Highlighting of the Antimicrobial Activities of Extracts of Morindamorindoides, a Medicinal Plant used in West and Central Africa

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Abstract

The renewal of the anti-infective arsenal has become over the last 10 years a public health priority. To this end, an ethno-pharmacological approach has been developed. From Traditional self-medication habits, Morindamorindoides was shown to be used throughout sub-Saharan Africa for its effects against the diarrhoea, malaria, skin infections, itching and fevers. Given the breadth of its scope, this Rubiaceae was investigated for antimicrobial potencies.After harvesting and packaging of leaves, stems and roots, extractions in water, methanol, acetonitrile, acetone, ethyl acetate or chloroform were performed. The antimicrobial activities of the extracts obtained were evaluated against eleven microbial strains. Extraction of roots using acetonitrile resulted in the higher antimicrobial activity against staphylococcus aureus.The cytotoxicity test of this crude extract exhibited an IC_{50} of 420.32 µg/mL on Vero cell line and 388.33 µg/mL on 3T3 cell line. The kinetics of inactivation reveals a loss of cultivability of Staphylococcus aureus and Candida albicans was modeled using the weibull model. The results of future work concerning purification and characterization of compounds of this acetonitrile extract including their cytotoxic activities could inform us of fields of application of these natural substances in human medicine, veterinary and cosmetology.

Keyword: *Medicinal plant, Morindamorindoides, antimicrobial activity, cytotoxic activity.*

I. INTRODUCTION

Since the late twentieth century, we are seeing an increasing appearance of pathogenic bacteria resistant to antibiotics. This emergence may firstly be explained by excessive and inappropriate use of antibiotics in human and veterinary medicine **[1, 2]**. On secondly, this could also be due to the reduction in antibiotic research since the 1980. The placing on the market of new antimicrobial molecules by pharmaceutical companies has thinned gradually since. Thus, from 1983 to 1987, 16 new antibiotics were placed on the market in the United States of America, followed by 7 (from 1993 to 1997) and 3 from 1998 to 2002 **[3]**. The bacteria thus exposed to the same antibiotics over a long period can only adapt and generate resistances and then evolve towards different multi-resistances type (MDR, XDR and PDR)**[4].** Faced with this recurrence of the infections difficult to treat induced by these pathogenic bacteria, the strengthening of the antimicrobial drugs is one of the major public health concerns. In this context, an ethno-pharmacological approach was initiated within the framework of collaboration between the biochemical pharmacodynamics laboratory and the university laboratory of biodiversity and microbial ecology. This approach was motivated by the fact that if the traditional medicine used since millennia continues today to be exploited by 80% of the world population **[5]**, this could also be explained by some effectiveness to it. Therefore, in this approach, traditional medicinal self-medication habits have been exploited in order to identify new sources of antimicrobial compounds. Among the many species of medicinal plants listed, *Morindamorindoides* known to treat infections of any kind in West and Central Africa has attracted our attention. The previous studies carried out on this plant only were concerned the leaves whereas other organs such as the roots are also traditionally exploited for the same pathologies. Indeed, for highlighting the presence of antimicrobial substances in *Morindamorindoides*, we have found it useful to carry out a comparative study on 3 parts of this plant (leaves, stems and roots).

II. MATERIAL AND METHODS

A. Harvesting and preparation of Morindamorindoidesextracts

The plant material used is composed separately of the leaves, stems and root of this medicinal plant. These different parts of the plant were collected during the months of April and May(2009 and 2010) in Ivory Coast. They were then sorted, washed, cut into small pieces and then dried

out of the sunlight for 8 days. After this time, each party was pulverized into fine powder. In order to extract the maximum compounds, six extracts per plant part were performed (aqueous extract, methanol extract, acetonitrile extract, acetone extract, Ethyl acetate extract and the chloroform extract). For each extraction, at room temperature, twenty-five grams of powder from each organ were homogenized separately in 300 mL of the various solvents (water, methanol, acetonitrile, acetone, ethyl acetate and chloroform) on a stirring table at 100 rpm for 24 hours. Each mixture was then filtered on Watman paper. The various filtrates are centrifuged at 11000G for 15 minutes at 5 ° C. The solvents contained in each supernatant are then removed by rotavapor (methanol, acetonitrile, ethyl acetate, acetone and chloroform) or by lyophilization (water). The 18 powders (extracts) obtained are stored at 4 ° C. in the absence of light and are then used to carry out tests of biological activities.

B. Comparison of Antimicrobial Activities by Diffusion Method in agar Medium

In order to identify the organ of the plant which exhibits the best antimicrobial activity and the solvent which better selects the antimicrobial substances, the diffusion method in agar medium was used for the antibacterial activity tests of the various extracts. The assessment tests antimicrobial activities were carried out against 11 pathogens including 7 Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Enterobacter aerogenes* 6086 IPC, *Citrobacterfreundii* NRRL B 2643, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiellaoxytoca* CIP 7932, *Salmonella enterica* CIP 8297 and *Pasteurellamultocida* ATCC 43137), 2 gram-positive bacteria (*Enterococcus faecalis* CIP A186 and *Staphylococcus aureus* ATCC 25923) and 2 yeast (*Candida albicans* ATCC 2091 and *Cryptococcus neoformans* ATCC 32045). The concentration range of the different extracts was performed according to a series of half dilution from 30 mg/mL to 0, 01 mg/mL for organic extracts using 70% DMSO as diluent. The aqueous extracts evolve from 150 mg/mL to 0.58 mg/mL with water as diluent. From a 24-hour culture of incubation of the targeted strains, a pre-culture of 3 to 4 hours was performed. The optical density at 600 nm (OD 600nm) of this pre-culture allows us to take an amount corresponding to 2.10^7 CFU which is homogenized in 20 mLof TSA maintained in melt at 50° C. In this inoculated and solidified agar, 9 wells of 6 mm in diameter are hollowed out and 70 µL of each concentration of plant extract is deposited in the wells obtained. When finished, the petri dishes are incubated at 30 or 37 ° C.

After 24 or 48 hours of incubation, the radius of inhibition from the edge of the well is measured.The values for the different concentration of each extract were used to plot the regression line between the dilution of the initial concentration and the radius of inhibition. From this regression line, the dilution of the initial concentration which induces in 1 mm of inhibition radius is determined. It is the arbitrary unity (UA). This value is used to calculate the specific activity of the extract which is expressed in AU/mg. It represents the inverse of the amount of extract which induces 1 millimeter of inhibition radius from the edge of the well**[6]**.

C. Inactivationkinetics

The inactivation kinetics of *Staphylococcus aureus* and *Candida albicans* were carried out with the extract exhibiting the highest antimicrobial activity. The test was to count the number of Colony Forming Units (CFU) of microorganisms depending on the contact time with different concentrations of plant extract. From a culture in broth of *Staphylococcus aureus* and *Candida albicans*, different inoculums of 10^5 CFU / ml are prepared and incubated in the presence of 800 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL And 0 µg/mL of plant extract. At regular intervals of time, 50 µL of each broth in incubation are taken and seeded in spiral on nutrient agar. After 24 or 48 hours of incubation, the colonies are counted. These data make it possible to represent kinetics of inactivation of microorganism by each concentration of extract. The modelling of the inactivation kinetics of the microorganisms was carried out by the Weibull model according to Equation 1:

$$Log\ (N) = Log\ (N_0) - \left(\frac{t}{\delta}\right)^p$$

With**N** : Number of colony forming units ; $\mathbf{N_0}$: Number of colony forming units at T_0; **t** : Time (hours); **δ** : Time required for decimal reduction in the number of colony forming units; **p** : Model Adjustment Parameter

The graphical representation by this Log (N) equation as a function of time makes it possible to determine the value of δ for each concentration of extract. The different values of δ (time required for a decimal reduction of the starting inoculum) determined are represented as a function of the concentration of the extract. The graph obtained makes it possible to evaluate and to quantify the effect of the concentration of the extract on the reduction of the target microorganisms.

D. Cytotoxic Activity Evaluation

The evaluation of this activity was carried out according to the method described by certain authors **[7, 8]**. Mouse fibroblast 3T3 cells and monkey epithelial VERO cells are cultured in RPMI and DMEM medium, respectively, in cell culture flasks at 37° C in a CO_2 incubator (5%) under sterile conditions and in a saturated atmosphere by water. Before their confluence, when the cells are in an

exponential phase of growth, the cell mat obtained is dissociated by trypsin. The medium is subtracted, the carpet is washed with 10 ml of a HBSS saline solution devoid of divalent cations and then coated with 1 mL of EDTA trypsin added with 2 mL of HBSS without Ca^{2+} or Mg^{2+}. The cells are incubated for 5 min at 37 ° C. and the complete dissociation of the carpet is monitored under a microscope. The cell suspension is mixed with culture medium containing SVF (fetal calf serum) to stop the trypsinization and then centrifuged for 5 min at 200 g. The cell pellet is taken up by culture medium and the cell concentration is estimated by counting on a Malassez cell. Cells were seeded at day 4500 per well in 96-well cell culture plates in a volume of 100 µl per well. To do this, a range of concentrations of this extract was previously carried out in cell culture medium. This extract being relatively hydrophobic, it was prepared in DMSO before being added to RPMI or DMEM so as not to exceed a final concentration of 10% of DMSO. Each point of the range is made in triplicate. At day +2, 20 µL of MTT solution are added to the 200 µL already present in the wells and the plates are still incubated for 4 hours at 37 ° C. Then the plates are emptied by suddenly reversing them, and then 80 µL of lysis buffer are added to dissolve the formazan crystals formed by the viable cells. After homogenization of the contents of each well, their optical density is read at 540 nm **[9]**. The viability percentage of the cells exposed to each concentration of extract is estimated relative to the control cells without extract. In case of cell toxicity, the concentration which induces 50% viability (IC_{50}) is determined.

III. RESULTS AND DISCUSSION

After filtration and removal of the solvents, the various extracts originating from the roots are in the form of a powder of amorphous appearance, of a yellow or orange-yellow color. At the level of the leaves and stems, the organic extracts are green with a more or less viscous appearance. The aqueous extracts exhibit a brown powder with amorphous appearance. The extraction yields were determined from the ratio of the mass of the extract to the mass of dry organ powder. These different yields are summarized in the Figure 1.

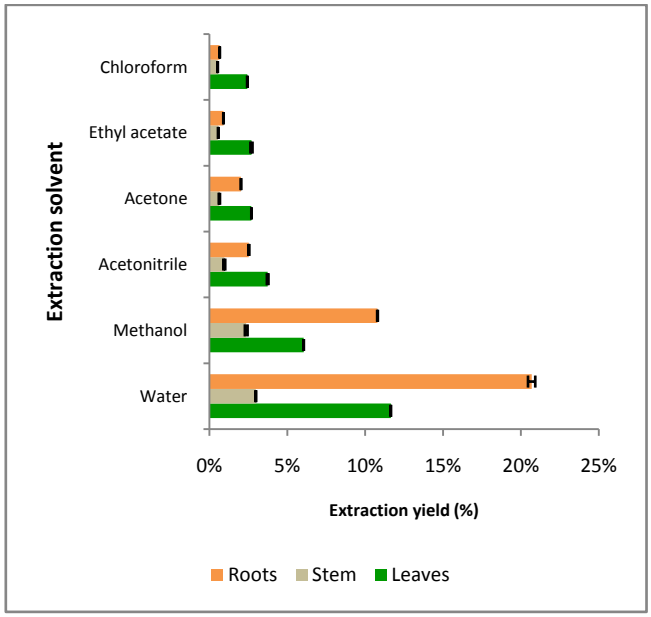

Figure 1: Extraction Yield for each Organ

The different extraction yields exhibit that the organs of this plant contain more polar compounds than apolar compounds. Moreover, the root is richer in polar substances than the other organs studied. The leaves contain more apolar substances than the root and the stem.

The antimicrobial activity of the various extracts is detected by the diffusion method in agar medium (Figure 2). The specific activities of each extract on all the 11 microorganisms are presented in Figure 3. This figure reveals specific antimicrobial activities vary depending on the organ of the plant and the target strain. The specific activities of leaves extracts vary from 0 to 1.11 AU/mg. The best activity was recorded with the methanolic extract on *Klebsiellaoxytoca.* Stems extracts have a specific activity evolving from 0 to 4.83 AU/mg. The root extracts recorded activities ranging from 0 to 51.64 AU/mg. The best activities are with the extracts made with solvents less polar than methanol. Their antimicrobial activities are more interesting on *Staphylococcus aureus, Candida albicans and Cryptococcus neoformans*. The root of

Morindamorindoides is therefore the organ that has the highest concentration of antimicrobial active ingredients. Previous studies of the antimicrobial activities of *Morindamorindoides* were conducted only on leaf extracts. From these previous studies, antiparasitic quercetin, steroids and iridoides were isolated [10, 11]. Despite antibacterial and antifungal activities highlighted on leaves extracts [12, 13], no compounds (antibacterial and antifungal) have been characterized from the leaves of *Morindamorindoides*. Moreover, like other study performed on species of *Morinda* genus, this study indicates the presence of higher activity in extracts from roots.

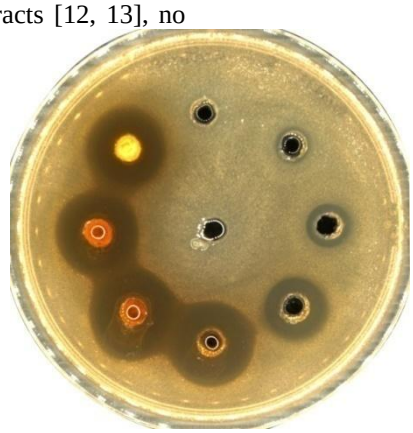

Figure 2: Inhibition Area by Diffusion in Agar Medium of Acetonitrile Extract with of the Root of *Morindamorindoides* on *Staphylococcus aureus*

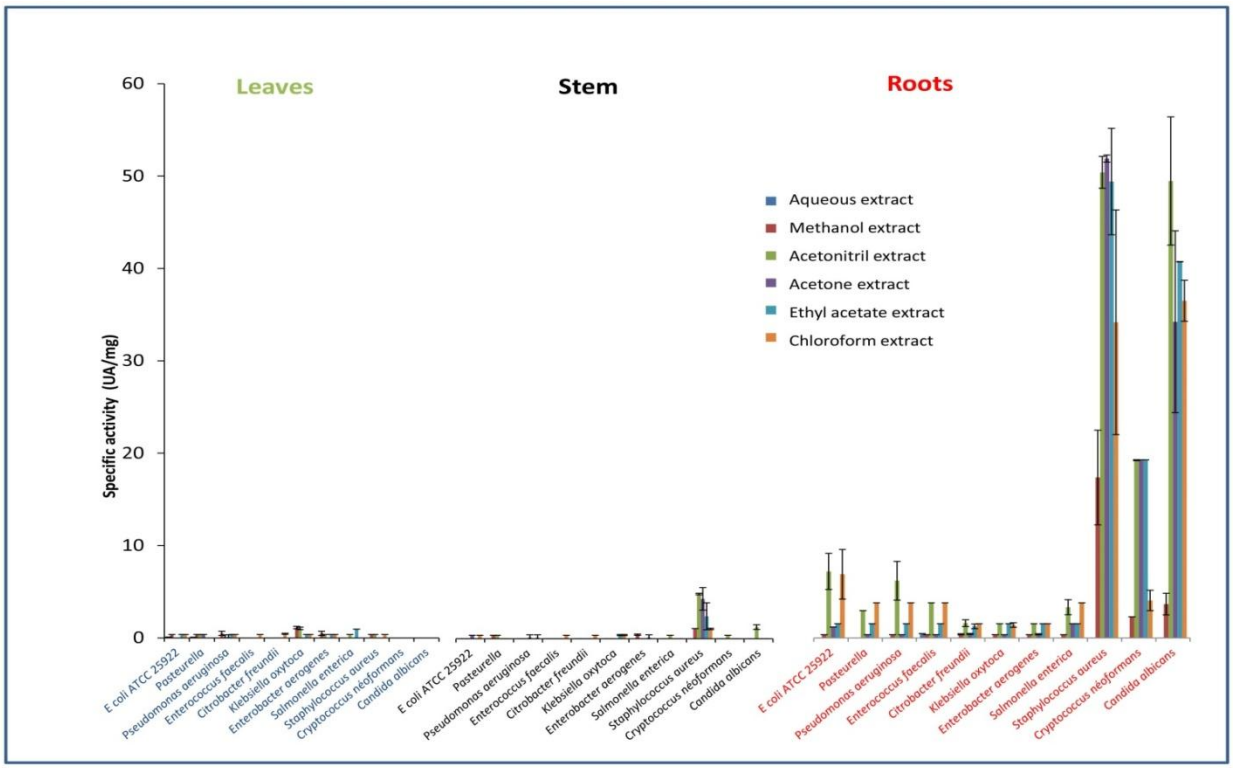

Figure 3: Specific Activity of Different Extracts on 11 Microorganisms

The inactivation kinetics of the acetonitrile extract of the *Morindamorindoides* roots was carried out on bacterium (*Staphylococcus aureus*) and yeast (*Candida albicans*). Microorganism viability in the presence of four different concentrations of acetonitrile extract of *Morindamorindoides* roots (800µg/mL, 400µg/mL, 200µg/mL and 100µg/mL) was measured as a function of time (Figure 4). The results exhibit a loss of cultivability over time. The inactivation kinetics obtained do not show a Log linear relationship between the populations and the contact time. These concave-shaped kinetics can be described and modeled by the cumulative function of the Weibull frequency probability distribution [14]. The modeling of the different kinetics of inactivation was used to determine No (number of initial colony

forming unit), δ (the time required for a decimal reduction) and p (adjustment parameter) values (

Tableau **1**). The evolution of the value δ (time required for a decimal reduction of the starting inoculum) depending of the concentration of the extract for each microbial strain is presented in Figure 5. The effects of the concentration of the root acetonitrile extract on the viability of the two microorganisms were compared. The representation of the time required for a decimal reduction (δ) as a function of the extract concentrations exhibit two different trends. On *Staphylococcus aureus*, beyond 200 µg/mL, the time required for a decimal reduction in the number of bacteria remains almost constant. Moreover, the increase of the concentration of the extract leads to a decrease of δ with*Candida albicans.* No saturation was observed for concentrations up to 800µg/mL

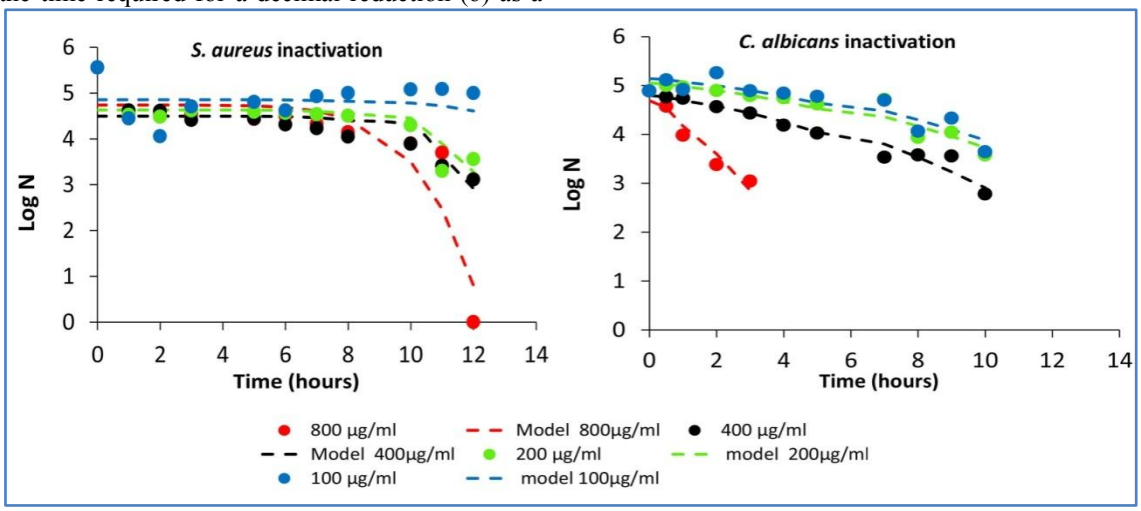

Figure 4: Inactivation Kinetics *S aureus* and *C albicans* for Different Concentrations of acetonitrile Extract from the Root of *Morindamorindoides*. Dotted Curves Represent the Correlation According to the Weibull Model

Tableau 1: N0, δ et p Valeurs résultant de la modélisation de Weibull

	Staphylococcus aureus			***Candida albicans***		
Concentration	N_0	δ	p	N_0	δ	p
	4.66	9,46	6,323	4,89	2,86	1,809
800 µg/ml	5,56	9,66	6,323	4,87	2,63	1,809
	5,61	10,31	6,323			
	4.66	9,44	6,323	4,89	7,03	1,809
400µg/ml	5,56	10,23	6,323	4,87	7,18	1,809
	5,61	10,34	6,323			
	4.66	10,29	6,323	4,89	8,58	1,809
200µg/ml	5,56	10,49	6,323	4,87	8,08	1,809
	5,61	10,91	6,323			
	4.66	18,57	6,323	4,89	8,79	1,809
100µg/ml	5,56	13,77	6,323	4,87	9,35	1,809
	5,61	11,83	6,323			

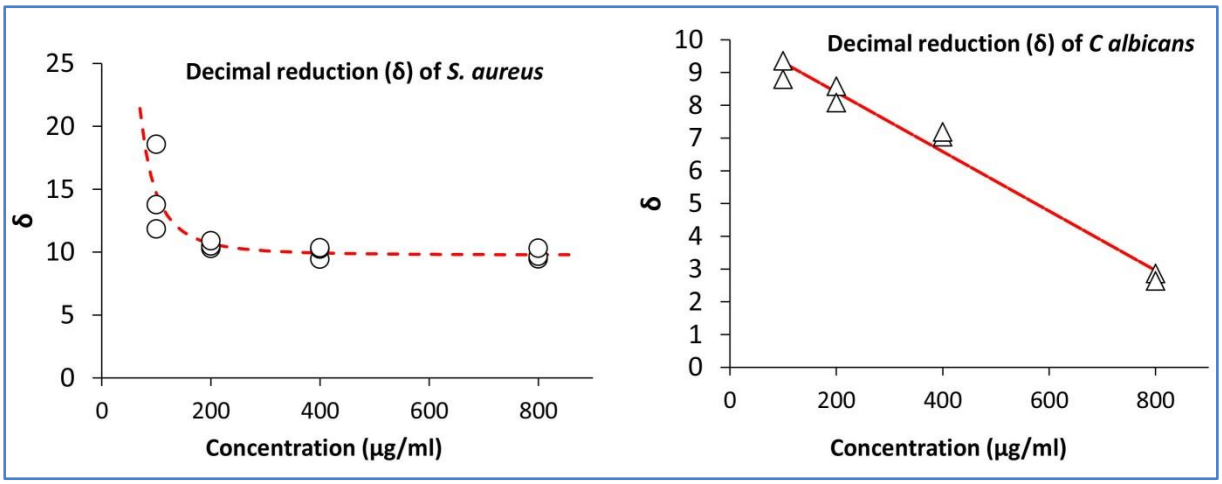

Figure 5: Evolution of the Decimal Reduction (Δ) of Microorganism as a Function of the Concentration Of The Extract With Acetonitrile Of The Roots Of *Morindamorindoides*

The cytotoxic activity tests of the acetonitrile extract of the root of *Morindamorindoides* carried out on the 3T3 and VERO cells revealed an absence of cytotoxicity at 250 µg/mL.At 500 µg/mL, there is a cytotoxic effect which affects 90% of the 3T3 cells. From cell viability percentage curve as a function of the concentration of the extract, the IC50s of 420.32 µg/mL for the VEROs and 388.33 µg / mL for the 3T3 cells were determined. It is a less pronounced cytotoxic activity compared to that obtained from the ethanolic extract of the leaves of*Morindaelliptica*, a medicinal plant of the same genus with an IC50 of 30 µg/mL**[7]**.

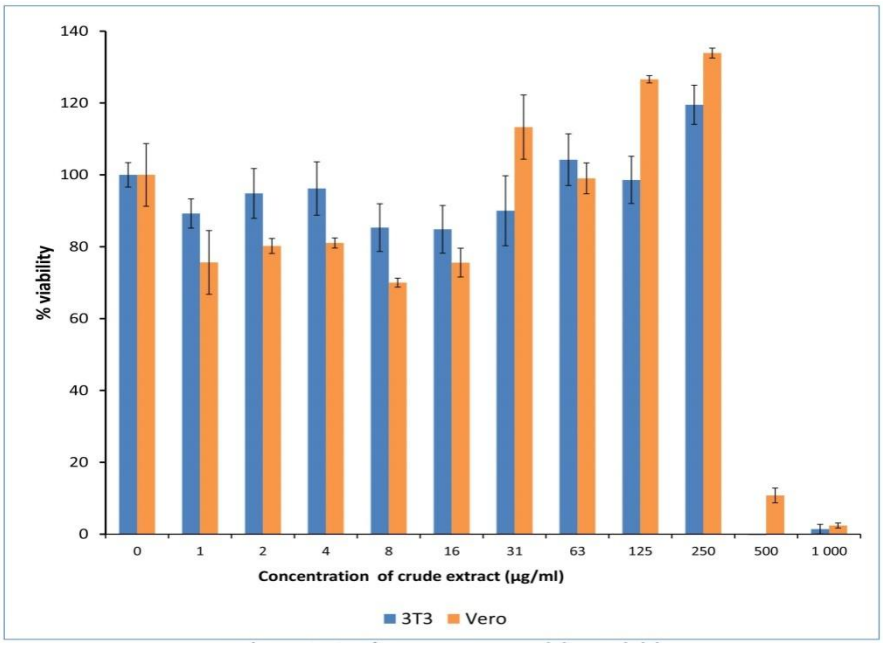

Figure 6: Viability of 3T3 and Vero Cells as a Function of the Concentration of Acetonitrile Extract from the Root of *Morindamorindoides*

IV. CONCLUSION

The strengthening of the arsenal of antimicrobials motivated this study which allowed an exploration at the level of the molecules synthesized by medicinal plants. This exploration was particularly focused on *Morindamorindoides*, a medicinal plant exploited in Côte d'Ivoire and other sub-Saharan African countries.The comparative study of the antimicrobial activity of the different organs of this plant revealed that its root is a source of antimicrobial plant molecule. So, the use of this plant in traditional medicine could be justified. For the next step in this study, we will focus on the plant molecule contained in the acetonitrile extract to evaluate its biological activities. The results of future work could inform us

of fields of application of these natural substances in human medicine, veterinary and cosmetology.

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