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Antisickling, Antioxidant and Antibacterial Activities of *Afromomum alboviolaceum* (Ridley) K. Schum, *Annona senegalensis* Pers. and *Mondia whitei* (Hook. f.) Skeels

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Abstract: In accordance with statistics from the World Health Organization, nearly 80% of populations depend on traditional medicine for primary health care. In order to scientifically validate the phyto-therapeutic wealth of the Democratic Republic of the Congo, three plants were chosen namely: *Afromomum alboviolaceum*, *Annona senegalensis* and *Mondia whitei*. The objective of our study was to assess the antisickling, antioxidant and antibacterial activities of the ethanolic extracts and the dichloromethane fractions of these three plants. Sickle cell blood was supplied from Centre de Médecine Mixte and Anémie SS of Yolo Sud, Kinshasa. Three bacterial strains were used including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739 and *Pseudomonas aeroginosa* ATCC 9027. The ethanolic extracts of *A. alboviolaceum* and *A. senegalensis* and dichloromethane fractions of *A. alboviolaceum* and *M. whitei* showed IC50 values < 100 µg/mL in the ABTS test. Only the dichloromethane fraction of *A. senengalensis* showed an IC_{50} value of less than 100 µg/mL for the DPPH test. The inhibitory concentration 50 (IC_{50}) values obtained from the ABTS test are lower than those of the DPPH test. All tested extracts possess a high antisickling activity and only soluble dichloromethane extracts are active vis-à-vis *Staphylococcus aureus* (MIC = 31.5 µg/mL). These results constitute a scientific evidence validating the use of these three medicinal plants for the management of sickle-cell anemia in the Democratic Republic of the Congo.

Keywords: Antisickling Activity, Antioxidant Activity, Antibacterial Activity, Inhibitory Concentration, ABTS, DPPH

1. Introduction

According to the World Health Organization statistics (WHO), nearly 80% of people depend worldwide on traditional medicine for their primary health care [1]. To this end, the study of plant chemistry is still a topical subject despite its age. This is mainly due to the fact that the plant kingdom represents an important source of

bioactive molecules. These compounds include, coumarins, alkaloids, phenolic acids, tannins, terpenes, flavonoid [2] that are endowed with interesting pharmacological properties including antioxidant activity.

In fact, sickle cell disease is a genetic disease linked to a structural abnormality of hemoglobin S [3]. This condition is characterized by severe anemia, vaso-occlusive seizures and a high susceptibility to both viral and bacterial infections. This latter constitute a danger for the patients given the chemoresistance of these pathogens to the usual antibiotics [4]. Sickle cell anemia is also characterized by an increased production of free radicals in plasma, which are considered as the initiator of hemolysis [5]. Exogenous antioxidants provided by food constitute a therapeutic alternative in the management of such pathologies. This is the case of secondary metabolites such as polyphenols, which are powerful antioxidants that may inhibit the formation of free radicals and oppose the oxidation of macromolecules [6-7]. For this purpose, the research of natural substances with antibacterial and antioxidant activities from plants would constitute an important scientific issue in the management of sickle cell anemia. Medicinal plants have been used since times immemorial to manage various ailments [17]. Traditional medicine is the oldest method of curing and treating human diseases and various plants have been found as a source of effective chemotherapeutic agents against several human diseases in different parts of the world. Henceforth, there is a growing interest in the development of drugs from plant origin [12] [17] [26]. In Africa, traditional medicine is of great value and more than 70% of African communities refer to traditional healers concerning health issues [17-20].

Several studies reported the use of medicinal plants for the treatment of various ailments as well as the evaluation of several activities including the antiplasmodic, anti-hyperglycemic, anti-inflammatory, antioxidant, antibacterial, anti-mycobacterial, anti-malarial, anti-parasitic, antimicrobial, antidiarrheal, anticonvulsant, anti-trypanosonal, anti-snake venom and antinociceptive properties and many other biomedical properties of pharmaceutical relevance [17-25]. These properties that plants possess is due to its important phytochemical constituents like triterpenes, anthocyanins, glucids, coumarins, flavonoids, alkaloids and many others [23].

In order to scientifically validate the phyto-therapeutic wealth of the Democratic Republic of the Congo, our choice was focused on three plants namely: *Afromomum alboviolaceum* (Ridley) K. Schum, *Annona senegalensis* Pers. and *Mondia whitei* (Hook. f.) Skeels following the hypothesis that they would contain secondary metabolites able of imparting to them the antisickling, antioxidant and antibacterial properties. The objective of the current study was to assess the antisickling, antioxidant and antibacterial activities of ethanolic extracts and dichloromethane fractions of these three plants.

2. Material and Methods

2.1. Material

2.1.1. Different Plants

The selection of plant species used in this study was based on previous ethnobotanical surveys [8]. Three plant species were used, precisely the root barks of *Aframomum alboviolaceum* (Ridley) K. and *Mondia whitei* Schum as well as the leaves of *Annona senegalensis* Pers. Systematic analysis of these plants was carried out at the Laboratory of Systematic Botany and Plant Ecology, Department of Biology, Faculty of Sciences, University of Kinshasa by the botanist Blaise Bikandu. These samples were dried out in room temperature (± 27°C) at the Molecular Bio-Prospection Laboratory, Department of Biology for two weeks and then ground to obtain fine powder for further analyses.

2.1.2. Blood

Sickle cell blood used in this study was provided by Centre de Médecine Mixte et d'Anémie SS located at Yolo-Sud, (Mabanga), Kalamu Commune, Kinshasa.

2.1.3. Bacterial Strains

In the current study, three bacterial strains were used namely *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739 and *Pseudomonas aeroginosa* ATCC 9027. These strains were provided by the Laboratory of Microbiology, Faculty of Pharmaceutical Sciences, University of Kinshasa.

2.2. Methods

2.2.1. Assessment of Antioxidant Activity

Ten mg of dry extract of each sample were dissolved in 1 mL of methanol for polar extracts and the mixture of dichloromethane-methanol (1:1) for apolar extracts (solution A: 10 mg/mL). The dilution was carried out with different concentrations as follows: 8 mg/mL, 6 mg/mL, 4 mg/mL and 2 mg/mL.

2.2.2. Evaluation of Radical Scavenging Activity

(i). ABTS Radical Scavenging Capacity

In reaction with potassium or sodium persulfate ($K_2S_2O_8$), the ABTS (2,2'-azino-bis-3-ethylbenz-Thiazoline-6-sulfonic acid) forms the cationic radical ABTS from blue to green color. The addition of antioxidant reduces this radical and causes the mixture decolorization. The decolorization of the radical measured by spectrophotometry at 734 nm is proportional to the concentration of antioxidants [9] [27].

Dissolve in 500 µL of distilled water a quantity of ABTS reagent corresponding to 20 millimoles: solution A. Then dissolve in 500 µL of distilled water a quantity of potassium persulphate ($K_2S_2O_8$) corresponding to 10 millimoles: solution B. Therefore, mix both solutions A and B at equal volume and keep the mixture away from light between 12 and 16 hours: this solution constitutes the stock solution for ABTS radical. Then dilute the stock solution of the radical with methanol x times in order to obtain an analytical

solution of which the absorbance ranges between 0.800 and 1.000.

In a test tube, place 20 µL of methanol with 1980 µL of solution of ABTS: control solution. On the other hand, in another test tube, place 20 µL of the sample solution for each concentration level, and add to this solution 1980 µL of ABTS radical working solution and incubate for 30 minutes in the absence of light. Successive reading of solutions for each concentration level is performed at 734 nm with the spectrophotometer: blank (methanol), control solution and samples.

The percentage inhibition of ABTS radical is determined as follows:

$$\% \, inhibition = \left[1 - \frac{A_x}{A_c}\right] x \, 100 \qquad (1)$$

Where, Ax: the absorbance of ABTS radical in the presence of the extract

Ac: absorbance of ABTS (control solution)

ABTS scavenging activity of extracts was expressed in IC_{50}. Different IC_{50} values were determined using Graph Pad Prism version 6.0 software. Each sample was measured in triplicate.

(ii). DPPH Radical Scavenging Capacity

This method is based on the degradation of DPPH radical (2,2 DiPhenyl-1-PicrylHydrazyl). The DPPH radical is a violet-colored radical, the addition of antioxidant reduces this radical and causes the mixture to discolor. This radical decolorization measured by spectrophotometer at 517 nm is proportional to the concentration of antioxidants [9] [26-27].

Dissolve 3.2 mg of DPPH in 100 mL of methanol (80%) and this solution is kept out in the dark for at least a hour. The absorbance of this solution must be adjusted to 0.7 ± 0.05 using methanol (80%). So, in a test tube, mix 20 µL of methanol with 1980 µL of DPPH radical solution: control solution. In another test tube, place 20 µL of sample for each concentration by adding to 1980 µL solution of DPPH radical working solution and incubate for 30 minutes in the absence of light. Successive reading of the solutions for each concentration is carried out using a spectrophotometer at 517 nm: blank (methanol), control solution and different sample solutions. The DPPH radical inhibition percentage for each sample was determined using the following formula:

$$\% \, inhibition = \left[1 - \frac{A_x}{A_c}\right] x \, 100 \qquad (2)$$

Where, Ax: the absorbance of DPPH radical in the presence of the extract

Ac: absorbance of DPPH (control solution)

DPPH scavenging activity of extracts was expressed in IC_{50}. Different IC_{50} values were determined using Graph Pad Prism version 6.0 software. Each sample was measured in triplicate.

2.2.3. Assessment of Antisickling Activity: Emmel's Test

All antisickling experiments should be carried out on freshly collected blood. In order to confirm their SS nature, blood samples taken from sickle cell volunteers should first be characterized for hemoglobin electrophoresis on cellulose acetate gel. Once the SS nature is determined, these blood samples will be stored in the refrigerator at a temperature of ± 4°C. The stock solutions of plant extracts were prepared by simple dilution of the lyophilisate in physiological saline (0.9% NaCl) at 1 mg / mL. Two successive dilutions were carried out in order to obtain different solutions at 0.5 mg/mL and 0.25 mg/mL. The sickle cell blood (0.5 mL) was previously diluted five times with 2 mL of NaCl (0.9%) - $Na_2S_2O_5$ mixture (v/v).

Microscopic preparations were carried out by placing on the slide a diluted blood drop mixed with a drop of the drug (extract). The solution was covered with a cover slide and the edges of the slides were covered with supercooled paraffin in order to create hypoxia conditions. These various preparations were observed under the light microscope (OLYMPUS Model CH10BIMF at 600X magnification, after 24h).

2.2.4. Assessment of Antibacterial Activity

The antibacterial activity was evaluated using the micro-dilution method in liquid medium as previously reported [10]. The extract to be tested (20 mg) is dissolved in 250 µL of DMSO and the final volume is adjusted to 5 mL with Mueller Hinton culture medium (final concentration in DMSO equals to 5%). The bacterial suspension is prepared by adding in 2 mL of saline solution for each strain, three colonies isolated from strains to be tested (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739 and *Pseudomonas aeroginosa* ATCC 9027) and a-24h incubation allowed to obatin 0.5 McFarland (10^8 cells/mL). Therefore, the bacterial suspension is diluted in order to have 10^6 cells/mL (1:100 dilution).

The micro-dilution assay was performed in a 96-well sterile polystyrene microplates. Briefly, 100 µL of culture medium were placed inside wells (A2 to A9, B2 to B9, C2 to C9, D2 to D9, E2 to E9 and F2 to F9 and then the 11^{th} and 12^{th} columns were considered as controls). Using a micropipette, 200 µl of each extract to be tested (1000 µg/mL) were placed in wells A1 (extract 1: *A. alboviolaceum* with ethanol), B1 (extract 2: *A. alboviolaceum* with dichloromethane fraction, C1 (extract 3: *A. senegalensis* with ethanol), D1 (extract 4: *A. senegalensis* with dichloromethane fraction, E1 (extract 5: *M. whitei* with ethanol), F1 (extract 5: *M. whitei* with dichloromethane fraction).

Thus, take 100 µL of each extract stock solution for serial dilutions of 2 to 2 up to the ninth column and the last 100 µL (column 9) are removed. Five µLs of the inoculum (10^8 CFU/mL) are aseptically removed with a micropipette and added to all wells of the microplate except for wells of the 11^{th} column used as control for the bacterial growth (inoculum and culture medium) and wells of the 12^{th} column which were used as control of sterility of culture medium. Microplates were incubated in an oven at 37°C for 24 hours.

After the incubation period, 3 µL of Resazurin dye (1%) (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) was added to each well and the microplates were kept for a second incubation for 7 hours. The minimum inhibitory concentration (first wells with no bacterial growth) was determined 24 hours later.

2.3. Data Analysis

Data were analyzed using Graph Pad Prism version 6.0 Software (Graph Pad Software, San Diego California, USA). The analysis of variance having a criterion of classification (One Way ANOVA) allowed us in the comparison of means. The significance level is $p \leq 0.05$.

3. Results and Discussion

3.1. Yield of Extraction

The yield of extraction of different drugs after a-48h maceration is presented in figure 1 below.

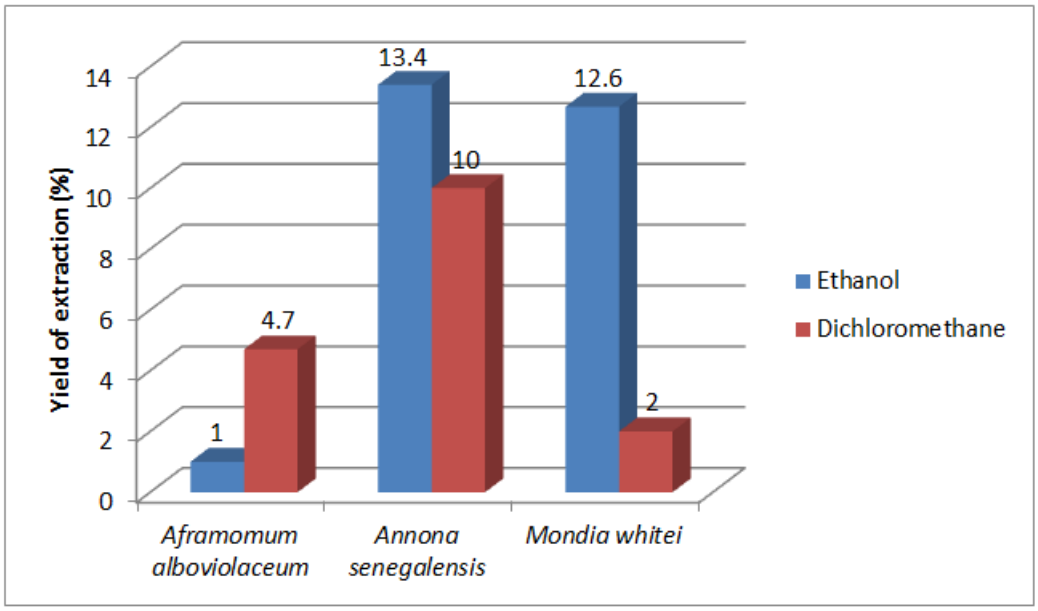

Figure 1. *Yied of extraction of Ethanolic extracts and Dichloromethane fraction of different drugs.*

From the above figure, it can be seen that the yield obtained with ethanolic extracts is higher than that of the dichloromethane fraction. By comparing the yield obtained between the dichloromethane fraction (apolar solvent), *A. senegalensis* and *A. alboviolaceum* have a higher yield compared to *M. whitei* which has a low yield (2%). The method used for obtaining the dichloromethane fractions is suitable for the extraction of terpene acids such as oleanic, betulinic and maslinic acids. For ethanolic extracts (polar solvent), *A. senegalensis* and *M. whitei* showed a good yield. In addition, it also shows that quantitatively important metabolites contain in the barks of our plants are those that pass easily in polar solvents, including polyphenols of which anthocyanins, tannins, flavonoids, but also saponins and alkaloids [15].

3.2. Antioxidant Activity

The antioxidant activity of tested extracts, determined by ABTS and DPPH assays is presented in Table 1 and is expressed as IC_{50} values. IC_{50} is the amount of antioxidant necessary to decrease the initial concentration of radical by 50%. A lower IC_{50} value indicates a higher antioxidant activity.

Table 1. *IC_{50} (µg/mL) values of plant extracts and Quercetin used as positive control. (Means ± SD, n=4).*

Extracts	IC_{50}			
	ABTS test		DPPH test	
	EtOH	DCM	EtOH	DCM
A. alboviolaceum	59.98 ± 2.54	43.25 ± 9.55	243.22 ± 83.36	263.03 ± 56.65
A. senegalensis	46.88 ± 1.43	381.07 ±154.73	112.46 ± 4.22	53.20 ± 0.97
M. whitei	132.43 ± 4.06	64.86 ± 3.06	279.89 ± 92.51	1056.8 ± 436.18
Quercetin	1.42 ± 0.40	NP	3.21 ± 0.99	NP

Legend: EtOH: ethanol, DCM: dichloromethane, NP: not performed

Ethanolic extracts of *A. alboviolaceum* and *A. senegalensis* as well as the dichlorormethane fractions of *A. alboviolaceum* and *M. whitei* showed different IC_{50} values <100 µg/mL with the ABTS test. For the DPPH test, only the dichloromethane fraction of *A. senegalensis* showed an IC_{50} value of less than 100 µg/mL. The capacities of various

extracts to inhibit radicals vary substantially in each type of test. This difference in activity can be explained by the qualitative or quantitative range in secondary metabolites of each extract. For ABTS test, the ethanolic extracts are active for *A. alboviolaceum* and *A. senegalensis* except *M. whitei* while for the DPPH test none of the extracts are active. However, only the dichloromethane fraction of *A. alboviolaceum* and *M. whitei* are active for the ABTS test and the dichloromethane fraction of *A. senegalensis* is inactive whereas the dichloromethane fraction of *A. senegalensis* is active for the DPPH test and inactive for *A. alboviolaceum* and *M. whitei*.

Furthermore, it should be noted that the inhibitory concentration 50 (IC_{50}) values obtained in the ATBS test are lower than those of the DPPH test. This difference in activity is attributed to the mechanisms of reactions. In fact, the ABTS radical reacts with hydrophilic and lipophilic compounds at the same time while DPPH° radical reacts only with hydrophilic compounds [11-12]. Therefore, it should also be noted that although our extracts showed a lower activity compared to the activity of quercetin used as positive control, this activity is interesting compared to other plants [12-13].

3.3. Antisickling Activity

Different micrographs of the SS blood alone and of the SS blood in the presence of various extracts are displayed in the figure 2 (a-d_2) below.

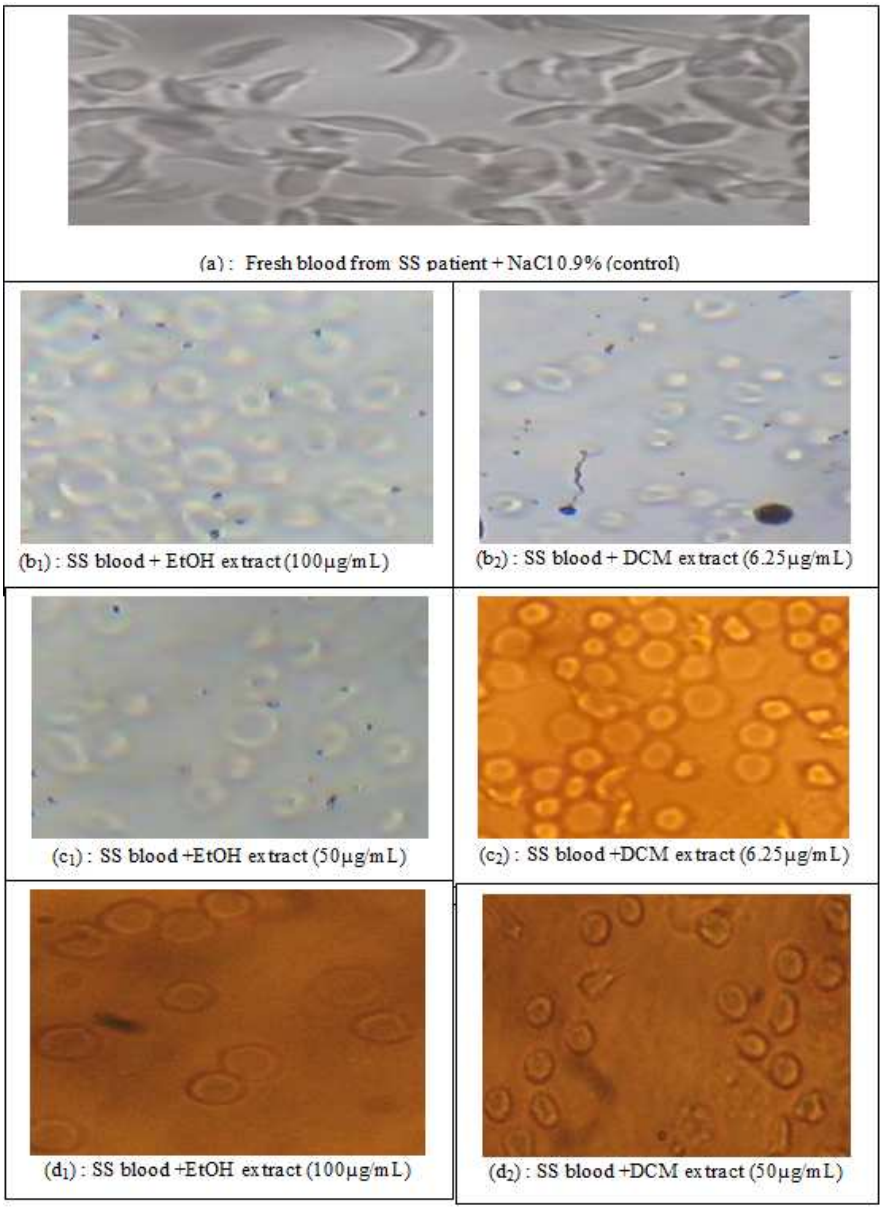

Figure 2. *Antisickling test Microscopy: untreated erythrocytes (a); Erythrocytes treated with EtOH extract 100 µg/mL (b1) and DCM fraction 6.25 µg/mL (b2) of A. alboviolaceum; Erythrocytes treated with EtOH extract 50µg/mL (c1) and DCM fraction 6.25 µg/mL (c2) of A. senegalensis; Erythrocytes treated with EtOH extracts at 100 µg/mL (d1) and DCM fraction at 50 µg/mL (d2) of M. whitei. (magnification X500), [NaCl 0.9]. EtOH: Ethanolic, DCM: Dichloromethane.*

Figure 2 (a) shows the sickle-red blood cells, indicating that it is SS blood. In our experimental conditions, this sickling was sustained by the hypoxia created by surrounding the preparations on microscope slides with paraffin. On the other hand, figures (b1) - (d2) show the aspect of erythrocytes which resume their normal biconcave form in the presence of different extracts and fractions of used plants while they are placed under the same hypoxia conditions with the control in the presence of saline solution.

As it can be noticed that (b1)-(d2) dichloromethane fractions are highly active (minimum normalization concentration 6.25 µg/mL) for *A. alboviolaceum*, *A. senegalensis* and *M. whitei* compared to the ethanolic extracts. This high activity observed with the dichloromethane fraction is due to the presence of terpene acids which are apolar molecules. Apolar molecules have a high capacity of cell penetration, this would confer to them the power to interfere with the cellular mechanisms at the base of the normalization of the sickled-cells leading to the restoration of their normal characteristic biconcave shape. These results also corroborate with Tshibangu and Ngbolua [14-15] who have reported that betulinic and maslinic acids are at the basis of *Callistemon viminalis* and *Syzygium cordatum* antisickling activity.

By comparing the antisickling activity of these three plants, it is found that *A. senegalensis* has a high activity compared to *A. alboviolaceum* and *M. whitei*. These results are similar to previous works of Ngbolua *et al.* [10], [12-13] whom demonstrated the antisickling activity of aqueous and ethanolic extracts of *A. senegalensis*. This activity could be explained by its richness in secondary metabolites. Moreover, it should be noticed that all three plants displayed an antiradical scavenging activity as well as the antisckling activity.

3.4. Antibacterial Activity

Table 2 gives the results of the antibacterial activity of various extracts.

Table 2. *Antibacterial activity of different extracts.*

Extracts	MIC (µg/mL)		
	***S. aureus* ATCC 25923**	***E. coli* ATCC 8739**	***P. aeroginosa* ATCC 9027**
EAA	4000	> 4000	4000
DAA	> 4000	> 4000	>4000
EAS	500	> 4000	4000
DAS	31.25	4000	4000
EMW	> 4000	> 4000	4000
DMW	4000	> 4000	4000

Legend: EAA: Ethanolic extract of *A. alboviolaceum*; DAA: dichloromethane fraction of *A. alboviolaceum*; EAS: Ethanolic extract of *A. senegalensis*; DAS: dichloromethane fraction of *A. senegalensis*; EMW: Ethanolic extract of *M. whitei*; DMW: Dichloromethane of *M. whitei*; ATCC: American Type Cell Collection; MIC: Minimal Inhibitory Concentration.

This table shows that only dichloromethane fractions are active against *S. aureus* (MIC = 31.5 µg/mL). The antibacterial activity of the dichloromethane fraction of *A. senegalensis* on *S. aureus* could be explained by the probable presence of organic acids (triperpenic acids) which are well known for their high antibacterial activity, in particular with the dismemberment of the microbial membrane [15]. Given that sickle-cell anemia is characterized by a high susceptibility to staphylococcal infections, the antibacterial activities of the studied plants constitute an alternative management and therefore reduce the risk of bacterial infections.

4. Conclusion and Perspectives

The main objective of the current study was to assess the antisickling, antioxidant and antibacterial activities of different extracts of three Congolese taxa in order to contribute to the development of local plants used in Congolese traditional medecine for the management of Sickle Cell Anemia. It was demonstrated that ethanolic extracts of *A. alboviolaceum* and *A. senegalensis* and different dichloromethane fractions of *A. alboviolaceum* and *M. whitei* showed good IC_{50} values <100 µg/mL with the ABTS radical test while *A. senengalensis* dichloromethane fraction showed an IC_{50} value of less than 100 µg/mL for the DPPH radical test. The inhibitory concentration 50 (IC_{50}) values obtained from the ABTS test are lower than those of the DPPH test. All tested extracts possess a high antisickling activity and only soluble extracts in dichloromethane were active to *Staphylococcus aureus* (MIC = 31.5 µg/mL).

All these results constitute a scientific evidence validating the use of these three medicinal plants for the management of sickle-cell anemia in the Democratic Republic of the Congo. Therefore, an assessment of the acute and/or chronic toxicity of different bioactive fractions is required as well as more advanced investigations are needed for the development of an improved traditional drug.

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