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Ethnomedical, phytochemical and biological investigations of *Margaritaria discoidea* (Baill.) Webster, a plant species widely used in Guinean traditional medicine

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Abstract: From an ethnomedical survey conducted in Conakry and Dubreka (Guinea), 12 traditional healers and 10 herbalists were interviewed. Their knowledge and experience along with the traditional uses of *Margaritaria discoidea* (euphorbiaceae) were recorded. The fractionation and purification of the leaf extract led to the isolation of a series of securinane-type alkaloids including the known ent-Phyllanthidine, 14,15-dihydroallosecurinine-15-β-ol, securinine, securinol, and viroallosecurinine. Their structures were elucidated on the basis of ^{1}H and ^{13}C-NMR data and comparison with published spectra. The biological activities of the methanol and chloroform leaf extracts along with the alkaloids Y were evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, *Mycobacterium chelonae*, the protozoa *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei brucei*,and *Trypanosoma cruzi* and/or HIV1 and 2. Although weak to moderate, these biological findings support partly the wide traditional use of *Margaritaria discoidea.*

Keywords: Ethnomedicine, Securinane-Type Alkaloids, Antimicrobial, Antiprotozoal

1. Introduction

Nowadays, the Guinean traditional medicine remains very popular. Traditional remedies are widely available in both rural and urban areas. Most of these remedies are plant species. Although the Guinean flora is reputed to be the richest one over West Africa [1], the intensive and anarchic exploitation of this vegetal resource could led to the extinction of some plant species. Consequently, it's urgent to make an inventory of the most exploited plant species in order to rationalize their use. Because of the diverse composition of the population, Guinea has a multicultural society with very often specific knowledge of medicinal plants. *M. discoidea* (euphorbiaceae) is well-known in the Guinean traditional medicine for the treatment of various illness including diabetes, helminthiases, wounds, diarrhea, malaria, gastric disorders, erectile dysfunctionement etc. [2-5]. Commercial exploitation of *M.discoidea* for medicinal purpose is very common in the capital Conakry and the prefecture of Dubreka. Aiming to give a rational support to the traditional uses of *M. discoidea*, an ethno-medical survey along with phytochemical and biological investigations was undertaken.

2. Material and Method

2.1. Ethnomedical Survey

The survey was carried out from october 2009 to april 2010 and targeted traditional healers and herbalists. The questionnaire and oral interviews were based on the standardized model which was designed by the "Centre de Recherche et de Valorisation des Plantes Médicinales (CRVPM) – Dubréka". The main questions focused on demographic data (age and gender), educational level, traditional medical knowledge on *M. discoidea*.

2.2. Site of the Study

The study was carried in Conakry the capital and Dubreka, a prefecture 50 km distant to Conakry. These two cities are located in Lower-Guinea which is one of the most densely populated regions of Guinea. The typical vegetation of this coastal area is characterized by the presence of dense mangrove forests and many woody climbers and bushes. The traditional medicine and remedies are well developed and are exerted by numerous traditional practioners and herbalists.

2.3. Plant Material

Preparation of crude extracts

Plant extracts were prepared by macerating 20 g of powdered dried plant material with 100 mL solvent of chloroform or methanol for 24h. The extracts were then filtered and each filtrate was evaporated *in vacuo* to dryness. 5 mg were weighed and submitted for biological testing.

2.4. Experimental

General experimental procedures

Thin Layer chromatography (TLC)

The analytical and preparative TLC were performed on pre-coated silica gel $60F_{254}$ plates (Merck; 0,25 and 1mm layer thickness, respectively). The mobile phase was chosen according to polarity of fractions. Visualization was accomplished with the UV lamp (254 and 366 nm), and spraying with Dragendorff reagent for alkaloids.

Column chromatography (CC)

The column chromatography was made over silica gel 60–200 mesh (Merck) with a mixture of 2 solvents as eluant in gradient polarity.

Spectroscopic method

NMR spectra (^{1}H and ^{13}C-NMR, DEPT-135 and -90) were recorded at 30°C on a Bruker DRX-400 instrument (Rheinstetten, Germany) operating at 400MHz for ^{1}HNMR and 100MHz for ^{13}CNMR, using standard software packages. Chemical shifts (δ) are reported in ppm units downfield from tetramethylsilane (TMS), using TMS or the solvent signal as the internal standard.

Extraction and isolation

Dried and powdered *P. discoidea* leaf (200 g) was wetted with 100 mL of Ammonia for 1hour, then, percolated with 500 ml of dichloromethane for 24h. The extractive solvent was filtered and evaporated under a vacuum. The residue was dissolved in H_2O/HCl (pH 2-3) and filtered. The filtrate was adjusted to pH 8 with ammonia and treated several times with dichloromethane (6×150 ml). The dichloromethane mixture was then evaporated and concentrated to dryness under reduced pressure to obtain crude alkaloids (428 mg). A portion of the crude alkaloids (408 mg; PdA) was subjected to a column chromatography (CC) eluted with $CHCl_3$/CH_3OH (gradient of polarity). Based on their TLC profile (mobile phase: Toluene/Chloroform, 1:1) similar fractions were combined to give sub-fractions PDA1 to PdA8 which all were positive to Dragendorff.

The fraction PdA1 (62.3 mg) was purified using repetitive column chromatography with hexane/ Chloroform (gradient polarity) to yield PdA1-1 to PdA1.4. The sub-fraction PdA1-1 (32 mg) was subjected to TLC preparative with Toluene/Chloroform (70:30) as mobile phase to give compounds 1 (8.2mg), 2 (6.1 mg) and 3 (7.4 mg).

The fractions PdA2 (27 mg) was purified by repetitive CC with Chloroform /Ethyl acetate (gradient of polarity) to yield three sub-fractions PdA2-1 to PdA2-3. The sub-fraction PdA2-1 (16mg) was subjected to TLC preparative with Toluene/Chloroform (40:60) as mobile phase to give two compounds 4 (6.3 mg) and 5 (7.4 mg).

- Ent-Phyllanthidine (1): amorphous powder. R_f = 0.90; $CHCl_3$:toluene (1:1); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 6.83 (d, J=9.3 Hz, 1H, H-15), 6.27 (dd, J=9.3; 6 Hz, 1H, H-16), 5.81 (s, 1H, H-13), 4.69 (d ,J=6 Hz; t; 1H, H-8), 3.15-3.17 (m, 1H, H-2), 2.75 (m, 1H), 2.57- 2.46 (m, 2H), 1.99 (m, 2H), 1.77–0.91 (m, $5CH_2$)
- 4,15-dihydro-allosecurinin-15-β-ol (2):. amorphous powder. R_f = 0.80; $CHCl_3$: toluene (1:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.62 (s, 1H, H-12), 2.74(m, 1H, H-2), 0.80 – 3.00 (m, CH, CH_2).
- Securinine (3): amorphous powder. R_f = 0.71; $CHCl_3$:toluene (1:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.43 (dd, J=8.9, 6.5 Hz, 1H, H-15), 6.61 (d, J=8.9 Hz, 1H, H-14), 5.56 (s, 1H, H-12), 3.83 (m, 1H, H-7), 2.4-2.96 (m, 2H, H-6), 1.77-2.50 (m, 2H, H-8), 2.10 (m, 2H, H-2), 1.24-1.88 (m, 2H, H-4), 1.48–1.67 (m, 2H, H-5 and 2H, H-3).
- Securinol (4): amorphous powder. R_f = 0.76; $CHCl_3$:toluene (1:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.68 (s, 1H, H-13), 4.36 (m, 1H, H-15), 3.00 (m, 1H), 0.87-3.00 (CH, CH_2)
- Viroallosecurinine (5): amorphous powder. R_f = 0.33; $CHCl_3$:toluene (1:1); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 6.62(d, J=9Hz, 1H, H-14), 6.79 (dd, J=9; 5 Hz, 1H, H-15), 5.69 (s, 1H, H-12), 3.86 (m, 1H, H-7), 3.63 (m, 1H, H-2), 2.73-1.06 (m, $5CH_2$).

2.5. Biological Testing

Antiprotozoal activity

For all protozoan strains studied, the selectivity index (SI) of each *M. discoidea* extract was calculated from the ratio of the IC_{50} value determined in normal lung tissue (MRC-5) cells over the IC_{50} value determined in each protozoa assayed.

Antiplasmodial assay

Extracts of *M. discoidea* were tested against the chloroquine-sensitive Ghanaian strain of *Plasmodium falciparum*. The parasite was maintained in continuous log phase growth in RPMI-1640 medium supplemented with 2% P/S solution, 0.37 mM hypoxanthine, 25 mM HEPES, 25 mM NaHCO3 and 10% O+ human serum together with 4% human O+ erythrocytes according to the method of [6]. All cultures and assays were conducted at 37◦C under microaerophilic atmosphere (4% CO_2 3% O_2 and 93% N_2). The *in vitro* antimalarial activity was assessed using an adaptation of the procedure described by Mackler et al. [7],

Results were expressed as the percent reduction in Plasmodium falciparum present in the extract treated wells compared with the untreated controls. The IC_{50} was calculated from the extract dose versus parasite growth curves [8]. Treatment of *Plasmodium falciparum* cultures with chloroquine was used as a positive control.

Antitrypanosomal and Antileishmanial Activity

All extracts were tested against *Trypanosoma brucei brucei*, *Trypanosoma cruzi* and *Leishmania infantum* blood stream forms from axenic cultures in HMI-18 medium obtained from Prof. L. Maes of the Laboratory of Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical Sciences, Biomedical and Veterinary Sciences of the University of Antwerp, Belgium. Assays were performed in 96 well tissue plates, each containing 10 µl aqueous extract dilutions ranging from 100 to 0.01 µg/ml together with 190 µl of the parasite suspension (5×10^4 parasites/ml) in Hirumi (HMI) medium supplemented with 10% foetal calf serum and a solution of 5000 units penicillin/ml and 5000 µg streptomycin/ml, final concentration 2% in medium (2% P/S solution). All plates were incubated for 4 days in humidified atmosphere at 37°C in 5% CO_2. Two hours before the end of the incubation, 10 µl of Alamar Blue® solution were added. Fluorescence was measured after 4 hours of incubation with the Alamar Blue® in a fluorescence plate reader at 530 nm excitation and 590 nm emission wavelengths. The IC_{50} values were calculated by linear interpolation selecting values above and below the 50% mark. Positive controls included chloroquine for *Plasmodium falciparum* (IC_{50} of 0.047 µM), miltefosine for *Leishmania infantum* (IC_{50}: 6.1 µM), suramin for *Trypanosoma brucei brucei* (IC_{50} 0.035µM), benznidazol for *Trypanosoma cruzi* (IC_{50} 2.0 µM) [8].

Antibacterial and antifungal evaluation

Antibacterial and antifungal testing used a liquid dilution method previously described by Vanden Berghe and Vlietinck [9]. Tested microorganisms included: *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231, *Mycobacterium chelonae*. The standards included: Flucytosine for C. albicans (0,34 µg/ml) , Doxycycline for *S. aureus* and *E. coli* (0.83 and 0.82 µg/ml, respectively), Rifampicine for *M. chelonae* (0.1 µg/ml).

Anti-HIV evaluation

The antiviral screening against HIV-1 (strain IIIB) and HIV-2 (strain ROD) of the plant extract and compounds was carried out as reported by Pauwels et al. [10] and Pannecouque *et al*.[11]. Results are expressed as IC_{50} (µg/mL) and CC_{50} (µg/mL) for cytotoxicity on the MT-4 cells as mean±SD. Azidothymidine (AZT) (purity >99%) was used as a positive control.

Cytotoxicity Assay

Cytotoxicity was evaluated on MRC-5SV2 cells (human fetal lung fibroblasts). Cell lines MRC-5 (human lung fibroblast) were cultured in MEM medium, supplemented with 20 mM L-glutamine, 16.5 mM $NaHCO_3$, 5% foetal calf serum and 2% P/S solution. All cultures were kept at 37°C and 5% CO_2. Niclosamide was used as standard for cytotoxicity on MRC-5 cells (IC_{50} 2.66±0.44-µM).

Assays were performed in sterile 96-well tissue culture plates, each well containing 10 µl of each sample dilutions together with 190 µl of cell suspension (2.5×10^4 cells/ml). After 7 days incubation, cell proliferation/viability was assessed after addition of MTT (Sigma) (50 µl of a 1/2.5 solution per well). After 4 hours of incubation at 37°C, the % absorbance reduction at 540 nm for the treated cultures and untreated control cultures were obtained and compared, and CC50 values (50% cytotoxic concentration) were determined [8].

3. Results and Discussion

3.1. Ethnomedical Data

A total of 22 participants (13 male and 9 female) were interviewed. Of these, 55% (12/22) were traditional healers (9 male and 3 female) and 45% (10/22) were herbalists (4 male and 6 female). The age of the respondents were ranged from 25 to 50 years old with a mean of 41± 6 years for male and 35 ± 9 years for female. 41% (8/22) of the interviewees were under 35 years old, indicating a relative resurgence of interest of the young people. The majority of the traditional healers assumed to benefit their knowledge and experience from a familial inheritance 83% (10/12). The traditional use of the plant species as medicinal purpose varied from 5 to more than 20 years. None of these were legally registered to the Health and Public Hygiene Ministry.

P. discoidea is called in the vernacular languages as Keeri in Pular, Kheeri or Mete in Susu, Sorokognense keri in Mandingo. Different parts of *P. discoidea* are used as medicine by the respondents. Among these, the leaves are most frequently used (68%) followed by stem-bark (15%) and root-bark (17%). The most common methods of preparation included boiling or soaking in hot or fresh water while the preferred route of administration was oral. These methods are typical in the Guinean traditional medicine [2, 4].

The different diseases treated with the *P. discoidea* were fever for 9 traditional healers and 4 herbalists, malaria for 3 traditional healers and 4 herbalists, wound in mouth for 4 traditional healers and 1 herbalist, VIH for 2 traditional healers, boils for 2 herbalists, wounds for 1 traditional healer and 1 herbalist, and diabetes for 2 traditional healers.

In Africa, *M. discoidea* is a well-known medicinal plant

used for the treatment of various diseases such as blennorrhoea (Ivory Coast), toothache (Cameroun), post-partum pains (Central African Republic), stomach and kidney complaints, parturition facilitation (Congo) [12], onchocerciasis (North West Cameroon) [13], wound healing and skin infections (Ghana) [14] etc. On the other hand, the dried leaves can be used as a food supplement for sheep [15].

3.2. Phytochemical Data

The gross structure of compounds **1-5** were deduced from extensive analyses of the ^{1}H, ^{13}C-NMR and DEPT experiments, indicating the presence of ester carbonyl, methines, oxyquaternary, oxymethines, quaternary and methylenes. The overall similarity of the ^{13}C-NMR spectra of all five compounds with those of known securinane-type alkaloids is strong evidence of their identifications.

Compound 1

The ^{1}HNMR exhibited a doublet at δ_H 6.83 (H-15, 1H) and a doublet of doublets at δ_H 6.27 (H-16, 1H) which was indicative for a double bond, a singlet at δ_H 5.81 (H-13, 1H). The multiplet at δ_H 4.67 – 4.70 was attributed to the oxymethine H-8. The multiplet at δ_H 3.15 – 3.17 was in accordance with a methine nearby an Nitrogen. The remaining proton signals were assigned to the methylene protons of the compound. As shown in Table 1, the ^{13}C-NMR of 1 supported the ^{1}H-NMR assignments and was quite superposable to that of ent-Phyllanthidine [16].

Compound 2

As in cpd1, the singlet signal at δ_H 5.62 (H-12, 1H) was characteristic of the securinane-type alkaloids. However 2 differed from 1 by the lack of a double bond. Due to the ^{13}C-NMR similarity of the spectrum of 2 with previous reported data [17], the compound was identified as 14,15-dihydro-allosecurinin-15-β-ol.

Table 1. *^{13}C NMR data of compounds 1-5 and known securinane-type alkaloids*

N°	Ent-Phyllantidine [16]	1	14,15-Dihydro-AlloSecurinine-15-β-ol [17]	2	Securinine [18]	3	Securinol [18-21]	4	Allo-Securinine [22]	5	ViroAllo Securinine [16, 18]
2	71.2	71.1	65.5	65.5	62.9	62.8	62.8	63.0	60.5	60.7	60.8
3	23.9	23.8	26.1	26.0	27.3	27.3	24.2	23.9	20.8	21.0	21.1
4	23.2	23.2	24.7	24.7	24.5	24.5	22.4	22.8	21.9	22.1	22.2
5	25.2	25.1	25.2	25.2	27.2	27.2	25.6	25.6	18.3	18.5	18.5
6	56.0	55.9	53.0	53.0	48.7	48.8	52.5	52.6	43.4	43.6	43.6
7	-	-	59.9	59.9	58.7	58.7	59.2	59.1	58.5	58.7	58.8
8	70.9	70.8	35.8	35.8	42.2	42.3	69.8	69.9	42.4	42.6	42.7
9	40.6	40.5	83.9	83.8	89.4	89.4	41.1	41.3	91.0	91.6	91.7
10	82.9	82.8	-	-	-	-	84.5	84.7	-	-	-
11	-	-	174.6	174.5	173.5	173.4	-		172.4	172.5	172.7
12	172.1	171.9	109.5	109.5	104.9	104.9	173.6	173.7	110.6	108.9	108.9
13	113.2	113.1	173.7	173.6	170.0	170.1	112.7	112.4	167.3	167.4	167.6
14	164.3	164.2	26.9	26.9	121.3	121.4	171.8	172.2	122.4	122.6	122.7
15	126.4	126.3	68.0	67.9	140.2	140.3	30.2	30.5	148.4	148.6	148.7
16	134.4	134.3	-	-	-	-	-	-	-	-	-

Compounds 3-5

The ^{1}H-NMR data of compounds 3-5 are summarized in the experimental part. These are in adequation with their ^{13}CNMR data which were compared with known securinane-type alkaloids [16, 18-21] (Table 1). From these comparisons, compounds 3-5 were identified as Securinine, Securinol and Viroallosecurinine (C-9 at δ91.7 instead of δ91.0 for allosecurinine [22]), respectively.

The Securinega alkaloids are a class of natural products isolated from plants such as

Securinega suffruticosa, S. durissima, S. fluggeoides, S. virosa (Euphorbiaceae) and the bark of *Securidaca longepedunculata* (Polygalaceae) [23] , *Phyllanthus amarus, P. niruri* (Phyllanthaceae) [24].

3.3. Biological Data

Based on the above traditional uses of *M. discoidea*, the *in vitro* antimicrobial, anti-VIH and antiprotozoal activities of the polar and apolar extracts of the plant were performed.

Antimicrobial

The plant extracts were devoid of any activity (IC_{50}> 64 µg/ml) against *Staphylococcus aureus, Escherichia coli, Bacillus cereus* and the yeast *Candida albicans*. Only the methanol extract inhibited *Mycobacterium chelonae* with an IC_{50} of 36.56 µg/ml. Previous works on the antimicrobial activity of the alkaloids indicated a minimal inhibition concentration (MIC) of 0.500 mg/ml against *S. aureus, E.coli* and *Mycobacterium smegmatis* for securinine, 0.48 µg/ml against *Pseudomonas aeruginosa* and *S. aureus* for Viroallosecurinine [25].

Anti-HIV

As shown in Table 2, the antiviral activity of all the tested extracts and the alkaloids Ent-phyllanthidine 1 and viroallosecurinine 5 were not significant. However, only the methanol extract exhibited a weak antiviral effect against HIV-1 III_B strain with a mean of IC_{50} of 86,05 ± 16.61µg/ml and a CC_{50} > 125 µg/ml. Except the methanol extract, the selective index of the chloroform extract and the compounds 1 and 5 were less than 1. Although too weak, the HIV- 2 ROD strain was more sensitive to the chloroform extract than HIV-1 III strain whereas the methanol extract was more potent against HIV-1 than HIV-2.

Antiprotozoal activity

Table 2. *Anti-HIV activity of extracts and alkaloids from Margaritaria discoidea*

Sample	Strain	IC_{50} (µg/ml)	CC_{50}(µg/ml)	SI
PdCHCl3	HIV-1 III strain	> 11,4	= 11,4	< 1
		> 11,9	= 11,9	< 1
	HIV-2 ROD strain	> 15,4	= 15,4	< 1
		> 13,9	= 13,9	< 1
PdMeOH	HIV-1 III strain	= 97,8	> 125	> 1
		= 74,3	> 125	> 2
	HIV-2 ROD strain	> 125	> 125	X 1
		> 125	> 125	X 1
Ent-Phyllanthidine (**1**)	HIV-1 III strain	> 53,6	= 53,6	< 1
		>74,8	= 74,8	< 1
	HIV-2 ROD strain	> 54,7	= 54,7	< 1
		> 60,6	= 60,6	< 1
Viroallosecurinine (**5**)	HIV-1 III strain	> 66,1	= 60,6	< 1
		> 54,7	= 54,7	< 1
	HIV-2 ROD strain	> 59,6	= 59,6	< 1
		> 62,2	= 62,2	< 1

All the tested extracts were not cytotoxic against MRC-5 cells (IC_{50}>64µg/ml) and were also inactive against *Plasmodium falciparum* and *Leishmania infantum*. Both the methanol and chloroformic extracts inhibited the growth of *Trypanosoma brucei brucei* at concentrations of IC_{50} of 23.02 and 29.46 µg/ml (SI >: 1.94, 31.84), respectively. Only the chloroform extract displayed an inhibition of *T. cruzi* with an IC_{50} of 41.02 µg/ml and SI >1.95. These results are in agreement with those previously reported by Traoré *et al.* [26]. On the other hand, previous pharmacological investigations depicted the potential source of new microfilaricidal (*Onchocerca ochengi*, a model parasite for O. volvulus) lead compounds of the non-polar extract of M. discoidea [27]. Miscellaneous activities include the anti-inflammatory activity and the suppression of allergy in mice [28], the cytotoxic effect against ovarian cancer cells of the stem bark extracts [29].

With regards to the pharmacological activities of the alkaloids, securinine was the most studied. Securinine has been reported to exhibit antimalarial, and antibacterial activities as well as apoptotic activity in human leukemia HL-60 cells [21]. It induces apoptosis in the human promyelocytic leukemia cell line HL-60 indicating its potential as an efficient natural antitumor drug with low toxicity [30]. The anticancer properties of securinine against colon cancer SW 480 cell and myeloid leukaemia cell lines have been also reported [27]. Securinine was indicated to stimulate CNS as a substitute for strychnine and was used for this purpose until the late 1990s. Moreover, due to its neuroprotective activity against neurotoxicity induced by β-amyloid protein (one of the pathological brands of Alzheimer's disease), Securinine has a great clinical potential not only in preventing erosion of neurons, but also in compensating neuron damages. This is of interest since the neurodegenerative diseases will become one of the greatest medical challenges [31].

On the other hand, Securinine inhibited spore germination of some plant pathogenic and saprophytic fungi such as Alternaria spp, Curvularia spp, Colletotrichum spp, Helminthosporium spp, Heterosporium sp, Erysiphe pisi [32, 33].

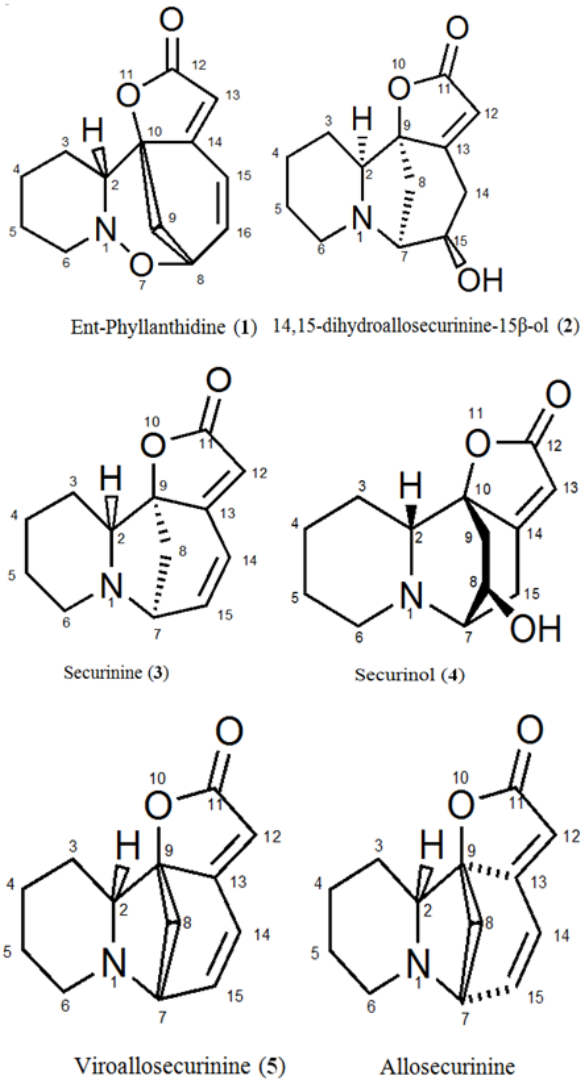

Figure 1. *Structures of Compounds 1 – 5*

4. Conclusion

Margaritaria discoidea is widely used within the traditional practioners and herbalists of Conakry and Dubreka, two cities of the Lower Guinea. A series of securinane-type alkaloids were isolated and identified. The leaf extracts exhibited moderate antimicrobial and antiprotozoal activities while the tested alkaloids were devoid of any anti-HIV activity. These preliminary results support even partly some traditional uses of *M. discoidea*. The presence of securinane-type alkaloids in particular securinine along with the moderate antimicrobial and antiprotozoal activities of the extracts provided a basis of further research and development of *M. discoidea* at least for the treatment of microbial and protozoa infections.

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