Physico-chemical and Microbiological Characterizations of the Leaf and Flower Powder of *Lippia multiflora Moldenk*, a Leaf Vegetable Consumed in Benin

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors HWD, AA and AD designed the study and supervised the work. Authors HWD, AT, JHH and ZB carry the experiments, analyzed and interpreted the data. Authors HWD, AA and GA wrote the manuscript. Authors AA and AD edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

**Background:** *Lippia multiflora Moldenk* is a shrub that is present in Benin. It is used as nutritious food and its leaves and flowers are rich in mineral salts and especially vitamin A and C. Also, these leaves and flowers transformed into powders, are sold and used to flavor sauces and for other purposes.

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Aim/Objectives: Our work aims to provide consumers with information on the sanitary and hygienic quality of the powders from the leaves and flowers of *Lippia multiflora* in order to determine their shelf life.

Methods: The study was carried out on three different powders, namely leaf-based powder; those from the flowers and those purchased from the sellers. Physico-chemical analyses and microbiological evaluation were made on these different powders at different dates for 18 months to see the growth of the microorganisms in these powders.

Results: These analyses revealed that the means of the physico-chemical parameters in the leaf-based powders, the flowers and those sampled are respectively: water content (7.42%; 9.89% and 11.67%); ash content (15.83%; 10.77% and 6.52%); pH (7.25; 6.22 and 5.95) and titrable acidity (0.55%; 2.46% and 1.84%). Microbial loads in total flora are below the standard for 18 months in leaf and flower powders but are above the standard in powders sampled after 12 months of storage. The growth of yeasts and molds was observed during the first 6 months and their load is well below the standards from the 7th month until the end of storage. Coliform, *Salmonella*, *Staphylococcus aureus*, faecal *Streptococcus* and *Clostridium* sulfito-reducers loads are below the detection threshold throughout the shelf life.

Conclusion: Therefore the powders based on the leaves and flowers can be kept for a period of eighteen months without risk to the consumer. But those sampled can be kept for up to 12 months.

Keywords: *Lippia multiflora*; physico-chemistry; microbiology; consumer and risk.

1. INTRODUCTION

Local leafy vegetables are cheap and easily accessible to many communities in rural, peri-urban and urban areas [1,2]. They are consumed worldwide according to their richness on nutritional component (vitamins, proteins and minerals) and medicinal properties [1-4] and also secondary metabolites that contribute to the well-being of the organism [5,6]. For this, leafy vegetables can be crucial for food, nutritional, health and income security for vulnerable urban populations [7,8]. The leafy vegetables are prepared in the form of sauces either with fresh leaves for some such as spinach (*Solanum macrocarpum* and *Celosia argentea*), or with dried leaves powdered for others such as *Cerathoteca sesamoides*, *Adansonia digitata*, *Corchorus tridens* and *Lippia multiflora* [2]. *Lippia multiflora Moldenke* is a frutescence aromatic plant up to 1-2 metres tall with whitish flowers and belongs to the verbenaecae family [9]. It is a species that grows naturally and preferentially on gravel soils and can be cultivated on various types of suns in different ecosystems [10]. *Lippia multiflora* is a plant whose inflorescences, leaves and roots are used as seasoning or medicine in Benin [11,12].

Considered as an accompanying vegetable, *L. multiflora* is used as a nutritious food with antibiotic, vermifuge and dysentery treatment [2,12,13]. As a result, the leaves and flowers of this leafy vegetable are dried and stored as powder in the preparation of multiple recipes [12]. In addition, powders from this plant are packaged in various packages and sold in rural and urban markets [12]. These powders obtained under questionable processing conditions do not always meet hygiene standards and therefore do not guarantee the health quality of consumers. To promote the consumption of *Lippia multiflora* powder, it is necessary to assess the health risks associated with the consumption of powders sold in our markets. It is within this framework that this research, which aims to determine the microorganisms contained in the samples of leaf and flower powders *Lippia multiflora* at different conservation dates, as well as the physico-chemicals of these powders in order to determine their likely shelf life for their use without risk of intoxication to consumers.

2. MATERIALS AND METHODS

2.1 Production of *L. multiflora* Leaf and Flower Powder

*Lippia multiflora* leaf and flower powders production design followed three major steps of simple unit operations. The leaves and flowers were sorted, washed and wiped. Then, they were dried in the room naturally ventilated. After drying, the leaves and flowers were ground, sifted and powdered. The resulting powders were immediately packaged in 15 kg opaque plastic buckets and left at room temperature. The powders produced did not undergo any other special treatment and were free of food additives. This method of production of foliar powders was
previously described by [14-16] in fruit and vegetable drying operations.

2.2 Sample Collection of *L. multiflora* Powder at the Market

Samples of powder packaged in bottles of *L. multiflora* were purchased from a saleswoman in Savalou market in central Benin. Samples stored in plastic bags previously crimped and kept at room temperature away from light at the laboratory of Food and Applied Nutrition Direction (DANA) in Porto-Nov. A total of three powder samples (leaf powder, flower powder and powder collected from the market) were analyzed to determine the micro-flora and physico-chemical characteristics of the powder from 7 days (T1), 6 months (T2), 12 months (T3) and 18 months (T4) after powder production or purchase.

2.3 Determination of Titrable Acidity, pH and Moisture and Ash Content of Powder

Titrable acidity and pH were measured on a suspension of 10 g powder in 90 mL distilled water as described by [17]. Inolab pH 730 device (WTW D-82362 Weilheim, Germany) calibrated with pH 4.01 (STP4, WTW, Germany) and pH 7.00 (STP7, WTW, Germany) standards was used for pH measurements. Titrable acidity expressed as a percentage of lactic acid was measured with a 0.1 N soda solution up to pH 8.5.

\[
\% \text{ lactic acid} = \frac{V (\text{mL}) \times \text{NaOH} \times 10^{-3} \times 90}{W (\text{g})}
\]

The moisture content was determined according to [18]. The ash content was determined by incineration according to the recommended standard [18].

2.4 Microbiological Analyses

Microbiological analyses were carried out in accordance with the requirements of the International Organization for Standardization (ISO). The stock solution was obtained using ISO 4833. Ten grams of the powders were aseptically collected from a sterile Stomacher bag and added 90 mL of Buffered Peptonized Water (FTE). The resulting mixture was homogenized in Lablemco brand Stomacher. Successive decimal dilutions were obtained by adding 1 mL of the stock solution to 9 mL of sterile ETP and so on until the desired dilution level was achieved.

2.4.1 Detection of total flora

The total flora was determined by the ISO 4831 method. Dilutions of $10^{-1}$ to $10^{-5}$ of the resulting mixture were seeded to the Plate Count Agar culture medium (PCA; Oxoid CM 325, England) and incubated at 30°C in a Memmert oven for 72 hours.

2.4.2 Detection of coliforms

Coliforms were determined using ISO 4832. The research is based on the use of the Most Likely Number (NPP). The Lactose soup with Glossy Green (BLBV; Oxoid CM 31) was used as an incubating medium and seeding was performed in the agar mass at 1 mL of the $10^{-1}$ and $10^{-2}$ dilutions. Incubation was done at 30°C ±1°C in a Memmert brand oven for 24 to 48 hours.

2.4.3 Detection of *clostridium*

They were searched according to ISO 7954. Five ml of the Bacto-Sulfite Agar medium and 1 mL of the stock suspension were pasteurized at 80°C in a Bosch autoclave for 10 minutes. The characteristic colonies of *Clostridium* are to be black (development of iron sulphide) and remain along or at the bottom of the tube.

2.4.4 Detection of positive coagulase *staphylococcus*

*Staphylococcus* was counted according to NF EN ISO 6888-1 (1999). 0.1 mL of the stock suspension was spread over the Baird-Parker agar (BP OXOID CM0275) supplemented with 50 mL egg yolk emulsion in 1000 mL culture medium. Incubation was conducted at 37°C for 24 h. *S. aureus* produces black, shiny, convex colonies, 1.5 mm in diameter, surrounded by a clear halo (proteolysis) from 2 mm to 5 mm.

2.4.5 Detection of faecal *streptococci*

The method used is that of ISO 7899. Faecal *streptococci* were tested in the stock solution and in dilution $10^{-1}$. They were grown in the middle of Rothe at 37°C for 24 hours. The positive tubes (the pellets remain at the bottom of the tube and disturbances) were transplanted into the Soup Ethyl Violet Azide and incubated at 37°C for 24 hours. The positive tubes were then confirmed on the Bile Esculine Azide (BEA) agar. Incubation was done at 37°C for 24 hours. The
characteristic colonies were small and translucent black.

### 2.4.6 Detection of salmonella

The method used is that of ISO 6579. It consists of adding to the EPT powder. Thus, the resulting mixture is considered to be the pre-enrichment that is incubated at 37°C ±1°C in a Memmert brand oven for 24 hours. Then, 1mL of the pre-enrichment mixture was incubated in 9 mL of Rapaport-Vassiladis and incubated at 37°C ±1°C in a Memmert brand oven for 24 hours. The resulting mixture was incubated at 37°C ±1°C for 24 hours on the HEKTOEN agar. The suspect colonies (colourless or colourless black center) were isolated again on the agar KLIGER (Hajna). Identification is performed using Xylose Lysine Decarboxylase (XLD) and Triple Sugar Iron (TSI) agar. The urease test was done to confirm Salmonella, knowing that Salmonella is negative urease. We finally move to serology for a specific serotyping.

### 2.4.7 Yeast and mold detection

Yeast and mold were determined according to ISO 7957. The culture medium used was oxytetracycline glucose d agar and seeding was done at 0.1 mL of the agar surface stock suspension and spread. Incubation was performed at 25°C in a Memmert brand oven for 5 days and was read daily. The yeasts, mucous and shiny colonies were taken over and reissued in a Memmert oven for 24 hours. The yeasts, mucous and shiny colonies were taken over and reissued in a Memmert oven for 24 hours. The yeasts were determined according to ISO 6579. It consists of adding to the EPT powder. Thus, the resulting mixture is considered to be the pre-enrichment that is incubated at 37°C ±1°C in a Memmert brand oven for 24 hours. Then, 1mL of the pre-enrichment mixture was incubated in 9 mL of Rapaport-Vassiladis and incubated at 37°C ±1°C in a Memmert brand oven for 24 hours. The resulting mixture was incubated at 37°C ±1°C for 24 hours on the HEKTOEN agar. The suspect colonies (colourless or colourless black center) were isolated again on the agar KLIGER (Hajna). Identification is performed using Xylose Lysine Decarboxylase (XLD) and Triple Sugar Iron (TSI) agar. The urease test was done to confirm Salmonella, knowing that Salmonella is negative urease. We finally move to serology for a specific serotyping.

#### Table 1. Titrable acidity, pH, moisture and ash contents of powders during storage

| Samples   | Parameters | Times | Mean     |
|-----------|------------|-------|----------|
|           |            | T1    | T2       | T3       | T4       |          |
| Leaves'   | Moisture   | 6.69±0.02 | 7.2±0.1 | 7.5±0.12 | 8.3±0.15 | 7.42±0.67 |
| powder    |            |       |         |          |          |          |
| Ash       | 13.7±0.01  | 15.02±0.04 | 17.06±0.1 | 17.54±0.01 | 15.83±1.79 |
| pH        | 7.4±0.01   | 7.3±0.02 | 7.2±0.03 | 7.1±0.04 | 7.25±0.13 |
| Titrable acidity(%) | 0.46±0.02 | 0.52±0.001 | 0.58±0.01 | 0.66±0.03 | 0.55±0.08 |
| Flowers'  | Moisture   | 7.71±0.03 | 9.23±0.02 | 10.34±0.4 | 12.10±0.5 | 9.86±1.85 |
| powder    |            |       |         |          |          |          |
| Ash       | 11.5±0.2   | 10.16±0.01 | 10.7±0.7 | 10.74±0.6 | 10.77±0.55 |
| pH        | 6.7±0.1    | 6.5±0.1 | 6.3±0.2 | 5.4±0.3 | 6.22±0.57 |
| Titrable acidity(%) | 2.05±0.02 | 2.25±0.02 | 2.55±0.01 | 3.02±0.01 | 2.46±0.42 |
| Market'   | Moisture   | 10.21±0.2 | 11.24±0.3 | 13.11±0.13 | 12.12±0.6 | 11.67±1.23 |
| powder    |            |       |         |          |          |          |
| Ash       | 5.6±0.4    | 5.4±0.2 | 7.26±0.4 | 7.83±0.1 | 6.52±1.2  |
| pH        | 6.5±0.01   | 6.2±0.01 | 5.6±0.02 | 5.5±0.01 | 5.95±0.48 |
| Titrable acidity(%) | 1.76±0.01 | 1.82±0.04 | 1.85±0.02 | 1.94±0.01 | 1.84±0.75 |

#### 2.5 Statistical Analyses

Difference between all parameters was evaluated using SNK test with STATISTICA V6. The values were accurate for a probability less than 0.05. Microbial load data were transformed into logarithm corresponding. Either N = a x b the microbial load expressed in Colony Forming Unit per gram (CFU/g) of product. Thus, the logarithmic transformation gives Log N = Log a + Log b.

### 3. RESULTS AND DISCUSSION

#### 3.1 Moisture and Ash Contents, Titrable Acidity and pH of Powders during Storage

The results of the physico-chemical analysis of the three different powders are presented in Table 1. No significant difference was observed between the three powders according to Student Newman Keul test. Nevertheless, the moisture content varies from 6.69% to 8.30% with an average of 7.42% in leaf powders and from 7.71% to 12.1% with an average of 9.89% in flower powders while in purchased powders, this rate is between 10.21% and 13.11% with an average of 11.67%. The moisture levels in the three powders are higher than that found by [16] in Moringa oleifera powder (2.72%) and lower than that obtained from Manihot esculenta powder (52.72%) reported by [20]. These relatively low levels of moisture in the three powders are an advantage for their preservation because low water content significantly limits the growth of microorganisms and thus the alteration of food [20].
Micro-organisms require mineral salts for their growth [21]. The high ash content is a reflection of the content of mineral salts in food [22]. The ash content also varies from 13.7% to 17.54% with an average of 15.83% in leaf powders, from 10.16% to 11.5% with an average of 10.77% in those of flowers and from 5.4% to 7.83% with an average of 6.52% in the powders purchased. These ash contents are high and are higher than that determined by [16] on Moringa oleifera powder (2.38%). These high levels could lead to the multiplication of certain microorganisms in powders.

The leaf powders are practically neutral with an average pH of 7.25 ± 0.13. This pH value obtained is substantially equal to that found by [16] on the base powder sheet of Moringa oleifera. Powders from flowers and those sampled have average pH of 6.22 ± 0.57 and to 5.95 ± 0.48, respectively. The values averages of three titratable acidities of the powders were from 0.55 ± 0.08% for sheets based powders; 2.46 ± 0.42% for those based on flowers; 1.84 ± 0.75% for those purchased. During storage, the pH decreases and the titrable acidity increases. This situation could result from growth of ferment microorganisms contained in large quantities in the different powders.

According to Amoa-Awua and Jacobsen [23], cassava is made to close majority of lactic acid bacteria, yeasts and of mold. Thus, the development of such microorganisms in cassava paste would accelerate the fermentation process, in particular the production of acid from the target fermenting sugars present [24,25].

3.2 Microbiological Characterizations of the Various Powders of L. multiflora

Evolution of microorganisms in the powders of the leaves, flowers, and those collected was monitored for 18 months of storage. Total aerobic mesophilic flora (FMAT) is a good indicator of contamination [26]. Thus, in the leaf powders, a total flora (Fig. 1) of 4.11 LogUFC / g was counted after 7 days of storage. It increases to 5.18 LogUFC / g after 6 months of storage then to 5.62 LogUFC / g after 12 months. After 18 months of storage, this total flora decreases to 5 LogUFC / g. The total flora after 7 days of storage is 4.24 LogUFC / g and drops after 6 months to 3.95 LogUFC / g. This flora goes back to 4.6 LogUFC / g and 5.69 LogUFC / g after 12 months and 18 months, respectively of storage in those based on flowers. In powders procured in market, the total flora increases gradually as the hard storage time and the end of the total flora storage is estimated at 5.9 LogUFC/g, value – above the norm tolerated. While the charges of the total flora in the powder leaves and flowers are below the standard which is of 5.7 LogUFC/ g but these powders purchased after 12 months of storage is - above the norm. According to [27], the aerobic mesophilic microflora is the set of microorganisms capable of multiplying in air and at average temperatures, more precisely those whose optimum growth temperature is between 25°C and 40°C. They are pathogenic bacteria for humans on the one hand, and spoilage microorganisms on the other. So the powders procured on the market are unhealthy for consumption after 12 months of storage.

From Fig. 2, yeast spends 1 LogUFC / g after 7 days of storage leaf powder 3 LogUFC / g value above the norm tolerated (2.7 LogUFC / g) after 6 months of storage. This trend is reversed and they are well below the norm until the end of storage in leaf-based powders. In flower-based powders, yeasts after 7 days of storage are estimated at 2.6 LogUFC / g. They drop after 6 months to 2 LogUFC / g and up to the end of the storage is the value of the yeast is below to the standard tolerated. In powders procured on market, this value after 7 days is estimated at 2.6 LogUFC / g. It increases after 6 months LogUFC 2.85 / g, this value is the - top of a standard. It falls and returns below the norm until the end of storage. So, there was the colonization of the three different powders by the yeasts and their total disappearance after 7 months of product storage. They remain such until the end of the observations. This phenomenon is explained by the fact of their rapid multiplication the first 6 months of storage. This multiplication is certainly inherent on the one hand to their commensality for plant products in general and on the other hand to the activity of the residual moisture of the product which gradually is eliminated and acts consequently on the survival of these microorganisms [28].

Referring to Fig. 3, in powder leaves, molds are estimated at 2 LogUFC / g after 7 days of storage and go after 6 months 3.46LogUFC / g value under the norm tolerated (5 LogUFC / g). Until the end of conservation their estimate is largely below the norm. As for the molds that are found in flowers powders, they pass 2.95 LogUFC / g, value under the standard after 7 storage days. They decrease after 6 months of storage to 2.47 LogUFC / g, value below the
standard. This decrease is observed until the end of storage. In bought powders, mold after seven days are estimated at 2.9 LogUFC / g. They increase after 6 months to 3.8 LogUFC / g, these values are under the norm tolerated. After 12 months of storage, they drop and remain well below tolerated standards until the end of storage.

Fig. 1. Evolution of the load on Flora Total Aerobic Mesophilic (FAMT) in the three powders
Fig. 2. Evolution of the load in yeasts in three powders.
Fig. 3. Evolution of the load in the mold in three powders
The high water and ash contents in powders of *Lippia multiflora* represent favorable conditions for the development of yeasts and molds. Because the humidity levels favorable to the evolution of molds remain in the range of 3.2% to 10.7% in relation to their water activity (aw) [29]. This significant development of microorganisms in these powders can affect the quality of the product. However, since yeasts and molds are not generally pathogenic, their presence is unlikely to affect the health quality of the product. Indeed, the loads below the coliform detection threshold for salmonella, of staphylococcus aureus, offaecal streptococci and clostridium sulphite-reducing which are pathogens recorded in the powders throughout the shelf life is due to the presence of lactic acid which would be unfavorable for the growth of these germs. Indeed, according to the work of [20,30], the presence of lactic acid constitutes a barrier to the growth of pathogenic microorganisms.

4. CONCLUSION

The powders from dried leaves and flowers of *Lippia multiflora* displays certain safety at the beginning of the storage until at least 18 months without risk of contamination for the consumer and has a grind of good hygienic quality. But powders bought from market cannot be stored for more than 12 months. The absence of pathogens (S. aureus, *Salmonella*) and flora indicative of faecal pollution (*Fecal coliforms* and reducing *Clostridium sulfito* spores) gives the product an acceptable quality. However, the presence of mold is not favorable for keeping the product for a longer period. The values of the physico-chemical parameters recorded during the storage of the different powders attest to a certain safety of these powders and confer quality assurance on them. Efforts to improve production conditions and standardization of the technological diagram of the powder must be continued in view of a longer storage.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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