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Anti-Sickling and Antibacterial Activities of Extracts from a Congolese Diplopod (*Tachypodoiulus sp.*, Arthropoda)

Koto-te-Nyiwa Ngbolua[1,*], S. Ngunde-te-Ngunde[1], Dipa J. Tshidibi[1], Moke E. Lengbiye[1], Tshimankinda P. Mpiana[2], Elumba G. Ekutsu[1], José Justin Mbimbi Mayi Munene[1], Zoawe B. Gbolo[1], Ngiala G. Bongo[1,3], Gbiangbada Nzemu[4]

1. Department of Biology, Faculty of Science, University of Kinshasa, P.O. Box 190 Kinshasa XI, Democratic Republic of the Congo.
2. Department of Chemistry, Faculty of Science, University of Kinshasa, P.O. Box 190 Kinshasa XI, Democratic Republic of the Congo.
3. Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro-Tanzania.
4. Institut Supérieur des Techniques Médicales de Yakoma, Nord-Ubangi, République Démocratique du Congo.

*Corresponding author: Koto-te-Nyiwa Ngbolua,
Department of Biology, Faculty of Science,
University of Kinshasa,
Kinshasa XI, Democratic Republic of the Congo.
Tel.: +243 81 68 79 527
E-mail: jpngbolua@unikin.ac.cd

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ABSTRACT

Zoo-pharmacognosy is a mean by which animal self-heal by using of plants and invertebrates. Because of their phylogenetic closeness and common neural pathways of chemosensory perception, humans and great apes, when displaying symptoms of illness learn to select some biological resources as medicine: e.g. diplopods secretions is used by great apes to heal against various skin bacterial infectious diseases which are also recurrent pathologies of the SCD. The present study evaluated the antisickling and antimicrobial activities of organic acids and alkaloids extracted from a Congolese diplopod using Emmel test and micro-dilution technique respectively.The chemical screening performed on the diplopods extract revealed the presence of phenolic compounds, alkaloids, quinones, terpenoids and organic acids. The results of bioassays revealed that organic acids and alkaloids crude extracts possess promising antisickling and antibacterial activities *in vitro* as revealed by both the observed normal biconcave form of sickle erythrocyte (normalization rate >80%) and the bacterial (*S. aureus* ATCC 25923) growth inhibition. The mean values of MIC are 61.5 µg/mL and 125 µg/mL respectively for organic acids and alkaloids extracts. Diplopods can be serving as a new source of antisickling and antibacterial bioactive molecules that could be duplicated by organic synthesis chemistry for formulating drugs to manage SCD. For the best of our knowledge, this is the first time report on the antisickling activity of organic acids and alkaloids extracts of animal origin.

Keywords: Sickle cell disease, Zoo-pharmacognosy, Diplopod, alkaloids, organic acids, Organic synthesis chemistry.

INTRODUCTION

There is a growing evidence that ethno-medicine is an efficient source of new pharmaceuticals [1, 2]. This approach using ethno-botany and folk medicine can provide useful information as a pre-screen to select plant for experimental studies. For thousands of years, traditional medicine has played a key role in the prevention and treatment of various diseases [3]. According to the World Health Organization, in Africa, the lack of access to modern health care as well as their socio-cultural background behaviour makes that over 75% of the population recourse to the traditional medicine for their relief [4]. The advanced research in the field of ethno-pharmacology has led research scientists to the discovery of conventional drugs such as artemisinin, quinine, taxol, morphine and codeine from medicinal plants [5]. However, the ethno-pharmacological approach which is based on field surveys has some limitations in its application particularly the reluctance of traditional healers to disclose their secret and the lack of consensus among healers relating to the use of certain medicinal plants. For this purpose, the alternative strategy uses zoo-pharmacognosy approach for identifying bioactive agents from plants or invertebrates [6]. It is a mean by which animal self-heal. The self-medicative behaviour is well documented in non-human primates' practice. As a matter of fact, the use of plants and invertebrates for therapeutic purposes is well established in great apes. Indeed, because of their phylogenetic closeness and common neural pathways of chemosensory perception, humans and great apes, when displaying symptoms of illness learn to select some biological resources as medicine [7, 8].

In order to discover new effective molecules against human diseases, some researchers have been studying for several years, the self-medication behaviour in non-human primates in their natural environments in order to identify plant taxa and/or animals that they use for their treatment [8, 9]. In the particular case of animal taxa, Simmen *et al.* [10] of National Museum of Natural History of Paris (France) reported that lemurs of Madagascar use diplopods secretions to heal against various skin diseases. This practice is well documented in some species of prosimians and simians, including some great apes. Initial observations have been reported in sapajous of Madagascar by Simmen and Tarnaud (*Cebus apella*, *C. olivaceus*, *C. capucinus*) [11,12], and more sporadically in other Plathyrrhiniens (spider monkeys : *Ateles geoffroyi*) [13] douroucouli in captivity (*Aotus spp*) [14], among anthropoids (Orang outan : *Pongo pygmaeus*) [15] (chimpanzee : *Pan troglodytes*) [9] and in several Lemurs in Madagascar (black lemur: *Eulemur macaco*) [16]; and other lemurs (*Fulvusrufus rubriventer*) [17].

The chemical composition of the diplopods secretions reported in the literature refers to the presence of natural products such as benzoquinone, hydroquinone, quinones, alkaloids and cyanogens compounds that would confer to them their therapeutic properties [18]. The overall hypothesis of this study is that some secondary metabolites contained in these secretions could display antisickling and/or antibacterial activities.

The aim of the present study was to evaluate the antisickling and antibacterial activities of organic acids and alkaloids extracted from the diplopods. Organic acids extracts and alkaloids were previously reported to possess antisickling and antibacterial activities respectively [8, 19]. Indeed, sickle cell disease (SCD) is a genetic and neglected disease for which current proposed therapies are quite expensive and have attendant risk factors and patients are currently exposed to bacterial infections due to the loss of their immune system. In addition to the painful crises and anaemia, infections are recurrent pathologies of the SCD. These include meningitis, septicemia and osteomyelitis. *Salmonella typhi* and *Staphylococcus aureus* are the bacteria most implicated in septicemia and osteomyelitis, while *Escherichia coli* K1 serotype is able of causing very serious neonatal infections that are potentially complicated by meningitis or septicaemia [20, 21]. In this regard, if the research hypothesis is validated, diplopods can be serving as a new source of antisickling and antibacterial bioactive molecules that could be duplicated by organic synthesis for managing SCD.

MATERIALS AND METHODS

Zoo-pharmacognosy survey

Information about the use of diplopods as medication by apes was obtained through online and library search (Google scholar, PubMed).

Samples collection

The tested material (fig.1) used in this study was collected in the "MONASTERE L'ASSOMPTION" forest located in the Mont-Ngafula commune (4°25' S latitude and 14°09' E longitude, fig.2). Its average altitude is 357 m above sea level.

Figure 1: Diplopods (*Tachypodoiulus sp.*, Arthropoda)

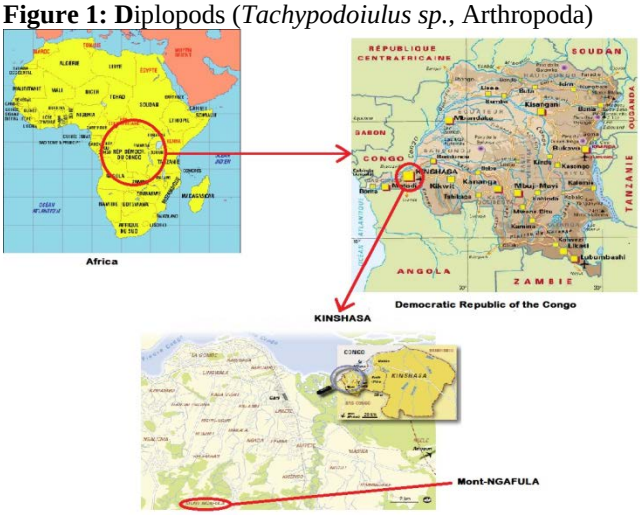

Figure 2: Map of the site of the diplopod sample harvest (Kinshasa, DR Congo)

Diplopods were stored in methanol (MeOH) (preservative solution) for three days, and then dried at room temperature (approximately 27 °C) for three days. Samples were crushed using a mortar, to obtain powder. The powder was then macerated for 72 hours. After filtration, the alcoholic extract was evaporated to dryness under reduced pressure using a rotary evaporator. The crude extract was used for chemical screening and extraction of organic acids and alkaloids.

Extraction and chemical screening

The crude extract (10 g) was repeatedly extracted by cold percolation with 95% ethanol (EtOH) and water (100 mL x 2) for 48 hours. Chemical screening was done in aqueous and organic extract. Extraction of alkaloids and organic acids were done using an established protocol as previously reported [8, 22].

Blood and antisickling assay

Blood samples used to evaluate the antisickling activity of the diplopods extracts in this study were taken from known drepanocitary adolescent patients attending the "Centre de Médecine Mixte et d'Anémie *SS*" and "Centre Hospitalier Monkole", both located in Kinshasa area, D. R. Congo. None of the patients had been transfused recently with Hb AA blood. All antisickling experiments were carried out with freshly collected blood. In order to confirm their *SS* nature, the above-mentioned blood samples were first characterized by

hemoglobin electrophoresis on cellulose acetate gel, as previously reported [8]. They were found to be *SS* blood and were then stored at ± 4 °C in a refrigerator. An informed consent was obtained from all the patients participating in the study. All the research procedures have received the approval of Department of Biology Ethics Committee.

Sickle cell blood was diluted with 150 mM phosphate buffered saline (NaH_2PO_4 30 mM, Na_2HPO_4 120 mM, NaCl 150 mM) and mixed with an equivalent volume of 2% sodium metabisulfite. A drop from the mixture was spotted on a microscope slide in the presence or absence of alkaloids or organic acids extracts and covered with a cover slip. Paraffin was applied to seal the edges of the cover completely to exclude air (Hypoxia). Duplicate analyses were run for each extract. The RBCs were analyzed by measuring various parameters including the area, perimeter and the radius of each RBC using a computer assisted image analysis system (Motic Images 2000, version 1.3; Motic Chine Group Co LTD) and statistical data analysis were processed using Microcal Origin 6.1 package software.

ANTIMICROBIAL ACTIVITY

Microbial strains

The activity of the diplopods extracts was tested toward *Staphylococcus aureus* (*S. aureus* ATCC 25923) and *Escherichia coli* (*E. coli* ATCC 25922). The tested strains were obtained from the American Type Culture Collection (ATCC, Rockville MD, USA).

Minimum inhibitory and minimum bactericidal concentrations

An aliquot (10 µL) of a 10^6 CFU/mL overnight culture was added to wells of a sterile 96-well micro-plate titer. Diplopods extract (DE) was diluted in Tryptone Soya Broth (TSB) (Becton Dickison) containing 0.1% (v/v) Tween 80 and added to wells to give final concentrations ranging from 0.976 to 500 µg/mL. The positive control wells contained TSB + bacteria suspension without DE while negative control wells contained TSB only. Optical density (OD) was measured at 630 nm using a microplate reader (Titertek Twin-reader, Finland) and again after incubation for 24 hours at 37 °C. The minimum inhibitory concentration (MIC) was determined as the lowest DE concentration at which the OD after 24 hours of incubation of the inoculum remained the same or reduced compared with the initial reading. MTT (30 µL) in aqueous solution (0.01%) was used to evaluate the bacterial viability. For minimum bactericidal (MBC) determination, 10 µL was taken from each well after incubation and spot inoculated on Tryptic Soy Agar (TSA) (Oxoîd) and incubated for 72 hours at 37 °C. The concentration at which no growth observed on subculture was determined as the MBC [23].

RESULTS AND DISCUSSION

Chemical screening

The chemical screening performed on the aqueous and alcoholic extracts of diplopods revealed the presence of phenolic compounds, alkaloids, quinones, terpenoids and organic acids. The presence of secondary metabolites such as polyphenols and alkaloids for which the antibacterial properties are well established [7, 8, 19] is a good indication because these invertebrates can be a source of new drugs for human health.

Antisickling activity of diplopods extracts

Figures 3, 4, and 5 show respectively the micrographies of SS blood alone in NaCl 0.9% solution (control, fig. 3) and the SS blood incubated with the organic acids extract (fig. 4) and alkaloids crude extract (Fig. 5) from the Congolese diplopods.

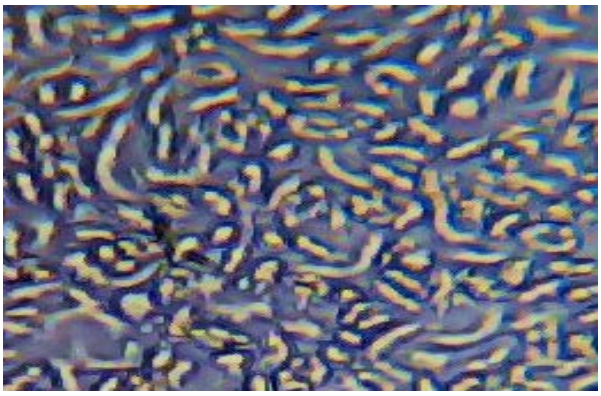

Figure 3: Morphology of drepanocytes of untreated SS blood (control) (x500) [NaCl 0,9% ; $Na_2S_2O_5$ 2%,].

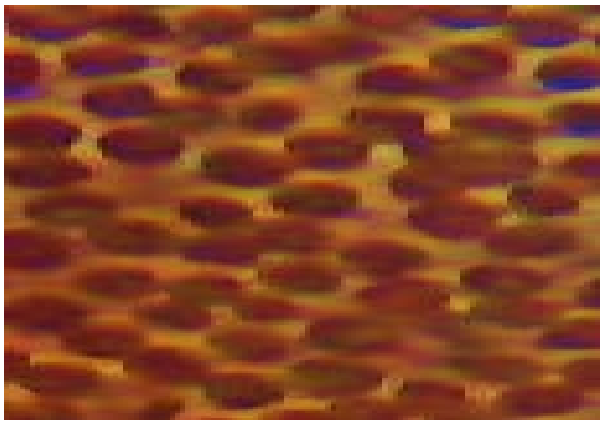

Figure 4: Morphology of drepanocytes treated with 50 µg/ml of organic acids crude extracts from diplopods (X500) [NaCl 0,9% ; $Na_2S_2O_5$ 2%,].

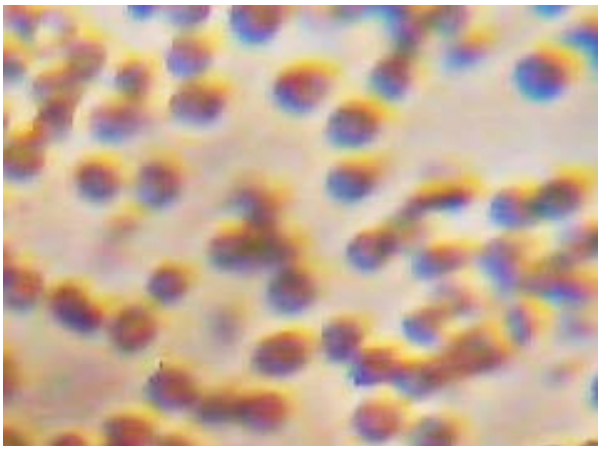

Figure 5: Morphology of drepanocytes treated with 50 µg/ml of alkaloids crude extracts from diplopods (X500) [NaCl 0,9% ; $Na_2S_2O_5$ 2%,].

Figure 3 shows that the control contains in majority sickle-shaped erythrocytes, confirming the SS nature of the tested blood. Mixed together with both organic acids and

alkaloids crude extracts from diplopods (Fig. 4 and 5), the majority of erythrocytes are reversed normal-shape. This indicates that diplopods extracts have antisickling effects (normalization rate > 80%). A similar result was already obtained for some medicinal plant species used for the management of SCD by Congolese traditional healers. The treated sickle erythrocyte demonstrated a remarkable similarity to normal blood values. This indicates that organic acids and alkaloids are the major antisickling agents in diplopods. These results confirm those already given by our research team with organic acids such as betulinic acid, maslinic acid and lunilaric acid from plants used in traditional medicine for the management of SCD [4,24]. For the best of our knowledge, this is the first time report on the antisickling activity of organic acids and alkaloids extracts of animal origin.

Antimicrobial activity

The antibacterial activity of alkaloids and organic acids crude extracts from diplopods is given in Table 1.

Table 1: Inhibitory effect of diplopods extracts against bacteria (expressed as the minimum inhibitory concentration MIC and the minimum bactericidal concentration MBC).

Diplopods	*S. aureus* (ATCC 25923)		*E. coli* (ATCC 2592)	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Alkaloids crude extract	125	> 125	500	>500
Organic acids crude extract	62,5	> 125	500	>500

As it can be seen from the table 1, the antibacterial activity of organic acids extract is higher than that of alkaloids extract. In fact, it can be observed that the *S. aureus* strains are more sensitive to organic acids extract than alkaloids extract. While *E. coli* strains are less sensitive to these two extracts because their MIC is higher than 125 µg/mL. This shows that the antibacterial activity of the diplopods depends firstly on the nature of the tested extracts and secondly on the nature of the studied strains. All the tested extracts have bacteriostatic effect by inhibiting the growth of tested bacteria strains. Because of the taxonomic proximity of human beings and great apes, compounds active against human pathogens likely inhibit infectious agents affecting great apes as it can be seen on figure 6. Recent findings have revealed that the antibacterial substances occurring naturally in the diplopods are lysozyme-like compounds and unstable when heated [25]. In the present study, we validate such properties and we can additionally postulate that diplopods active extracts could also act against microbes probably by targeting cell's communication system (quorum sensing). The breakdown of this system causes an attenuation of microbial pathogenicity [26]. The anti-quorum sensing effect of organic acids from diplopods may reduce pathogenicity and biofilm formation in great apes. The new antibiotics of animal origin which targets pathogenesis instead of killing the microbial organism may provide less selective pressure and therefore decreased emergence of resistant strains in human especially in SCD patients.

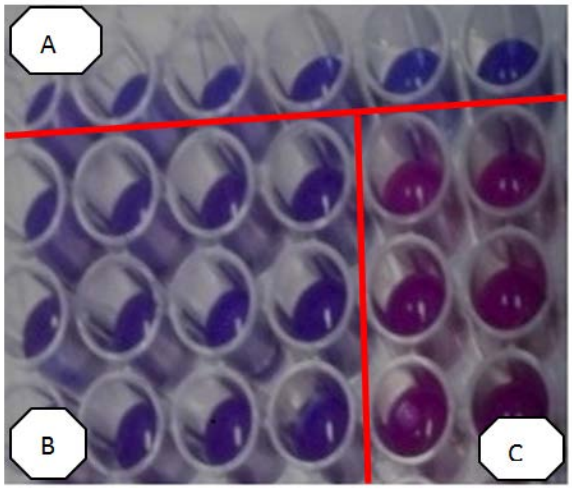

Figure 6: Effect of diplopods extract on the bacterial growth as revealed by the micro-dilution technique (A: The negative control ie wells containing TSB only; B. TSB + bacteria suspension + DE: Inhibition of growth; C. The positive control ie wells containing TSB + bacteria suspension without DE: Growth).

CONCLUSION

The present study evaluated the chemical composition and the in vitro antisickling and antibacterial activities of diplopods extracts. The extracts obtained from this invertebrate displayed promising antisickling and antibacterial effects in vitro. Total organic acids extracts inhibit the sickling of sickle erythrocytes in hypoxic conditions. The antibacterial effects of extracts of organic acids are greater than that of alkaloids extracts. The ability of diplopods extracts to display antibacterial properties may represent a rational explanation for the use of such invertebrate species in the great apes self-medicative behaviour to heal skin diseases. These results suggest that the study of animal behavior might be a promising and complementary approach for identification of potentially bioactive compounds of medicinal benefits (antisickling and antibacterial agents).

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