Original Research Article

MICROBIOLOGICAL CONTROL OF THIRTY TRADITIONAL HERBAL FORMULATIONS MARKETED IN DIVO CITY IN THE CENTER WESTERN OF CÔTE D'IVOIRE

ABSTRACT

Herbal medicinal products are widely used by the population. Unfortunately, the majority of these remedies in Côte d'Ivoire were not tested for efficacy, safety and quality. This work aims to investigate the microbial quality of some traditional herbal formulations produced and sold in Divo city. For this study, 30 samples of liquid remedies were collected in 2017 and analyzed in the goal to check potential contaminations by pathogens. Microbial analysis was achieved according to method based on standard procedures for food safety in specific culture media. The results showed that the contamination level of yeasts and molds were evaluated between $1.3x10^4$ to $7x10^5$ CFU/g. The level of contamination of total coliforms, aerobic mesophilic flora and fecal streptococci varied from $1.5x10^4$ to $7.5x10^5$ CFU/g; $1.6x10^4$ to $5x10^5$ CFU/g then $1.6x10^4$ to $4.3x10^5$ CFU/g respectively. Nine (30%) of the samples were contaminated by total coliforms, one (3.33%) was contaminated by mesophilic aerobic mesophilic microorganisms, fourteen (46.66%) were contaminated by fecal *streptococci* and fourteen (46.66%) were contaminated by yeasts and fungi. Therefore, the use of these herbal remedies could generate risks of toxi-infection for consumers. It is necessary to train practitioners of traditional

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medicine on Good Manufacturing Practices in order to produce quality and safety herbal remedies for the market

Keywords: Herbal formulations, Contamination, microorganisms, Standard

1. INTRODUCTION

Since the earliest days of the human species, plants have been essential elements in human life through their various uses including construction, nutrition and health. These uses became cultural for some populations who usually take them for their different needs [1, 2, 3]. In these last decades, the interest of the populations for the herbal remedies is more and more increasing. Recognition of their clinical, pharmaceutical and economic growth continues to grow. Indeed, according to World Health Organization, 80% of the world population particularly in the developing countries rely on non-conventional medicines mainly of herbal sources in their primary healthcare [4]. WHO has described traditional medicine as one of the alternative means to achieve total health care coverage of the world's population. WHO encouraged member states to develop sector of traditional medicine uses by proposing technical guidelines for the assessment of herbal medicine [5].

In Côte d'Ivoire, there are numerous herbal medicinal products in the market but there isn't data on their efficacy, safety and pytochemical composition. To ensure surest promotion of these herbal formulations in the country, the Ivorian parliament voted the law number 2015-536 which organizes activities of practitioners of traditional medicine. Unfortunately, National Program of Promotion of Traditional Medicine (PNPMT) of health ministry noticed that the majority of practitioners didn't perform their herbal formulations. This situation could provoke some healthy risks due to factors such as adulteration, substitution, contamination, lack of standardization and incorrect preparation of these remedies [6]. The consumption of hazard remedies could cause diseases such as hepatic, renal, cardiovascular diseases and some infections lied to pathogen germs. With the prevalence in the Ivorian market of herbal formulations, it would be a great interest to investigate some parameters of safety of these products.

Thus, in the present study, the microbial quality of 30 herbal remedies sourced from recognized and non-recognized practitioners by NPPTM in Divo in South western Côte d'Ivoire has been evaluated.

Microbial quality of some herbal medicines have been already carried out in the district of Abidjan in Côte d'Ivoire by Kroa *et al*. [7] and Bakary *et al*. [8]. However, data relating to the microbiological control of herbal formulations sold in the town city Divo renowned for being an area of strong practice of traditional medicine were little documented. Firstly, this work plans to identify some herbal formulations produced by practitioners of this city and secondly numerate potential germs present in their products.

Data collected from this study could be used to organize seminar and workshop in the goal to train practitioners on Good Manufacturing Practices (GMP) of herbal medicinal products

2. MATERIAL AND METHODS

2.1. MATERIAL

It is composed of herbal medicines (liquid) produced by a traditional medicine center of Divo identified by the National Program for the Promotion of Traditional Medicine (PNPMT) and those which sold in the street. The sampling selected 30 traditional remedies produced by six practitioners. According to the manufacturer, the samples were codified as below:

- AGO code: 12 samples from a center recognized by the PNPMT;
- AKOUA code: 10 samples from a center recognized by PNPMT;
- PRO code: 01 sample from a center recognized by PNPMT;

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- OTA code: 01 sample from center not recognized by PNPMT;
- ASSA code: 02 samples from a center not recognized by PNPMT;
- and DIFI code: 04 samples from a .

2.2. METHODS

2.2.1. Collection

The drug samples were taken from various neighborhoods in the town of Divo, a town located in center western Côte d'Ivoire in the Lôh-Djiboua region.

2.2.2. Framework and period of the study

This study took place from November 2017 to February 2018, i.e. a period of four (04) months. It took place in two phases: The first phase consisted on collecting samples in the town of Divo and the second phase consisted on analysis of the samples in laboratory of PNPMT.

2.2.3. Sampling

2.2.3.1. Choice of criteria

The traditional remedies analyzed were selected according to the following criteria:
- Traditional remedies produced by traditional medicine centers recognized by the PNPMT (23 samples);
- Traditional remedies produced by practitioners unidentified by PNPMT and sold in the street (07 samples).

2.2.3.2 Sample size

A total of thirty (30) samples of traditional medicines in liquid form were collected from six (6) traditional medicine practitioners

2.2.4. Microbiological analysis

The detection and enumeration of germs were carried out according to the method based on standard procedures AFNOR [9] which use standard microbiology techniques. The enumeration focused on the mesophilic aerobic microorganisms (NF.V08-51), total coliforms (NF.V08-50), yeasts and molds (Standards XP-V08-059-Oct 1996) and the search for enterococci (fecal streptococci) (NF EN ISO 7899-1). Specific culture media where the germs grow better were used. Thus, the quantity of germs counted gives an idea of the microbial load. The control focused yeast and molds (YM), mesophilic aerobic microorganisms (MAM), fecal streptococci (FC) and total coliforms (TC).

2.2.4.1. Method of preparing culture media

The media which were used in this study are: Plate Count Agar (PCA, EMD MILIPORE CORPORATION, Darmstadt, Allemagne), Violet Red Bile Lactose Agar (VRBL, LABORATOIRE CONDA S.A, Madrid, Espagne), Sabouraud –Chloroamphénicol (LABORATOIRE CONDA S.A, Madrid, Espagne) and Bile Aesculine Azide (BEA, LIOFILCHEM SRL, Teramo, Italie). Buffered Peptone Water (BPW, (LABORATOIRE CONDA S.A, Madrid, Espagne) was used to prepare stock solution (SS) for enrichment. The techniques used are classic methods and refer to French (AFNOR) and international (ISO) standards. Culture media were prepared according to the manufacturer's instructions.

2.2.4.2. Preparation of samples

2.2.4.2.1. Preparation of stock solution (SS) and serial dilution

For its realization, 25 mL of the solution to be analyzed was taken aseptically and added to 225 mL of sterile Buffered Peptone Water (BPW) and homogenized for 2 minutes, and this solution was the stock solution (SS). For decimal dilutions, according to AFNOR standard, 1

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mL of SS was taken and added to 9 mL of sterile distilled water to have a 10^{-1} dilution. The successive decimal dilutions made it possible to obtain dilutions of: 10^{-2}; 10^{-3}; 10^{-4} 10].

2.2.4.2.2 Culture media inoculation method

To carry out this inoculation, 1 mL of the suspension from each decimal dilution was transferred aseptically into sterile Petri dish. Media as Plate Count Agar (PCA) or BEA or VRBL or Sabouraud-Chloramphenicol cooled in a 45°C liquefied water bath was added to the sample at the rate of 15 mL per dish. Then, rotary movements of the Petri dish were made in order to have a homogeneous distribution of the sample in the Petri dish. The dishes was subsequently closed and then left to stand on a perfectly horizontal surface until complete solidification of the first layer. After solidification, a second layer of 7 mL of media was added for further solidification. Finally, the dishes were turned over, and then incubated respectively at 30°C for 72 h for MAM (Procedure NF.V08-51), at 30°C for 24 h for the TC (Procedure NF.V08-50) and FC (Procedure NF EN ISO 7899-1) , then at 25°C for 5 days for YM (Procedure XP-V08-059-oct1996) [10, 11].

2.2.4.3. Expression of results

According to AFNOR standard [9], only dishes with more than 30 colonies have been selected for the calculation of the number of microorganisms in CFU/mL. Then, a comparison was made with acceptance criteria in microbiology for herbal medicinal products [12]. Depending on the values obtained, the traditional remedy could be deemed compliant or not compliant. The number of microorganisms was calculate with expression below:

$$N = \frac{\sum C.}{V\,(n1 + 0{,}1\,n2)\,d}$$

N: Number of microorganisms in CFU/mL
V: Volume of inoculum applied
ΣC: Sum of colonies in Petri dishes selected
n1: number of Petri dishes selected at the first dilution
n2: number of Petri dishes selected at the second dilution
d: dilution rate corresponding to the first dilution considered

3. RESULTS AND DISCUSSION

3.1 Microbiological analysis of remedies codified AGO

This analysis was carried out through Petri dishes in which each dilution was seeded on each respective medium at the surface. The results of the microbiological analysis of the samples codified AGO were presented in Table 1. The test showed absence of yeasts and molds in two (02) samples. On the other hand, eight (08) samples exhibited yeasts and molds in higher quantity than the acceptable limits while two (02) samples have a presence of yeasts and molds in acceptable values. At the level of fecal streptococci, seven (07) samples presented absence of streptococci while five (05) samples showed streptococci with a quantity greater than the acceptable values. As concern mesophilic aerobic, all the samples showed presence of these microorganisms in a number less than or equal to the acceptable values. All the samples (12) were contaminated by total coliforms.

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Table 1: Determination of the total viable bacterial and fungal counts (CFU/mL) in herbal medicine samples codified AGO

	Number of germs (CFU/mL)			
	Yeasts and molds 48 à 72 H 37°C	**Fecal streptococci 48 à 72 H 37°C**	**Mesophilic Aerobic Microorganisms 48 à 72 H 37°C**	**Total coliforms 48 à 72 H 37°C**
AGO 1	$5.9x10^5$	Absence	$5x10^5$	$7.5x10^5$
AGO 2	$2.9x10^5$	$2.3x10^4$	$3.9x10^5$	$5.3x10^5$
AGO 3	$1.8x10^5$	Absence	$2.7x10^5$	$1.5x10^5$
AGO 4	3.10^5	Absence	$3x10^5$	$1.4x10^5$
AGO 5	$1.1x10^5$	$2.6x10^5$	$3x10^5$	$1.3x10^5$
AGO 6	$3.6.10^5$	$2.6x10^5$	$3.8x10^5$	$4.6x10^5$
AGO 7	$7x10^4$	$4.3x10^5$	$4.2x10^5$	$2.5x10^5$
AGO 8	$3.7x10^4$	Absence	$2.3x10^5$	$27x10^4$
AGO 9	$2.9x10^5$	Absence	$2.1x10^5$	$5.6x10^4$
AGO10	Absence	Absence	$2.2x10^5$	$5.9x10^4$
AGO11	Absence	$1.0x10^5$	$2.6x10^5$	$3.2x10^4$
AGO12	$3.6x10^4$	Absence	$2x10^5$	$7.9x10^4$

3.2 Microbiological analysis of herbal remedies codified AKOUA

The results on microbiological analysis of these remedies were presented in Table 2 for ten (10) samples. Yeasts and molds were absent in seven (07) samples; they were present in two (02) samples in acceptable values but they were in unacceptable limits for one sample. At the level of fecal streptococci, the results showed that streptococci were absent in four (04) samples, and present in six (06) with values superior to the acceptable limits. Regarding mesophilic aerobic, three (03) samples showed absence of these microorganisms and seven (07) samples showed them in acceptable values. Total coliforms are absent in (05) samples while five (5) samples were strongly contaminated by these microorganisms. The remedy codified AKOUA 5 didn't show any microbial contamination.

Table 2: Determination of the total viable bacterial and fungal counts (CFU/mL) in herbal medicine samples codified AKOUA

Number of germs per millimeter of medication (CFU/mL)

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	Yeasts and molds 48 à 72 H 37°C	Faecal streptococci 48 à 72 H 37°C	Mesophilic aerobic microorganisms 48 à 72 H 37°C	Total coliforms 48 à 72 H 37°C
AKOUA 1	$2.7x10^4$	$1.9x10^5$	$2x10^5$	$5.2x10^4$
AKOUA 2	$1.3x10^4$	$6.5x10^4$	$1.6x10^4$	Absence
AKOUA 3	$5.6x10^4$	Absence	$2.66x10^4$	$3.5x10^4$
AKOUA 4	Absence	Absence	$7.05x10^4$	$5.8x10^4$
AKOUA 5	Absence	Absence	Absence	Absence
AKOUA 6	Absence	Absence	$4.4x10^5$	$1.2x10^5$
AKOUA 7	Absence	$3.1x10^5$	$4.5x10^5$	$9.9x10^4$
AKOUA 8	Absence	$3.4x10^5$	Absence	Absence
AKOUA 9	Absence	$2.4x10^5$	Absence	Absence
AKOUA10	Absence	$1.9x10^5$	$1.5x10^5$	Absence

3.3 Microbiological analysis of herbal remedies codified DIFI

This analysis concerned four (04) samples and data were mentioned in Table 3. It showed an absence of yeasts and molds in one (01) sample while three (03) samples were highly contaminated. Fecal Streptococci were absent in three (03) samples and present in one (01) sample in an unacceptable value. Regarding mesophilic aerobic microorganisms, three (03) samples showed a presence of these germs in acceptable values and one (01) sample exhibited the mesophilic germs in a strongst quantity. All samples showed the presence of total coliforms in acceptable values.

Table 3: Determination of the total viable bacterial and fungal counts (CFU/mL) in herbal medicine samples codified DIFFI

	Number of germs per millimeter of medication (CFU/mL)			
	Yeasts and molds 48 à 72 H 37°C	Fecal streptococci 48 à 72 H 37°C	Mesophilic aerobic microorganisms 48 à 72 H 37°C	Total coliforms 48 à 72 H 37°C
DIFI1	$1.25x10^5$	$1.6x10^4$	10^6	$4.9x10^5$
DIFI2	$7x10^5$	Absence	$3x10^5$	$5.2x10^5$
DIFI3	Absence	Absence	$3.5x10^5$	$9.3x10^5$
DIFI4	$2.7x10^5$	Absence	$2.4x10^5$	$4.3x10^5$

3.4 Microbiological analysis of herbal remedies codified ASSA, OTA and PRO.

This analysis concerned four (04) samples collected from three (03) traditional practitioners and data were presented in Table 4. The herbal products codified ASSA were represented with 02 samples. These samples didn't contaminated by Mesophilic aerobic microorganisms and total coliforms, one (01) sample showed absence of yeast/molds and fecal streptococci while these microorganisms were found in a highest quantity in the another sample. ASSA1 didn't show any contamination. The single herbal remedy codified OTA was contaminated only by total coliforms. The remedy codified PRO was strongly contaminated by Yeast/Molds and fecal streptococci, the level of mesophilic aerobic microorganisms was acceptable and total coliforms were absent in this sample.

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Table 4: Determination of the total viable bacterial and fungal counts (CFU/mL) in herbal medicine samples codified ASSA, OTA and PRO

	Number of germs per millimeter of medication (CFU/mL)			
	Yeasts and molds 48 à 72 H 37°C	**Fecal streptococci 48 à 72 H 37°C**	**Mesophilic aerobic microorganisms 48 à 72 H 37°C**	**Total coliforms 48 à 72 H 37°C**
ASSA1	Absence	Absence	Absence	Absence
ASSA2	$1.5x10^5$	$2.1x10^5$	Absence	Absence
OTA	Absence	Absence	Absence	$1.5x10^4$
PRO	$1.1x10^5$	$3.2x10^5$	$1.6x10^5$	Absence

Figure 1 presented the contamination (not compliant) level of the thirty remedies. Yeast and molds contamination was around 46.67% i.e for fecal streptococci, those of mesophilic aerobic microorganisms and total coliforms were 3.34% and 73.33% respectively. In addition, only two (02) remedies (6.67%) amongst the remedies didn't show any contamination due to the microorganisms, there were AKOUA 5 and ASSA 1. On the other hand, 28 samples marketed were contaminated with most of the germs. The mesophilic aerobic microorganisms were less implied in the contamination of these herbal products than the other microorganisms. The most important microbial contaminant was total coliforms.

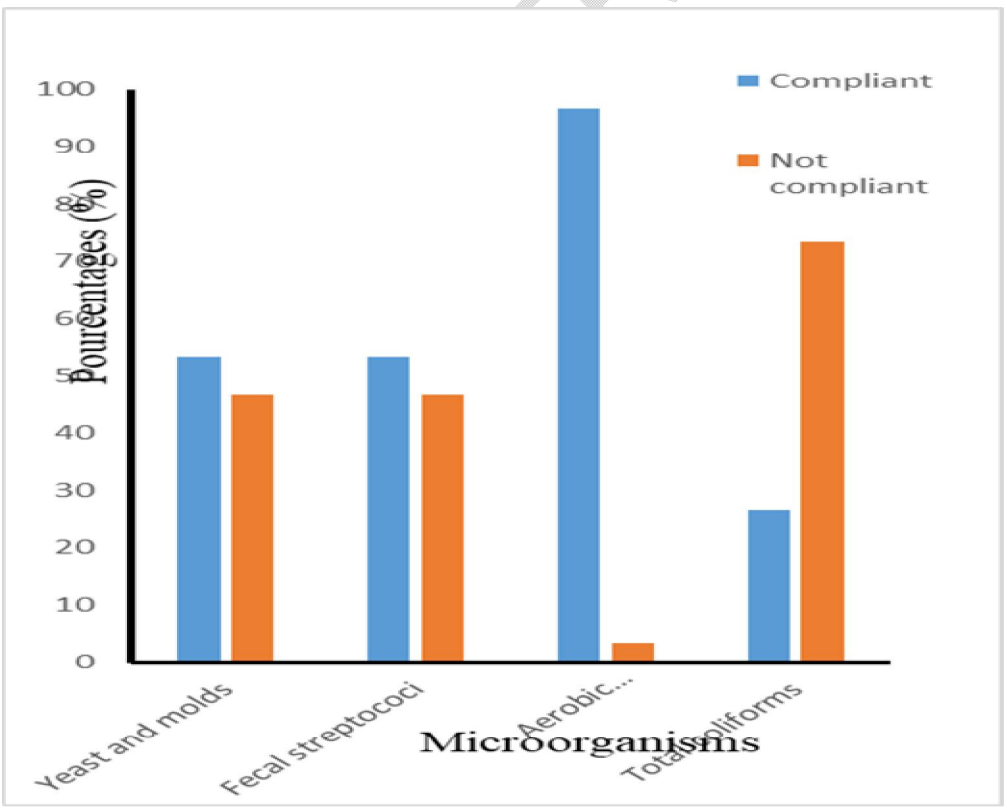

Figure 1: Histogram of contamination level of the thirty (30) remedies sampled

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The herbal medicinal products selected for this study were liquid forms. The choice to explore these forms was that herbal medicines in liquid pharmaceutical form for oral use presented the highest microbial contamination, and they were also the most consumed products. Presumably, the proliferation of microorganisms may result from the failure to control moisture levels of herbal medicines during transportation and storage, as well as from the failure to control the temperatures of liquid forms and finished herbal products. Moreover, most medicinal plants are prepared in an open environment in unhygienic conditions that gradually lead to contamination with enteric pathogens with public health importance [13]. All the samples were within their shelf life at the time of investigation. Twenty six (26) of remedies around 86.66 % were produced by traditional recognized by PNPM against 13.34% for center not identified by PNPMT. Herbal medicinal products usually contain bacteria and molds from soil and atmosphere. In addition, the quality of the water used in the preparation of herbal medicines may have contributed to the high level of microorganisms contamination observed for the herbal medicines analysed. Drinking water should be free from pathogenic microorganisms and bacteria that indicate fecal contamination [14, 15].

In this study, the limits of acceptable microbial contamination are: total mesophilic aerobic micro-organisms $5x10^5$CFU/g, yeasts and molds $5x10^4$ CFU/g, fecal streptococci and total coliforms should be absent [16,10].

In most cases, the presence of mesophilic aerobic microorganisms (MAM) in the samples could be explained by the absence of sanitary and hygienic condition during the production and a bad storage. This kind of contamination due to MAM could allow a rapid deterioration of the remedies and constitutes a risk for consumers [17]. In other studies, Agassounon et al [18] showed presence of mesophilic aerobic in herbal medicines with a load ranging between $1.04x10^2$ CFU/mL to $1.2x10^2$ CFU/mL. Coulibaly *et al*., [19] found also presence of these microorganisms in some herbal products collected in Abidjan (Côte d'Ivoire). These results demonstrated that MAM were usually present in herbal medicines sometimes above to limit. Thus, efforts have to be done by producers to limit contamination by MAM.

The important contamination observed was due to total coliforms which were intestinal bacterium and is an indicator of fecal contamination, revealing poor hygiene conditions in the preparation and storage of these herbal medicines. It could occur during production by contact with the producer or intermediate environment. A study that also evaluated the microbial quality of herbal medicines showed similar results, with 47.6% of samples contaminated with E. coli [20]. Recently in 2021; Allali *et al*. [21], analyzed some herbal medicines products marketed in Daloa (Côte d'Ivoire) and showed also presence of these micro-organisms.

Fecal streptococci are also indicators of fecal contamination like coliforms. They are important indicator of hygienic precariousness, inadequate processing or post-processing contamination. Pathogens of this family are potential causative agents of foodborne diseases, increasing the risk for consumers to develop intestinal tract infections [22, 23]. Since the detection of *E. coli* and fecal streptococci are indicative of fecal contamination, it can be concluded that the herbal medicines that contained these microorganism were contaminated directly or indirectly by human or animal feces and were therefore unsuitable for consumption [24]. It was known that contamination of herbal remedies with fecal streptococci and total coliforms is believed to be due to non-compliance with the application of Good Manufacturing Practices (GMP). Efforts therefore remain to be made in strengthening the capacities of craft production units through specific training.

The presence of Yeast and molds in the samples could be due to their presence on the raw material. This material was not probably well washed and dried before using for the production. The presence of fungal in herbal medicinal products were shown by Coulibaly *et*

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al., [19] and Allali *et al*. [21]on samples marketed in Abidjan and Daloa respectively with a load above limit. These contaminants were lied to important rate of humidity [25].
Apart to two (02) herbal products AKOUA 5 and ASSA 1, for which any microbial contamination was not observed, the others products were contaminated sometimes at level threshold compliant or not compliant. Soil, harvesting, drying, storage conditions and improper handling influence the microbiological quality of herbal drugs. The presence of microbial contaminant in unsterile herbal products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients taking the medicines [26]. Some infectious outbreaks have been associated with the use of heavily contaminated raw materials of natural origin.
Good Manufacturing Practices (GMPs) were observed during production of AKOUA5 and ASSA1. Unfortunately, the most of herbal product produced by traditional centers (AGO, AKOUA, PRO) recognized by PNPMT were contaminated, this suggested that these centers didn't apply guidelines to prepare herbal products (harvest, storage, treatment so on…). Thus; manufacturers should ensure the lowest possible level of microorganisms in the raw material, finished dosage forms and the packaging components to maintain appropriate quality, safety and efficacy of the products.
In addition, PNPMT has to reinforce their action in this town and follow activities of these centers in the way to improve their production. For the others centers (ASSA, PRO, OTA) not yet recognized by PNPMT, they need to be identified by PNMPT, and producers must be trained in Good Manufacturing Practices of herbal products. These actions could reduce the risk of microbial contamination or toxi-infection to consumers. As herbal medicinal products are complex mixtures which originate from biological sources, great efforts are necessary to guarantee a constant and adequate quality by carefully selecting the plant material and a standardized manufacturing process.
Regarding risk of intoxication for consumers, it is also essential to monitor establishments that market herbal medicines by checking that they have a license from the health authority for this trade and that the products are registered and authorized for consumption, as cases of falsification or commercialization of irregular herbal medicines may occur

CONCLUSION

Herbal remedies are substances or composition from vegetables used to treat or prevent diseases. This study aimed to carry out a microbial control on herbal products marketed in Divo city (Côte d'Ivoire) by evaluating quantity of Yeast/molds, mesophilic aerobic microorganisms, fecal streptococci and total coliforms.
It showed that on thirty (30) samples analyzed, only 02 products didn't present any contamination. The others herbal remedies were contaminated by the microorganisms at different level with compliant or not compliant values. Total coliforms were the most important contaminant with a percentage of contamination around 73.33%. Mesophilic aerobic microorganisms were less implied in the contamination than yeast/molds and fecal streptococci. In this study, remedies produced by the centers recognized by PNPMT or not presented a high level of contamination, therefore the majority of producers didn't follow Good Manufacturing Practices. There is, therefore, the need for constant monitoring and quality control of herbal medicinal products manufactured, marketed, advertised and used in Divo city. These results demonstrate important risks for consumers with the use of herbal medicines and the need for surveillance and the establishment of stricter control procedures in the production/ preparation and marketing of these herbal medicines to guarantee quality.
In the goal, to secure using herbal remedies, PNPMT must organize workshop training on GMPs for the different traditional medicine centers in Divo and raise awareness on the risk of consumption contaminated remedies. Microbial quality has to be built into the whole process beginning from the selection of propagation material to the final product reaching the consumer.

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