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Antiviral and antiparasitic activities of clovamide: the major constituent of *Dichrostachys cinerea* (L.) Wight et Arn

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ABSTRACT

The application of different chromatographic and spectroscopic techniques to the aqueous alcoholic leaves extract of *Dichrostachys cinerea* (L.) Wight et Arn. (Mimosaceae) led to the isolation and identification of ten phenolic compounds. Among them, apigenin-7-*O*-apiosyl (1→2) glucoside, chrysoeriol-7-*O*-apiosyl (1→2) glucoside and clovamide were isolated for the first time from the plant. The rest of the compounds are: flavonol glycosides (quercetin-3-*O*-rhamnopyranoside, quercetin-3-*O*-glucopyranoside, myricetin-3-*O*-rhamnopyranoside and myricetin-3-*O*-glucopyranoside), and three aglycones (myricetin, apigenin and kaempferol). The crude extract of *D. cinerea* showed a significant antitrypanosomal and antiviral effects. Clovamide as a major constituent was investigated for its antiviral and antitrypanosomal activities. It showed a significant antiviral effect against H5N1 influenza A virus with inhibition rate (74%) and a momentous trypanocidal effect against *Trypanosoma evansi* with IC_{50} value of 3.27 µg/ml, compared with the standard drug; diminazene aceturate (IC_{50}=0.72µg/ml). Therefore, clovamide is playing an important role in antitrypanosomal and antiviral activities for *D. cinerea* extract and it can be considered a new candidate for the treatment of these two infections.

INTRODUCTION

Dichrostachys cinerea (L.) Wight et Arn. (Mimosaceae) known as Sicklebush, Bell mimosa, Chinese lantern tree or Kalahari Christmas tree, is a semi-deciduous to deciduous fast growing tree, typically grows up to 7 meters in height. It characterized by strong alternate smooth spines (up to 8 cm long), dark grey-brown fractures on old branches and stems and bark on younger branches (Zeid *et al*., 2009).

D. cinerea is one of the very useful wild medicinal plants used in folk medicine across Africa and Asia (Aworet-Samseny *et al*., 2011). Its bark is used to prepare concoction for treatment of dysentery, headache and elephantiasis. The root infusions are used to treat gonorrhea coughs, syphilis and sore eye and also used as laxative, anthelmintic and strong diuretic. Leaves are good fodder for domesticated animals and seeds are edible (Neondo *et al*., 2012). Pharmacological studies on *D. cinerea* have shown antibacterial, antiviral, antilice, antiplasmodial, and antitrypanosomal effects (Aworet-Samseny *et al*., 2011; Vijayalakshmi *et al*., 2010; Atindehou *et al*., 2004). Phytochemical studies on *D. cinerea* revealed the isolation of various constituents, such as sterols, alkaloids, tannins, triterpenes, polyphenols, phenolic acids, flavonoids (Zeid *et al*., 2009) and cardiotonic heterosides (Tillement *et al*., 1977).

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Trypanosoma evansi, an animal-pathogenic protozoan parasite, infects various big animals including buffaloes, camels, equines, cattle, goats, and sheep causing great economic losses in the infected areas, where thousands of animals die from *T. evansi* infections (Luckins 1988). Diminazene aceturate and suramin are the most commonly used drugs for the treatment of *T. evansi* infection. However, they cause severe side effects and the development of drug resistant trypanosomes has occurred in many regions (Elkhateeb *et al*. 2012).

Influenza, a respiratory disease caused by influenza A viruses, is still one of the major epidemics worldwide that are highly transmissible pathogens for humans and some animals (Droebner *et al*., 2007). H5N1 is one of the highly pathogenic avian IV strains. It is rarely infected humans and poses a severe threat because of its high pathogenicity (Pleschka *et al*. 2009). The development of influenza A viruses resistant to antiviral drugs draw attention to the need for other antiviral drugs. Although several antiviral compounds have been developed against influenza virus, they are of limited efficacy due to toxicity and/or the appearance of drug-resistant virus mutants (Droebner *et al*., 2007).

The crude extract of *D. cinerea* showed a remarkable antitrypanosomal and antiviral effects (Atindehou *et al*., 2004; Aworet-Samseny *et al*., 2011). Therefore, it was of a great importance to check the antitrypanosomal and antiviral activities of its major constituents which my lead to an alternative drug with least side effects and higher efficacy for the treatment of *T. evansi* and H5N1infections.

MATERIAL AND METHODS

General

Mass spectra were measured on a JEOL JMS-AX500 spectrometer, Graduate School of Agriculture, Hokkaido University, Japan. NMR was recorded on a Bruker AM-500 FT-NMR spectrometer**,** Graduate School of Agriculture, Hokkaido University, Japan. UV spectrophotometer (Shimadzu UV-240/ National Research Center/Egypt)

Plant material

Fresh leaves of *D. cinerea* were collected from Wadi Halfa, South Egypt, in April 2013. The plant was authenticated by Prof. Dr. S. Kawashty, Department of Phytochemical and Plant Systematics, NRC. A voucher specimen was deposited in the Herbarium of NRC (CAIRC, Cairo, Egypt).

Extraction and isolation

The shade dried leaves (450 gm) were extracted by percolation for 48 hrs in 70 % methanol. The extract was chromatographed on a polyamide 6S (Riedel-De-Haen AG, SeelzeHaen AG, SeelzeHanver, Germany) column (500 gm) (180 × 5 cm) and eluted with H_2O/MeOH mixtures of decreasing polarities to yield four fractions (I-IV), which were individually subjected to column chromatography on sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden); using 50 % MeOH or n-butanol water saturated as an eluent, and/or preparative paper chromatography; using BAW (*n*-BuOH-HOAc-H_2O 4:1:5, upper layer) or 15 % acetic acid as an eluent. Compounds (**1**); 32 mg and (**2**); 24 mg were isolated and purified from fraction II, compounds (**3**); 76 mg, (**4**); 29 mg, (**5**); 41 mg, (**6**); 38 mg and (**7**); 33 mg were obtained pure from fraction III, finally fraction IV yielded compounds (**8**); 19 mg, (**9**); 17 mg and (**10**); 23 mg. Each isolated compound was purified using Sephadex LH-20 column. The structures were elucidated by chemical (partial and complete acid hydrolysis) and spectroscopic analysis (UV, ^{1}H and ^{13}C NMR, HMQC, HMBC, COSY and MS).

Antiviral activity

Madin-Darby Canine Kidney (MDCK) cells were maintained in the Center of Scientific Excellence for influenza viruses at the National Research Centre/Cairo/Egypt. The cells were propagated till confluence in multi-well plates. The highly pathogenic avian influenza (HPAI) virus A/Chicken/Egypt/M7217B/2013 (H5N1) used in the current study was isolated from the infected chickens in Egypt in 2013 and characterized at immunologic and molecular levels. MTT assay was used to determine the LC_{50} of the extract and clovamide according to the reported method (Mosmann *et al*., 1983). Briefly, the cells were cultured in 96 well-plates and incubated for 24 hrs at 37 ºC in 5% CO_2. Then, they were treated with triplicates of the tested compounds. One day later, the supernatant was discarded and monolayers of cells were washed three times with sterile phosphate buffer saline. MTT solution was added to each well and incubated for 4 hrs at 37 °C. The formed formazan crystals in each well were dissolved in acidified isopropanol (200 µl). Absorbance of formazan solutions was measured at λmax 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined as following: % of cytotoxicity = [(Absorbance of cell without treatment - Absorbance of cell with treatment)/ Absorbance of cell without treatment] x 100. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (TC_{50}). Plaque reduction assay was used to determine the antiviral activity for both of the extract and clovamide at safe concentrations (Hayden *et al.*, 1980).

Antitrypanosomal activity

Trypanosoma evansi (H3 strain, isolated from deer in Thailand) were provided by Dr. Onuma, Graduate School of Veterinary Medicine, Hokkaido University, Japan. Trypomastigote forms of the parasite *T. evansi* were maintained in HMI-9 medium supplemented with 20% heat-inactivated horse serum (Sigma-Aldrich/Germany). *In vitro* antitrypanosomal test was performed to determine the 50% inhibitory concentration (IC_{50}) on parasite growth for clovamide (Bawm *et al.*, 2008). Briefly, tests were performed in a 96-well microtiter plate using clovamide and standard trypanocidal drug, diminazene aceturate (Sigma-

Aldrich/Germany). Both of the standard and the tested compound were dissolved in dimethyl sulfoxide (DMSO). Three-fold serial dilutions were prepared in HMI-9 medium. Trypomastigotes of *T. evansi* were incubated in each well in the presence of three-fold serial dilutions of tested compound. The plates were incubated at 37 ºC in 5% CO2 atmosphere for 72 h and the number of motile parasites was counted using hemocytometer

RESULTS AND DISCUSSION

Identification of the isolated compounds

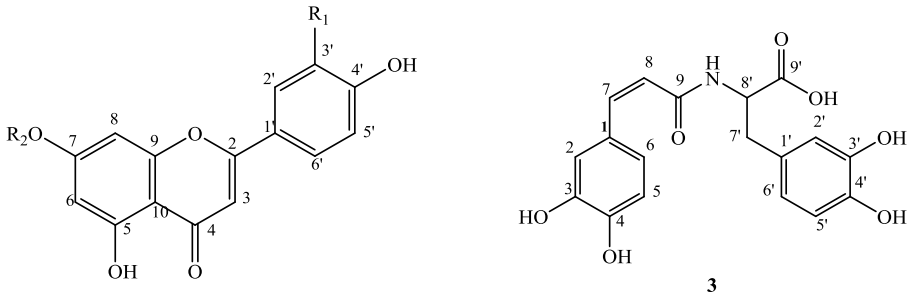
The application of different chemical and spectral techniques led to the isolation of ten compounds (Fig. **1**). They were identified as; apigenin-7-*O*-apiosyl (1 → 2) glucoside (**1**) (Li *et al.*, 1997), chrysoeriol-7-*O*-apiosyl (1 → 2) glucoside (**2**) (Lin *et al.*, 2007) and the major compound clovamide (N-caffeoyl-L-DOPA) (**3**) (Sanbongi *et al.*, 1998), were isolated for the first time from the plant. Quercetin-3-*O*-rhamnopyranoside (**4**) (Zeid *et al*., 2009), myricetin-3-*O*-rhamnopyranoside (**5**) (Kassem *et al.*, 2016), myricetin-3-*O*-glucopyranoside (**6**) (Zeid *et al*., 2009), quercetin-3-*O*-glucopyranoside (**7**) (Zeid *et al*., 2009), myricetin (**8**) (Kassem *et al.*, 2016), apigenin (**9**) (Marzouk *et al*., 2016) and kaempferol (**10**) (Marzouk *et al*., 2016) were previously reported to occur in the plant (Zeid *et al*., 2009).

Compounds (**1**-**3**) were isolated for the first time from the plant. Compound **3**, the major phenolic constituents of *D. cinerea* was elucidated and full characterized by 1D and 2D NMR (H-H COSY, HMQC and HMBC). The ^{1}H NMR spectrum showed two pairs of doublets at δ 6.41 and 7.17 ppm exhibiting a coupling constant of 15.7 Hz assigned to H-7 and H-8, respectively, which indicate two olefinic protons and their stereochemistry in trans.

Four aromatic doublets [δ 6.91 (1H, d, *J*=2.0 Hz), 6.71 (1H, d, *J*=8.0 Hz), 6.62 (1H, d, *J*=1.8 Hz) and 6.57 (1H, d, *J*=8.0 Hz] and two doublets of doublets [δ 6.79 (1H, dd, *J*=8.5, 2.0 Hz) and 6.44 (1H, d, *J*=8.0, 1.8 Hz)] corresponding to six protons, indicating the presence of an axial symmetry of the two aromatic rings.

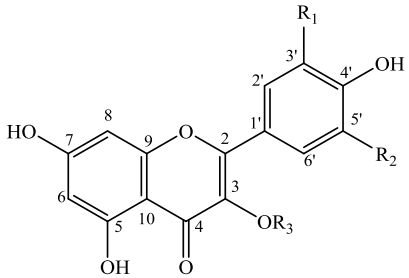
The 1H NMR spectrum also showed two doublets of doublets at δ 2.89 (1H, dd, *J*=18.5, 5.0 Hz) and 2.71 (1H, dd, *J*=22.5, 8.5 Hz), due to the position in α of the asymmetric carbon (C7′), which indicates the presence of one methylene unit. H-8' appeared as multiplet at 4.37 ppm due to the presence of methyne group.

The 1H and 13C-NMR data together with the HMBC corelation of compound 3 (Table 1) are in a good accordance with those previously published data (Sanbongi *et al*., 1998). Therfore, compound 3 was identified as clovamide [N-(3′,4′-dihydroxy-trans-cinnamoyl)-3-(3,4-dihydroxyphenyl)-L-alanine].



3

	R_1	R_2
1:	H	api(1 → 2) glc
2:	OCH_3	api(1 → 2) glc
9:	H	H



	R_1	R_2	R_3
4:	H	OH	Glc
5:	OH	OH	Rha
6:	OH	OH	Glc
7:	H	OH	Rha
8:	OH	OH	H
10:	H	H	H

Figure 1: Chemical Structures of the isolated compounds from *Dichrostachys cinerea*

Table 1: ^{1}H-NMR, ^{13}C-NMR and HMBC data of clovamide in DMSO-d_6.

H/C	^{1}H-NMR (500 MHz, DMSO-d_6, δ, ppm)	^{13}C-NMR (100 MHz, DMSO-d_6 δ, ppm)	HMBC
1	-	126.4	-
2	6.91 (1H, d, *J*=2.0 Hz)	114.3	C-4, C-6, C-7
3	-	144.8	-
4	-	147.4	-
5	6.71 (1H, d, *J*=8.0 Hz)	115.8	C-1, C-3
6	6.79 (1H, dd, *J*=8.5, 2.0 Hz)	120.4	C-2, C-4, C-7
7	6.41 (1H, d, *J*=15.7 Hz)	118.4	C-2, C-6, C-9
8	7.17 (1H, d, *J*=15.7 Hz)	139.5	C-1, C-7, C-9
9		165.2	-
NH	8.1 (1H, d, *J*=8.0 Hz)	-	-
1′		128.7	-
2′	6.62 (1H, d, *J*=1.8 Hz)	116.6	C-4', C-6'
3′	-	145.6	-
4′	-	143.7	-
5′	6.57 (1H, d, *J*=8.0 Hz)	115.3	C-1', C-3'
6′	6.44 (1H, d, *J*=8.0,1.8 Hz)	119.9	C-2', C-4'
7′	2.89 (1H, dd, *J*=18.5, 5.0 Hz, H-7′a) 2.71 (1H, dd, *J*=22.5, 8.5 Hz, H-7′b)	38.5	C-2', C-6', C-9
8′	4.37 (1H, m)	54.4	C-1', C-9
9′	-	173.6	-

Antiviral activity

The cytotoxicity of the extract and clovamide LC_{50} were found to be (30 and 68 µg/ml), respectively. Both showed a remarkable virus inhibition activity against influenza A virus (H5N1) infection up to 73% for the extract and 74% for clovamide at concentration (20 µg/µl). The pure compound clovamide showed higher inhibition rate than the extract at the used concentration.

Antitrypanosomal activity

Clovamide was also tested *in vitro* against *T. evansi* and showed a strong potential for antitrypanosomal activity with (IC_{50}=3.27 µg/ml) which compared well with the standard trypanocidal drug diminazene aceturate (IC_{50}=0.72 µg/ml). Although diminazene aceturate still show a stronger anti-trypanosomal activity against *T. evansi* than clovamide, its fatal side-effects (Peregrine *et al.*, 1993) together with the comparatively low toxicity of clovamide determined by the relatively high LC_{50} (68 µg/µl) nominate the later as a promising safe compound for further *in vivo* investigation.

CONCLUSION

Ten phenolic compounds were identified from *D. cinerea*, among them; compounds **1-3** were isolated for the first time from the studied taxa. To the best of our knowledge, this is the first report for the antiviral and antitrypanosomal activities of clovamide, which showed remarkable activity against H5N1 influenza A virus and a strong trypanocidal activity against *T. evansi* nominating it as a relatively safe promising antitrypanosomal compound for further investigation.

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Conflict of Interests: There are no conflicts of interest.

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