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EVALUATING THE ANTIBACTERIAL ACTIVITY OF MORINDA MORINDOIDES EXTRACTS ON THE IN VITRO GROWTH OF THE CLINICAL STRAINS OF *Shigella sp*.

KOFFI ALLALI EUGÈNE[1*], DOSSO MAMADOU[2], ABBA P. OBOUAYEBA[1], DJAKO S. T. AKRÉ[1], N'GUESSAN KOUADIO MICHAEL[1], A. SAKIRIGUI[3,4], EHOUMAN MOCKET ADOLPHE[5] AND A. B. ACKAH JACQUES AUGUSTE[1]

[1]College of Agroforestry, Agrovalorisation Laboratory, Department of Biochemistry and Microbiology, Jean Lorougnon GUEDE University, P.O Box 150 Daloa, Côte d'Ivoire.

[2]Biological Sciences, College of Biochemistry and Genetics, Peleforo Gon Coulibaly University, P.O Box 1328 Korhogo, Côte d'Ivoire.

[3]Laboratory of Pharmacognosy and Essential Oils, Faculty of Sciences and Techniques/University of Abomey-Calavi (UAC) Cotonou, Republic of Benin.

[4]Aromatic, Food and Medicinal Plants Research Laboratory, Faculty of Sciences and Techniques, UNSTIM/Abomey Republic of Benin, République du Bénin.

[5]Olopam Pharma and Research & Development, Côte d'Ivoire.

AUTHORS' CONTRIBUTIONS

This study was carried out in collaboration with all authors. Authors KAE, DM and APO are the main authors of the review. Author AS is the focal point of a sub-regional collaboration. He provided suggestions to the working group of the study. All other authors have equally contributed to literature review and editing process. The final manuscript has been read and approved by participating authors .

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ABSTRACT

For a couple of years, strains of *Shigella* resistant to aminopenicillins and cotrimoxazole have developed and have become predominant.

The purpose of paper is to show the social contribution in the care of gastroenteritis. As such, the antibacterial activity of the total aqueous and 70% ethanolic extracts of the leaves of *Morinda morindoides*, a plant from the Ivorian pharmacopoeia was evaluated.

In this paper, bacteriological tests were carried out on two pathogenic strains of *Shigella sp*. (EEQ and 1055).

The methods of diffusion, either in agar medium or in liquid, were used for the search for Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC).

The results show that both extracts tested have *in vitro* antibacterial bactericidal efficiency on both *Shigella sp*. strains. However, the 70% ethanolic extract which is twice as effective as the total aqueous extract is more active against strains of *Shigella Sp* tested, with a MBC of 25 mg/mL.

The finding suggest that antibacterial properties of this plant could also useful for caring shigellosis.

Keywords: *Morinda morindoides*; aqueous extract; ethanolic extract; bacterial strains; *Shigella sp*.; MBC; MIC.

**Corresponding author: Email: koffiallali@yahoo.fr;*

1. INTRODUCTION

Nowadays, herbal or traditional medicine is an accepted and recognized form of medicine in the whole world [1]. According to the World Health Organization, for many millions of people, herbal medicines, traditional treatments, and traditional practitioners are the main source of health care, and sometimes the only source of care [2]. It is known that between 65 and 80 % of the world's population use herbal medicines as their primary form of health care [3] and around 80% of African population use traditional medicine for basic treatment [4, 5, 6]

In Africa, as in Côte d'Ivoire, medicinal plants have a prominent place in the treatment of various diseases, especially where modern medicine has shown its limits [7]. The exploration of the medicinal flora begins with plants known for their therapeutic powers, and used traditionally [8] and whose properties have already been proved. Among these plants is *Morinda Morindoides*, from the traditional Ivorian pharmacopoeia. Numerous works have been done to show biological properties of this plant: thus, *in vitro* studies showed that *M. morindoides* possessed anti-malarial [9], anti-amoebic [10, 11]., anti-fungal [12], anti-bacterial [13-17]….etc….Moreover, those effects were emphasized in animal studies. *M. morindoides* showed its safety on animals' organs as the liver and the kidneys [18- 19].

Faced with the growing phenomenon of emerging diseases in the world, we are witnessing the development of bacteria resistant to antibiotics and their spread in hospitals and cities [20-21]. Also, the spread of diseases related to water and other health issue is not ignored by health officers, and according to the World Health Organization (WHO), these diseases generally result from the uncontrolled development of urbanization and the extension of slums, where million people still lack access to improved drinking water sources, and billion drink water from sources that are faecally contaminated and are exposed to the risk of water borne diseases in general, including cholera [22].

Under those conditions, gastroenteritis is part of the most recurrent pathologies. Generally, it is provoked by one or several viral or parasitic enteropathogenic agents, and causes diarrheal diseases [23]. Those diarrheal diseases are responsible for million diarrhea patients per year in the world causing the death of 525,000 among children aged between 0 and 5. [23]. However, among the bacteria species causing gastroenteritis, *Shigella* is the one which requires an antibiotic treatment. Most of the countries where shigellosis is endemic and/or epidemic have also a high number of HIV patients [24]. So far, classical treatments have been either the aminopenicillin or the Cotrimoxazole. Yet, for many years, strains of *Shigella* resistant to Aminopenicillin and to Cotrimoxazole have emerged and have become predominant [25-26].

Based on the above data, could not medicinal plants be sources of active biomolecules that can be exploited, available and affordable for our populations?

This issue thus prompted the objective of this study to research the *in vitro* activity of the aqueous and hydroethanolic extracts of *Morinda morindoides* on two clinical strains of *Shigella sp*.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Plant material

The plant material consists of the leaves of *Morinda morindoides*, washed beforehand and then slowly out from sunlight, at room temperature. This plant species was selected and collected in its natural habitat around the university Jean Lorougnon Guede in Daloa, a town in the Haut Sassandra region , in October 2018.

2.1.2 The microbial material and its culture medium

The microbial medium consists of two strains of *Shigella sp*. (1055; EEQ), involved in the gastro-enteritis and isolated from the stool of patients. They were provided by the laboratory of bacteriology and virology of the Regional Hospital Center of Daloa.

Some Mueller Hinton® agar (BK048) was used for the bacteriological tests, because they are recognized as the culture medium of reference for the study of the sensitivity of the bacteria to antibiotics [27].

2.2 Methods

2.2.1 Extraction of plant extracts

The dried leaves of the plant were smashed by a IKA® type mechanical pulverizer (LabortechniK Staufen; Germany: Janke & Kunkel).

One hundred grams (100 g) of plant powder was extracted in a liter (1 L) of distilled water by maceration in a BLG®-450 (blender), for 2 minutes. The resulting homogenate is filtered on cotton wool then on Whatman 3 mm filter paper successively. The resulting filtrate is dewatered with a Prolabo type oven at a temperature of 70°C to obtain the total aqueous extract (E. Taq.) [28].

As for the 70% ethanolic extract (E.ETH70 %), it is extracted from an absolute ethanol-water (70 :30 ; V/V), in the same conditions as the E.Taq..

For each obtained extract, the output was determined with the following formula [29-30]:

r_i (%) = (m_i/ m_0) x 100

ri : Output of the extract in percentage (%),
mi : Mass of the extract collected after the extraction (in g), (i=1: E.Taq.; i=2 : E. ETH 70%).
m0 : Mass plant powder used for the extraction (in g).

2.2.2 Preparation of the culture medium and the inoculums

- According to the maker, 38 g of dewatered powder of Mueller Hinton® agar (BK048) was dissolved in a liter (1 L) of distilled water. The mixture was boiled slowly and kept till the total dissolution and sterilized with an autoclave for 15 minutes at a temperature of 121°C. The agar was poured into Petri dishes and kept at a temperature of 4°C for later use.
- Preparation and numeration of the inoculum

From a stock culture, the strains of *Shigella sp*. codified (EEQ and 1055) were separately pricked out following the method of stria on Mueller Hinton (M.H) agar plates, then incubated for 18 to 24 hours [31]. The inoculum was prepared from a young colony of 18 to 24 hours perfectly isolated and emulsified in 10 mL of sterile distilled water in order to have a pre-culture. Following the homogenization, 1 mL of that pre-culture is added to 9 mL of sterile distilled water. That bacterial suspension was assessed in Colony Forming Units (CFU/mL) and is the 10^0 dilution or pure inoculum.

The numeration of the inoculum was carried out by successive decimal dilutions from the 10^0 dilution (pure inoculum) enabling to have the following dilutions 10^{-1}, 10^{-2}, 10^{-3} et 10^{-4}. Those various dilutions as well as the pure inoculum were sowed on the M.H agar (or box A) using a 2 µL caliber handle by 5 cm long stria, then incubated at 37° C for 24 hours. The microbial load of the 10^0 dilution is calculated following the formula below:

Stria of 10^0dilution → N_i (number of colonies)
Stria of 10^{-4} dilution → n_i

$N_i = n_i . 10^4$

Therefore, we obtain ni.10^4 colonies in 2 µl collected with a platinum loop. i.e. a microbial load of 10^7.ni/2 CFU/mL. Then the microbial load in each concentration range is: 10^7.ni/4 CFU/mL

n_i : Number of colony obtained on the stria of the 10^{-4} dilution of the box A.
N_i : Estimating the number of colonies on the stria of the 10^0 dilution of the box A.

2.2.3 Determining the Minimum Inhibitory Concentration (MIC)

The determination the MIC stemmed from the method described by Okou et al. [32]: from the two plant extracts obtained, concentration ranges (from 100 to 1.56 mg/mL) were prepared (following the method of two-fold dilution). In seven test tubes numbered 1 to 7 with sterile distilled water. Then, 1 mL of bacterial pure inoculum (10^0 dilution) was added to each concentration range: these are test tubes. A tube named negative or sterility reference was prepared with just 2 mL of distilled water.

At the same time, other concentration ranges were prepared in the same conditions to which 1 mL of distilled water was added. Those ranges are used as reference for each tube test. Following the incubation of the tube-tests and reference tubes at 37° C for 18 to 24 hours, some turbidity of cultures in the test tubes compared with reference-tubes was noted macroscopically.

2.2.4 Determining the Minimum Bactericidal Concentration (MBC)

The MBC is the lowest concentration of plant extract that leaves at most 0.10% of bacteria alive. Using a 2 µL caliber handle, the content of seven (7) tubes was collected and sown on a Mueller-Hinton agar plate (Box B). The sowing was carried out in the same conditions as those of box A. The colonies on the stria were counted 18 to 24 hours following the incubation in an oven at a temperature of 37°C.

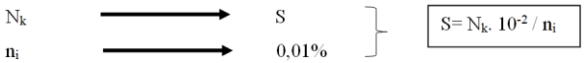
The number of the obtained colonies was expressed in percentage (%) in relation with the number colonies noted on the stria of the numeration 10^{-4} of box A (that corresponds to 0.01% of survivors). Thus, the concentration of the first experimental tube which number of colonies present on the stria is inferior or equal to that of the 10^{-4} dilution corresponds to the MBC. The ratio MBC/MIC was determined after the determination of those two antibacterial parameters (MIC, MBC).

In sum, three tests were carried out for each test in the same condition for the determination of the MIC and the MBC. That corresponds to six (6) numeration tests for each bacterial strain.

According to Marmonier, [33], if the ratio MBC/MIC ≤ 4, the tested substance is bactericidal; and the

ratio MBC/MIC > 4, the tested substance is bacteriostatic.

The calculation percentage method (S) of the survival originated from the one described by Zirihi et al. [34]. However, in this study, the percentage was calculated in relation to 100% of colonies on the stria of the numeration 10^0, contrary to Zirihi et al. [34] who assessed that percentage in relation to 100% of survival of a growth reference. That percentage is calculated following the formula below:



N_k: Number of colonies counted on each stria of box B.
n_i : Number of colonies obtained on the stria of 10^{-4} dilution of box A.
S : Percentage of survival

- Data processing

The percentages obtained after the counting of colonies on the stria of box A were computerized and processed by the Excel Software 2016.

3. RESULTS AND ANALYSIS

3.1 Output and Aspects of the Extracts Obtained

From one hundred (100) grams of powder of *M. morindoides*, it was prepared with distilled water and 70% ethanol, E.Taq. and E.ETH70% respectively. The obtained extracts are brownish with a spicy smell. The resulting masses are, m_1 = 12,18 g corresponding to the average output r_1 = 12.18 % for the E. Taq. and m_2 = 7.28 g, with an average output r_2 = 7.28 % for the E.ETH70 % respectively. The outputs of those various extractions, with their corresponding masses are presented in Table 1.

3.2 Result of Numeration

The numeration of the inoculum consisted in judging the shape of colonies and in determining the number of colonies contained in the pure inoculum of two strains of *Shigella sp*. (EEQ et 1055). The shapes of colonies and the number of colonies were compared for each strain. Out of the six numeration tests, the number of colonies decrease gradually, from the stria of the pure inoculum (10^0 dilution) till 10^{-4} dilution for the two strains. The number of average colony obtained on the stria of 10^{-4} dilution is n_1 = 4 colonies for strain 1055 and n_2 = 5 colonies for strain EEQ. The number of colonies calculated for the stria of the inoculum is $N_1 = 4.10^4$ colonies for the strain 1055 i.e. a bacterial load of 2. 10^7 CFU/mL in the pure inoculum, and 10^7 CFU/mL in each test tube. For the strain EEQ, $N_2 = 5.10^4$ colonies, i.e. a bacterial load of 2.5. 10^7 CFU/mL and a bacterial load of 1,25. 10^7 CFU/mL in each test tube. A table has been drawn to show the average number of colonies of each dilution (Table II). The observation of the colonies of the two strains of *shigella* revealed two round shapes, smooth, with regular edges after 18 to 24 hours in agar medium.

3.3 Determining the Minimum Inhibitory Concentration (MIC) in a Liquid Medium

During this work, the antibacterial activity was assessed by observing the inhibitory power of the E.Taq. and of the E.ETH 70% at various concentrations, on two clinical strains of *shigella* (1055 and EEQ). Turbidity due to the growth of bacterial germs in the experimental tubes was macroscopically noted. That turbidity indicating the presence of *shigella*, decreases gradually as the concentration of extracts increase, in relation to the reference tubes. Three tests were carried out in the same conditions and the absence of turbidity was noted for the three tests from a concentration of 50 mg/mL for the action of the E.Taq and of 25 mg/mL (MIC values) for the action of the E.ETH 70% on the two bacterial strains under study (*shigella* sp. 1055 and EEQ) up to the highest concentration of the two-fold dilution (i.e. 200 mg/mL).

Concerning the concentration inferior to 50 mg/mL of the E.Taq. and at 25 mg/mL for the E.ETH 70% , it was noted that the turbidity increases until the lowest concentration of the two-fold .

3.4 Determining the Minimum Bactericidal Concentration (MBC) on Agar

Following reading (determining) the MIC, the contents of the experimental tubes were sown on agar on petri dish (Box B) by stria of 5 cm.

Colonies were counted by means of direct counting following 18 hours of incubation in percentage (%) in relation to the colonies present on the stria of numeration 10^{-4} , corresponding to 0.01 % of the number of colonies present on the stria of numeration of the pure inoculum. Those results indicate a gradual decrease in the number of colonies of each strain, of the lowest concentration until 50 mg/mL for the E.Taq and 25 mg/mL for the E.ETH70 % (MBC values).

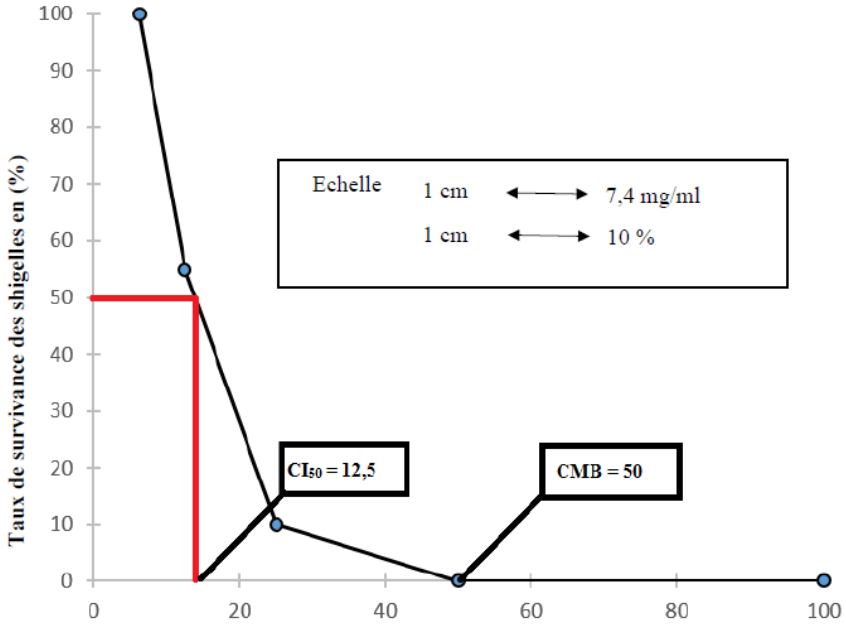
Table 1. Output of extracts

***Morinda morindoides* extract**	**Mass of plant powder : m_0 (g)**	**Average of the masses of each extract: m_i (g)**	**Output (%)**
(E.Taq.)	100	12,18	12,18
(E.ETH70 %)	100	7,28	7,28

Table 2. Average number of colony in each dilution

Shigella sp Strains	**Numeration of the inoculum**				
	Pure Inoculum (dilution d = 10^0)	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**
			Colonies		
Strain 1055	++++	+++	++	50	4
Strain EEQ	++++	+++	++	42	5

+ : lurking colonies: impossible to count



E. Taq Concentration. (mg/mL)

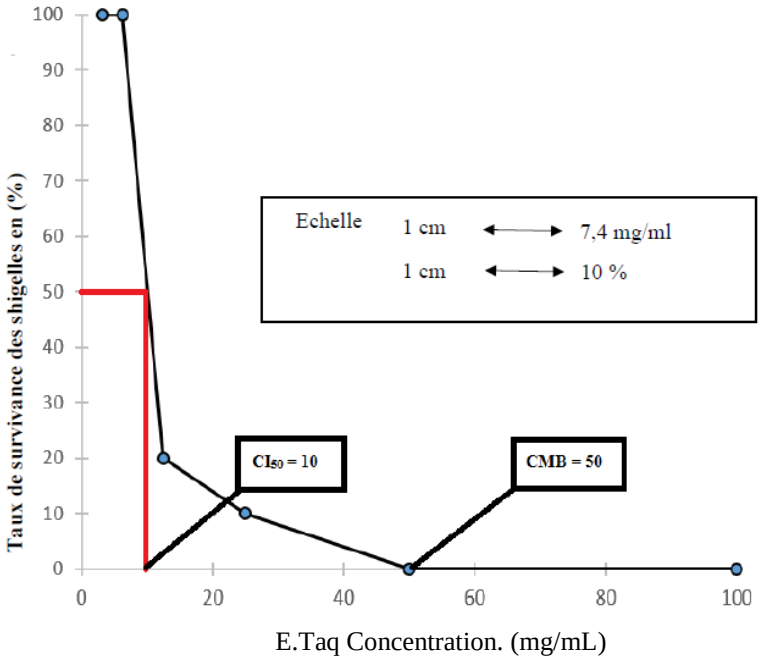
Graph 1. Sensitivity of the *shigella* EEQ to the E.Taq of *Morinda morindoides*

Table 3. Values of the Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentration (CMB) of E.Taq .and 70% E.ETH of *Morinda morindoides* in reltion to the strains of *Shigella* sp. (EEQ et 1055)

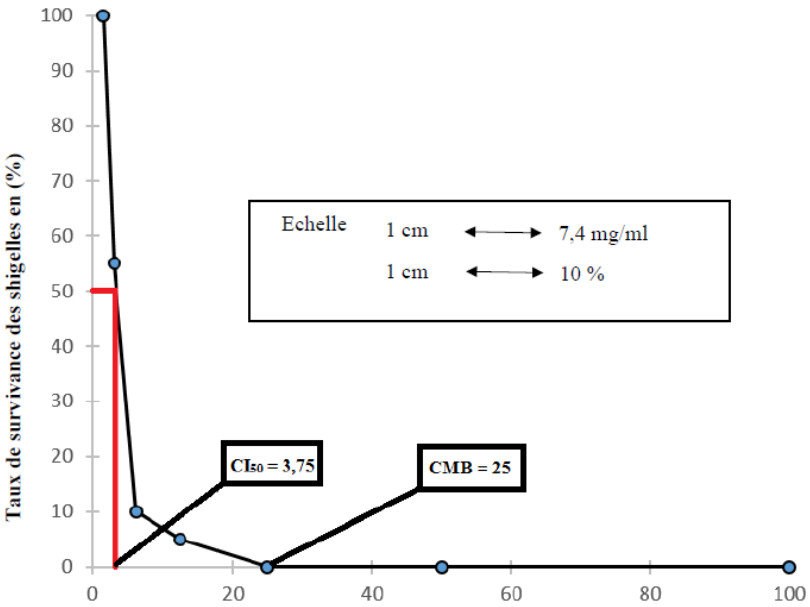
Extract	***Strains of Shigella sp.***	**Antibacterial Parameters (mg/mL)**		**Report**	**CI_{50}**	**Report**
		MIC	**MBC**	**MBC/MIC**	**$MBC_{E.Taq}$ /$MBC_{E.ETH\ 70\%}$**	
E.ETH 70%	*Strains 1055*	25	25	1	6	2
	Strains EEQ	25	25	1	3,75	2
E.Taq.	*Strains 1055*	50	50	1	10	2
	Strains EEQ	50	50	1	12,5	2

E.ETH 70%: *70% Ethanolic extract of Morinda morindoides. E.Taq : Total aqueous extract of Morinda morindoides*

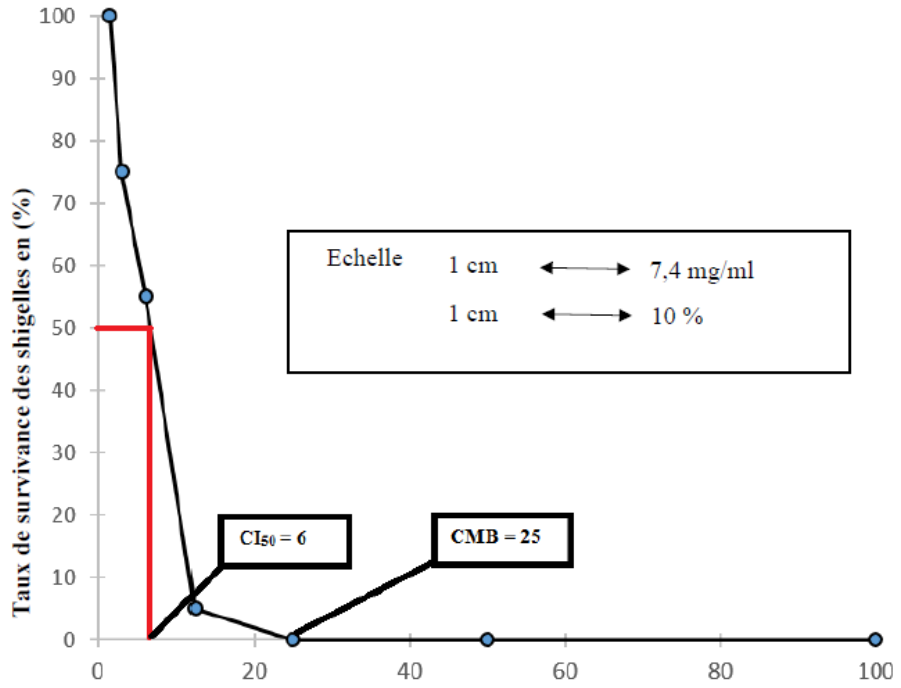
To better judge the antibacterial activity, the experimental data were presented in form of graph of survival. The activities of all the extracts were also compared on the basis of the MBC and MIC. The same values of antibacterial parameters of each extract of *Morindoides*, i.e the Minimal Inhibitory Concentration (MIC) and the Minimal Bactericidal Concentration (MBC), being experimental values were also obtained graphically. To those two values was added another antibacterial parameters obtained from the graph: That's all about the $CI_{50,}$ which indicates the concentration of plant extract for 50% of inhibition (i.e. CI_{50} = 12,5 mg/mL and CI_{50}= 10 mg/mL for E.Taq ; CI_{50} = 3.75 mg/mL and CI_{50} = 6 mg/mL for E.ETH 70% on the strains of EEQ and 1055 respectively. (Table 3).



Graph 2. Sensitivity of the *shigella* 1055 to the E.Taq of *Morinda morindoides*



Graph 3. Sensitivity of the *shigella* EEQ to ETH70% of *Morinda morindoides*



Graph 4. Sensitivity of the *shigella* 1055 to the ETH70% of the *Morinda morindoides*

The reports of efficiency between the tested extracts were assessed, on the basis of MBCs and also the ratio MBC/MIC which certify the efficiency of each extract. Those experimental data described under the forms of antibiogram graphs are outlined by graphs 1, 2, 3,4.

Generally speaking, all the graphs obtained outline a regular decrease, with more or less rise depending on the extracts. All the graphs cross the X-axis at various levels, corresponding to different values of MBC of each extract from a strain (*Shigella* EEQ or 1055).

4. DISCUSSION

In this study, two types of extractions were carried out and from obtained extracts, the average output was calculated in relation to the plant powder. The subsequent output for the E. Taq (r = 12.18 %) is higher than that of the E.ETH70% (r = 7,28 %). That could mean that the E.ETH70% contains less macromolecules than the E. Taq . The output values of the E. Taq and of the E.ETH70%, taken in this context confirm those of Koffi et al. [35]: their studies on this same plant species which average output are 13.915 % and 8.66 % respectively for the E.Taq and the 70% E.ETH ~~is~~ are nearly identical.

The counting of the colonies on box (A) of the numeration helped determine an approximate value of the microbial load of 10^0 dilution and the microbial load present in the different tubes containing the various tested concentrations or experimental tubes. Thus, the values obtained are higher than the minimal of the infecting *Shigella.* That number is 10^2 bacteria after oral penetration [36]. That helps us state that the quantity of *shigella* present in the concentration ranges is enough to infect a person.

The morphology (round, smooth, regular edges) of the strain colonies of *shigella* noted during this study is identical with the one described by Ibrahim [37].

The analysis of the results from the antibacterial activity of the E.Taq and the 70% E.ETH on the *in vitro* growth of the clinical strains of the *shigella* (EEQ and 1055) revealed that those bacterial germs (*shigella*) are sensitive to these two extracts of *M. moriendoides* tested at various levels. That sensitivity is outlined by a gradual decrease in the rate of the survival of the *shigella*, backed by a gradual decrease in the sensitivity curve of the bacterial germs to the tested extracts when their concentrations increase, as proved by KOFFI et al. [35]. That gradual decrease in the survival rates outline dose-dependent actions of the tested extracts.

This analysis also reveals a better antishigella activity of the 70% E.ETH in relation to the E.Taq, supported by the antibacterial parameters. In fact, the 70%

E.ETH, having the best inhibitory activity (against the EEQ and 1055 strains respectively), reveals the lowest values of MIC, of MBC or of CI_{50} (i.e. CMI = CMB = 25mg/mL and CI_{50} = 3,75 ; CI_{50} = 6 mg/mL vs.CMI = CMB =50 mg/mL and CI_{50} = 12,5 ; CI_{50} = 10 mg/mL) for the E.Taq.. That better antishigella activity of the 70% E.ETH in relation to the E.Taq is supported by the rise of the sensitivity graph of *shigella* to that extract, as proved by ACKAH et al. [20] : this rise is sharper (concerning the curve that outlines the sensitivity of the *shigella* [EEQ ; 1055] to that extract), compared with the rise of the other sensitivity curves of the bacterial germs to the E.Taq. They decrease gradually, because they depart gradually from the Y-axis.

On the other hand, the reports on efficiency of those extracts based on the MBC, ($CMB_{E.Taq}$/ $CMB\ 70\%_{E.TH70\ \%}$), reveal that the E.ETH70 % is two times more active that the E.Taq..That difference in the activity can be linked to the difference in the concentration of the various active chemical groups present in those extracts. It should be recalled that the use of water as extraction solvent is the first method of use of that plant [9]. Thus, the association of the ethanol with water would help increase the therapeutic potential of the extracts.

Considering the results of the studies carried out by other authors on that same plant and on the clinical strains of *Escherichia coli*, responsible for childhood gastroenteritis, our results are in harmony with theirs, on the antibacterial power of that plant [13]. But these works present some MBCs which are superior to the MBC obtained by those researchers (i.e. CMB = 7.5 mg/mL). That difference can be linked to the conditions of extractions or more, the difference in the sensitivity of the *Escherichia coli* and of the *Shigelles* to drugs. It should be recalled that the activity of a plant substance depends on many factors including the mode of extraction and the concentration in active ingredient [38].

According to Marmonnier [25], those two extracts have bactericidal antibacterial activity because the ratio MBC/MIC= 1 is inferior to four (4). That antishigella activity can also be explained by the intercalating power of the *Morinda morinddoides* compounds as Moroh [39] has already proved according to a qualitative and quantitative approach by the methods of intercalation competition with genomic DNA of compounds with the Syber Gold.

Besides, the 70% ethanol which was the extracting solvent of the extract with the best antishigella activity, is one of the most conducive for the concentrations of some compounds with antishigella properties.

5. CONCLUSION

The reinforcement of the antimicrobial gear motivated this very study and helped show the antibacterial activity of the two extracts of *Morinda morindoides* tested on the two clinical strains of *shigella* under study: EEQ and 1055.

The results obtained revealed that the two tested extracts of *Morinda morindoides* have a bactericidal antibacterial activity on the strains under study. That bactericidal action noted is dose dependent because it is linked to the increase in concentrations of extracts. The values of MBC and of MIC of the two extracts obtained are identical for each *shigella* strain (EEQ and 1055). Besides, the activity of the E.ETH 70% is twice superior to that of the E.Taq. Thus, it would be quite possible to state that, of the two types of extracts of the leaves of *Morinda morindoides*, 70% E.ETH has more bioactive molecules than the E.Taq on the two strains of *shigella* EEQ and 1055.

This proven activity on those enterobacteria (*shigella* EEQ and 1055) therefore justifies the traditional use of those leaves to treat gastroenteritis, and in the context of the diarrheic syndrome in the area of Daloa (Côte d'Ivoire).

6. IN PERSPECTIVES

On the basis of the promising findings presented in this paper, work on the remaining issues is continuing and will be presented in future papers. Thereby, it would be convenient to carry out other investigations, in particular:

- Further study of the issue of chromagraphy on column and on thin layer of the extract of E.Taq and of 70% E.ETH would be of interest in order to isolate the active molecule and improve the antishigella activity.
- Compare the activity of the active molecule found with that of the reference classical molecules.
- Extend the antibacterial study to other bacterial germs in order to determine the specter activity of this plant.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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