antibacterial and cytotoxic properties. **Materials and Methods:** The extract was obtained using 20% aqueous ethanol and further subjected to fractionation with 1:1 n-butanol/water. Chromatographic analyses of the n-butanol fraction led to the isolation of compounds (1-3). The compounds (1-3) were assayed for antibacterial activities using two-fold microdilution methods and cytotoxicity using brine shrimps lethality assay. **Results:** Following spectroscopic analyses the compounds were established as 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene (1α) and 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene (1β). Compound 2 was derived from 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene condensation with methyl acetate while Compound 3 was derived from 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene condensation with methyl propionate. These compounds (1-3) were active against *Pseudomonas aeruginosa* with minimal inhibitory concentration-value of 0.16 mg/ml. The compounds (1-3) also exhibited significant toxicity with LC_{50} (95% confidence interval [CI]) of 12.11 (7.32-20.05) μg/ml compared to standard anticancer drug, cyclophosphamide which had LC_{50} (95% CI) value of 16.37 (12.01-22.31) μg/ml. **Conclusion:** These compounds add for a novel structure that can be synthesized, further screened for *in vitro* and *in vivo* models and clinical trials in order to evaluate its potential for further development as new anticancer agent.

KEY WORDS: Antimicrobial, cytotoxicity, brine shrimps lethality test, *Combretum adenogonium*, substituted phenanthrenes

INTRODUCTION

Phenanthrenes are a class of phenolic compounds with three fused rings, which are presumably formed by oxidative coupling of the aromatic rings of stilbene precursors. A large number of differently substituted phenanthrenes are known to occur in more than 10 plant families and mainly have been isolated from orchidaceae, combretaceae, dioscoreaceae and betulaceae families [1]. Phenanthrenes and stilbenes are well-recognized as phytoalexins [2] and have been reported to possess various biological activities, including antitumor [1,3] antibacterial [4] and anti-inflammatory activities [5]. Phenanthrenes and their derivatives from the genus *Combretum* have been isolated from *Combretum hereroense*, *Combretum apiculatum*, *Combretum collinum* and *Combretum molle* [6-11]. The phenanthrenes and dihydrophenanthrenes from *Combretum caffrum* possessed good activity against murine P388 lymphocytic leukemia cell lines [3]. Structure-activity relationships of phenanthrenes and stilbenes are important in activity because substituents determine the planarity of the molecules, which is essential for drug-receptor interactions [6,12]. Although *Combretum adenogonium* is widely used in African traditional medicine, there is no existing report on the bioactive compounds from the plant that provides such pharmacological effects to humans. The current study investigated the bioactive agents from the roots of *C. adenogonium*, which led to the isolation of compounds (1-3). Herein, we report the isolation and structure elucidation of the compounds by means of nuclear magnetic resonance spectroscopy (^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90). The signals were measured on 400 MHz spectrophotometer using tetramethylsilane as internal standard and dimethyl sulfoxide (DMSO) as NMR solvent.

MATERIALS AND METHODS

Materials

Acetone, n-Butanol, petroleum ether were purchased from Kas medics (Kas Medics ltd, Tanzania). Dichloromethane was purchased from UNILAB (UNILAB®, Nairobi, Kenya), ethanol (absolute) was bought from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands) whereas DMSO was purchased from Sigma® (Poole, Dorset, UK). *Staphylococcus aureus* (NCTC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 29953) were obtained from the Department of Microbiolgy, the Muhimbili University of Health and Allied Sciences (MUHAS). Nutrient broth was purchased from Tulip Diagnostic (P) Ltd (Microxpress™, Goa, India). Iodonitrotetrazolium chloride was bought from SIGMA® (Sigma-Aldrich®, St Louis, USA). The Brine Shrimps eggs were purchased from Aquaculture innovations (Grahamstown 6140, South Africa) and sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast.

Collection and Extraction of Plant Materials

One sample of roots of C. *adenogonium* was collected from Handeni district, Tanga region, Tanzania in May, 2010. The plant was identified by Haji O. Seleman of the University of Dar es Salaam, Botany Department. Herbarium specimen (voucher specimen collection number LBM 965) is deposited in the Herbarium of the Botany Department, University of Dar es Salaam. The collected plant material was air dried, pulverized and extracted with 20% aqueous ethanol at room temperature for 24 h. The extract was dried under vacuo, followed by freeze-drying before analysis.

Isolation of Compound (1-3)

The root extract (111.20 g) was dissolved by shaking in 1:1 mixture of n-butanol and water and the two phases were separated in a separating funnel. An amount of 35.59 g of n-butanol fraction was packed into a 4.5 cm × 50 cm silica gel column and eluted successively with petroleum ether, petroleum ether/dichloromethane and dichloromethane/acetone. A total of 65 fractions were collected and analyzed using thin layer chromatogram (TLC) and each fraction which displayed similar TLC profiles was combined. Fractions 57-67 were combined, and further eluted in a small column to give semi-pure sub-fractions 11-16. These sub-fractions were combined and subjected to preparative TLC using 2% acetone/dichloromethane to yield 7.8 mg of compounds 1α and 2. These compounds which were yellowish amorphous powder, UV-positive with R_f value 0.5 in 2% acetone/dichloromethane were identified as 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene (1α) and substituted phenanthrenes (2) respectively. Compounds (1β) and (3) were obtained from further purification of compounds (1α) and (2) and re-analyzed with NMR spectroscopy.

Determination of Antibacterial Activity

Antibacterial activities of the compounds (1-3) were determined against three strains of bacteria (2 Gram-negative and 1 Gram-positive) and their minimum inhibitory concentrations (MICs) were assayed through two-fold microdilution method using sterile 96-well microtitre plates [13]. Each well of the plates were first preloaded with 50 μl of the broth media followed by an addition of 50 μl of the compounds (1-3) (=0.8 mg/mL) into the first wells of each row tested. The resulting mixture were serially two-fold diluted with tryptone soya broth media (made by dissolving 7.5 g of tryptone soya broth in 250 ml of sterilized distilled water) for each case 50 μl were drawn from the first row wells and transferred into the next and subsequent row wells. The remaining 50 μl from the last row well were discarded. Thereafter, 50 μl of the bacterial suspension (0.5 McFarland standard turbidity) was added in each well. Gentamycin sulfate was used as a positive control, DMSO as a negative control while the rows with tryptone soya broth and bacteria only were used as growth controls. Both plates were incubated at 37°C for 24 h. MIC-values were determined by adding 20 μl of 0.02% p-iodonitrotetrazolium (INT) chloride dye in each well, followed by incubation for 1 h at 37°C. The MIC-values of the compounds (1-3) was read at the concentration where a marked reduction in color formation due to bacterial growth inhibition was noted.

Brine Shrimps Lethality Test (BST)

Cytotoxicities of compounds (1-3) were evaluated by using BST as previously reported [14]. Briefly, a stock solution (5 mg/ml) of the compounds was prepared by dissolving them in DMSO. Different levels of concentrations (240, 120, 80, 40, 24 and 8 μg/ml) were prepared by drawing different volumes from the stock solutions and then added into vials, each containing ten brine shrimps larvae. The volume was adjusted to 5ml with artificial sea water prepared by dissolving 3.8 g of sea salt in 1 L of distilled water. Each level of concentration was tested in duplicate. The negative control contained brine shrimps, artificial sea water and DMSO (0.6%) only. The vials were then incubated under light for 24 h. The dead larvae were counted, and the mean was subjected to analysis using Fig P computer program (Biosoft Inc., USA).

RESULTS

Compound (1α) and (2)

The ^{1}H NMR spectrum of compound 1α and 2 had three aromatic proton singlets at δ_H 9.08 (H-5, *s*), 7.08 (H-7, *s*) and 6.97 (H-1, *s*) whose corresponding ^{13}C NMR signal were δ_C 109.07 (C-5), 99.45 (C-7) and 111.63 (C-1), respectively. The ^{13}C NMR spectrum of these compounds indicated signals due to oxygenated quaternary aromatic carbons at δ_C 153.22 (C-4), 157.75 (C-6), 147.68 (C-2), 144.91 (C-3) and 122.45 (C-8) as in Fig. 1α. Two of these carbons have methoxyl substituents as established by two sharp singlets at δ_C 55.7 (4-OCH_3) and 55.31 (6-OCH3) in the DEPT 135 and DEPT 90 respectively, while the remaining were due to hydroxyl substituent's. By comparing the peaks with those reported in the literature [Tables 1 and 2], the main skeleton of compound 1α was

found to be that of phenanthrene moiety [3,15,16]. Peaks appearing at δ_C 74.49 (CH) and 79.98 (CH_2) were assigned to C-11 and C-13, respectively as in structure 2. The ^{13}C NMR spectrum also showed a peak at 176.85 that are carbon signals for carbonyl (C-12) of structure 2. Since these peaks were seen as minor signals in the ^{13}C NMR, it is likely that compound 1α was reacting with impure molecule (a) to give compound 2. Thus, compound 1α was established as a major constituent whereas the other compound 2 was in minor quantity and its peaks overlapped with those of 1α. The structure of Compound 1α was established as 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene and the impure molecule (a) was identified as methyl acetate. Compound 2 was derived from 2,3,8-trihydroxy-4,6-dimethoxyphenanthrene condensation with methyl acetate.

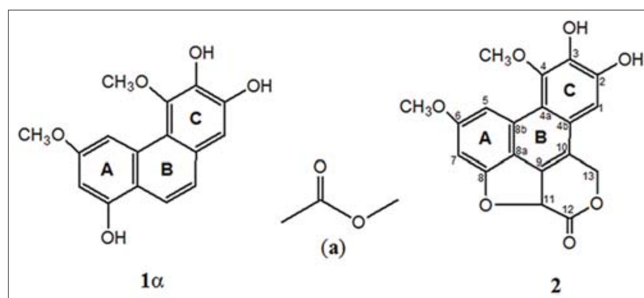


Table 1: ^{13}C NMR data of phenanthrenes (1α)[a] and (2)[a]

Position	Type of ^{13}C NMR	δ_C Observed (1α)	Type of ^{13}C NMR	δ_C Observed (2)	δ_C Reported [3,15]
1	CH	111.58	CH	111.58	110.42
2	C	147.68	C	147.68	147.63
3	C	144.91	C	144.91	144.96
4	C	153.22	C	153.22	153.23
4a	C	117.22	C	117.22	118.42
4b	C	110.95	C	110.95	
5	CH	109.03	CH	109.03	108.88
6	C	157.75	C	157.75	154.11
7	CH	99.41	CH	99.41	99.45
8	CH	122.45	CH	122.45	121.54
8a	C	133.74	C	133.74	134.05
8b	C	124.04	C	124.04	124.03
9	C	114.04	C	114.04	117.1
10	CH	126.22	CH	126.22	126.23
11			CH	74.49	
12	C		C	208.29	
13			CH_2	74.98	
4-OCH_3	CH_3	55.70	CH_3	55.70	60.12
6-OCH_3	CH_3	55.31	CH_3	55.31	55.77

[a]NMR data were measured on 400 MHz. The assignments were based on ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Table 2: ^1H NMR chemical shifts (in ppm) of phenanthrenes (1α)[a] and (2)[a]

Position	Multiplicity	δ_H Observed (1α)	δ_H Observed (2)	δ_H Reported [3,15,16]
1	*s*, 1H	6.96	6.96	6.96
5	*s*, 1H	9.07	9.07	9.07
7	*s*, 1H	7.07	7.07	6.74
10	*s*, 1H		2.11	
11	*s*, 1H		4.14	
13	*s*, 2H		3.95	
6-OCH_3	*s*, 3H	3.99	3.99	3.99
4-OCH_3	*s*, 3H	3.97	3.97	3.97

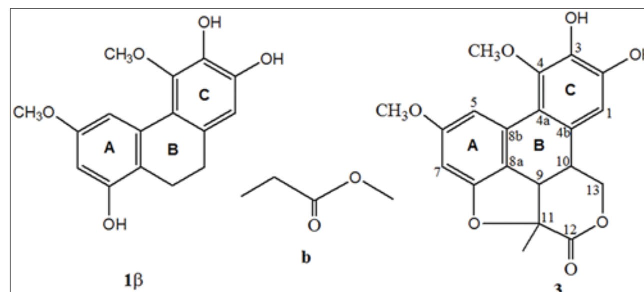
[a]NMR data were measured on 400 MHz. The assignments were based on ^1H NMR, ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Compound (1β) and (3)

Spectra for this compound exhibited almost similar pattern as it was for compound (1α) except that it indicated many major peaks at high field region of both ^1H and ^{13}C NMR.

The ^1H NMR spectrum of compound 1β exhibited three aromatic proton singlets at δ_H 9.07s (H-5, *s*), 7.07 (H-7, *s*) and 6.96 (H-1, *s*) whose corresponding ^{13}C NMR signal appeared at δ_C 109.03 (C-5), 99.41 (C-7) and 111.58 (C-1). Further characteristic peaks in ^{13}C NMR appeared at δ_C 29.55 and 32.03 corresponding to C-9 and C-10 as it is for the dihydrophenanthrene [3]. In DEPT-135 experiment signals resonating at 29.55 and 32.03 were CH signals instead of CH_2 as commonly reported for dihydrophenanthrenes, a factor which provides evidence for presence of substituent in C-9 and C-10 as it is in structure 3. The ^{13}C NMR and DEPT 135 showed peaks at δ_C 208.29 (C-12), 68.45 (C-11) and 55.79 (C-13, 2H) which are carbon signals for carbonyl and oxygenated aliphatic carbons. These signals were assigned to C-12, C-11 and C-13 in the molecule as it is in structure 3. These peaks had low intensity indicating that, an impurity (b) was thought to have been involved in the reaction with the major compound 1β to form compound 3. The ^{13}C NMR and ^1H NMR signals for the mixture of major compound 1β and minor compound 3 overlapped as indicated in Tables 3 and 4. The structure of Compound 1β was established as 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene and the impure molecule (b) was identified as methyl propionate. Compound 3 was derived from 2,3,8-trihydroxy-4,6-dimethoxy-9,10-dihydrophenanthrene condensation with methyl propionate.

Since these compounds were in mixed form, altogether were subjected to an uniform bioscreening study. Cytotoxicities of



compounds (1-3) were done by using the brine shrimps lethality assay and results indicated that the compounds (1-3) had LC_{50} (95% confidence interval [CI]) of 12.11 (7.32-20.05) μg/ml. These compounds (1-3) were also tested for antimicrobial activities against three bacterial strains namely *P. aeruginosa*, *E. coli* and *S. aureus*, and they were active only for *P. aeruginosa* with MIC-value of 0.16 mg/ml.

The standard anticancer drug, cyclophosphamide used had LC_{50} (95% CI) value of 16.37 (12.01-22.31) μg/ml.

DISCUSSION

The isolated compounds (1-3) were evaluated for their cytotoxic and antimicrobial activities. The compounds (1-3) exhibited potent cytotoxic activities against BST with LC_{50} value of 12.11 μg/ml and mild antimicrobial activities against *P. aeruginosa* with MIC-value of 0.16 mg/ml. The LC_{50} value of the standard anticancer drug, cyclophosphamide was found to be 16.37 μg/ml. Potent cytotoxic phenanthrenes both *in vitro* and *in vivo* have also been reported by other researchers [19]. Stemophenanthrene isolated from *Stemona tuberosa* were found to possess moderate cytotoxic activities against four cancer cell lines [23]. Numerous studies have also reported on the significant cytotoxic properties of phenanthrenes isolated from different species against various cell lines [1]. In contrast, the current study used a rapid, reliable, inexpensive and convenient in-house brine shrimp toxicity assay. Nevertheless, it has been demonstrated that BST correlates well with cell lines studies and other biological properties [14,20]. Plants that are rich in phenanthrenes such as *Cremastra appendiculata* have a long history of use in Chinese traditional medicine for treatment of various cancers [21]. The current study also showed mild antimicrobial activities of the isolated phenanthrenes against *P. aeruginosa* with MIC-value of 0.16 mg/ml. Previous antimicrobial studies from ethanolic root extract of *C. adenogonium* showed MIC-value of 1.25 mg/ml against *P. aeruginosa* [22]. These findings indicate that antimicrobial activities of the roots might be contributed largely by the presence of phenanthrenes compound. Previous findings have reported antimicrobial activities from phenanthrenes isolated especially from orchidaceae and combretaceae family although most of them were found to possess weak to moderate antimicrobial activities against the test strains [1]. *C. adenogonium* is used by African traditional healers for treatment of leprosy, cough, syphilis, snakebite, aphrodisiac, new and chronic wounds, malaria, septic wounds and fungal infection of the scalp [22]. Thus, the antimicrobial and cytotoxic properties of phenanthrenes from *C. adenogonium* that have been reported further lends support to its application by African traditional healers for treatment of such ailments.

On the other hand, following overlapping multiplicities from the NMR experiments, literature analysis was done to determine the possible impurities and their integral peaks. Similarities were observed for the most common laboratory solvents which are CH_3COOH, CH_3COCH_3, $CH_3COOCH_2CH_3$ and D_2O. As shown in Table 2, the ^{1}H NMR spectrum of compound 2 exhibited three proton singlets at δ_H 2.11 (H-10, *s*) and at δ_H 4.14 (H-11, *s*) which correspond to signals at δ_H 2.10 (CH_3COO^-, *s*) of the acetic acid as solvent impurity and at δ_H 4.14 ($RCOOCH_2R$, *q*) of the ethyl

Table 3: ^{13}C NMR data of phenanthrene (1β)[a] and (3)[a]

Position	δ_C Observed (1β)	Type of ^{13}C NMR	δ_C Observed (3)	Type of ^{13}C NMR	δ_C Reported [3,15]
1	111.63	CH	111.63	CH	110.42
2	147.73	C	147.73	C	147.00
3	144.95	C	144.95	C	144.96
4	153.26	C	153.26	C	153.23
4a	117.26	C	117.26	C	118.00
4b	111.00	C	111.00	C	140.37
5	109.07	CH	109.07	CH	108.88
6	157.80	C	157.80	C	154.11
7	99.45	CH	99.45	CH	99.45
8	122.49	CH	122.49	CH	121.54
8a	133.78	C	133.78	C	134.05
8b	124.09	C	124.09	C	124.03
9	29.55	C	29.55	C	29.74
10	32.03	CH	32.03	CH	30.34
11			68.45	C	
12			176.85	C	
13			55.79	$-CH_2$	
14			22.02	CH_3	
$4-OCH_3$	55.74	CH_3	55.74	CH_3	60.12
$6-OCH_3$	55.34	CH_3	55.34	CH_3	55.77

[a]NMR data were measured on 400 MHz. The assignments were based on ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Table 4: ^{1}H NMR data peaks of phenanthrene (1β)[a] and (3)[a]

Position	Multiplicity	δ_H Observed (1β)	δ_H Observed (3)	δ_H Reported [3,15,17]
1	s, 1H	6.97	6.97	6.96
5	s, 1H	9.08	9.08	9.07
7	s, 1H	7.08	7.08	6.74
10	s, 1H	2.11	2.11	2.12
13	s, 2H		1.13	
$6-OCH_3$	s, 3H	3.99	3.99	3.99
$4-OCH_3$	s, 3H	3.97	3.97	3.97

[a]NMR data were measured on 400 MHz. The assignments were based on ^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard. DMSO was used as NMR solvent. TMS: Tetramethyl silane, DMSO: Dimethyl sulfoxide

Table 5: ^{1}H NMR chemical shifts of common laboratory solvents [18][a]

Residual solvents	Proton	Multiplicity	$CDCl_3$	D_2O
Acetic acid	CH_3	*s*	2.10	2.08
Acetone	CH_3	*s*	2.17	2.22
Ethyl acetate	CH_3CO	*s*	2.05	2.07
	CH_2CH_3	*q*, 7	4.12	4.14
	CH_2CH_3	*t*, 7	1.26	1.24

[a]NMR data were measured on 400 MHz. The assignments were based on ^{1}H NMR, ^{13}C NMR, DEPT 135 and DEPT 90 experiments. TMS was used as internal standard

acetate as solvent impurity [Table 5]. The proton singlets at δ_H 2.11 (H-10, *s*) which corresponds to signals at δ_H 2.10 (CH_3COO^-, *s*) of acetic acid was also observed in compound 3 [Table 4]. In ^{13}C NMR spectrum, signal appeared at δ_C 208.29 (C-12) in compound 2 [Table 1] corresponds to δ_C 207.07 (R-CO-R) of the acetone as a solvent impurity. The ^{13}C NMR signals at δ_C 176.85 (C-12) and at δ_C 22.02 (C-14) of compound 3 corresponds to δ_C 175.99 (RCOR) of the acetone as solvent impurity and δ_C 21.03 (CH_3COO^-) of the acetic acid as solvent impurity [Table 6]. These substitution patterns observed in the molecules may have occurred during chromatographic or NMR analysis.

CONCLUSION

The preliminary cytotoxic activities exhibited by the substituted phenanthrenes add for a novel structure that can be synthesized, further screened for *in vitro* and *in vivo* models and clinical trials in order to evaluate its potential as new anticancer agent.

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