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ORIGINAL ARTICLE

Effects of aqueous and hydro-ethanolic *Moringa oleifera* Lam leaf extracts on the cultivability of 2 *Bacillus* strains isolated from rainwater

Morelle Raïsa Djiala Tagne[a] / Mireille Ebiane Nougang[b] / Claire Stéphane Metsopkeng[a] / Geneviève Bricheux[c] / Florence Donnadieu[c] / Paul Alain Nana[d] / Isabelle Ripoche[e] / Nehemie Donfagsiteli Tchinda[f] / Gabriel A. Agbor[f] /Pierre Chalard[e] / Télesphore Sime-Ngando[g] /Moïse Nola[a]*/

Authors' Affiliation

[a]Faculty of Sciences, Laboratory of Hydrobiology and Environment, University of Yaounde I, Yaounde, Cameroon

[b]Zoology Laboratory, Faculty of Sciences, University of Maroua, Maroua, Cameroon

[c]Laboratoire « Microorganismes: Génome et Environnement » (LMGE), UMR CNRS 6023, Université Clermont Auvergne, Campus Universitaire des Cézeaux, 1 Impasse Amélie Murat, TSA 60026 - CS 60026, 63178 Aubière Cedex, France

[d]Department of Oceanography and Limnology, Institute of Fisheries and Aquatic Sciences, University of Douala, Douala, Cameroon

[e]ICCF UMR 6296, SIGMA Clermont, 20 avenue Blaise Pascal - TSA 62006, 63178 Aubière Cedex, France

[f]Institute of Medical Research and Medicinal Plants Study, P. O. Box 13033 Yaounde, Cameroon

[g]Laboratoire « Microorganismes: Génome et Environnement » (LMGE), UMR CNRS 6023, et Laboratoire Magmas et Volcans (LMV), UMR IRD 163. Campus Universitaire des Cézeaux, TSA 60026 - CS 60026, 63178 Aubière Cedex, France

Corresponding author

Moïse Nola

Email:

moise.nola@yahoo.com

Tel:

(237) 699 43 26 39

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Abstract

In most developing countries, rainwater is used by many people to meet their water needs. Many *Bacillus* species known to be harmful to humans have also been identified in rainwater samples. Improving the microbiological quality of the rainwater using modern techniques seems expensive in rural areas for poor people. Extracts from several medicinal plants are often used in rural areas to treat certain bacterial infections. Water and alcohol are often used in traditional therapy to extract the principles of medicinal plants. But little is known about their functional differences or similarities. This study aims to assess the effects of aqueous and hydro-ethanolic leaf extracts of *Moringa oleifera* Lam on the cultivability of *Bacillus cereus* and *Bacillus thuringiensis* isolated from rainwater. Leaf extracts of concentrations 2, 1.5, 1 and 0.5 g/L in contact with cells were incubated at 23°C and 37°C for 3 h, 6 h, 9 h and 24 h. The results showed a decrease in cultivable cells abundance. In most cases, cell abundances between the aqueous extract and hydro-ethanolic extract were not significantly different ($P>0.05$). A significant difference ($P<0.05$) was noted amongst cell abundances from each incubation period to another. The effectiveness of each extract for cell inhibition was evaluated and then expressed as a decrease percentage in the number of cultivable cells. A decrease in cultivable *B. thuringiensis* abundance varied from 75.09 to 99.68% in hydro-ethanolic extract and from 70.75 to 97.94% in aqueous extract. That of *B. cereus* varied from 0 to 98.86% in hydro-ethanolic extract and from 41.09 to 92.20% in the other. This difference could be linked to the differences in the cell species intrinsic properties. It could also be linked to the presence of some compounds identified. Some of these compounds would act as nutrients for cell growth, counterbalancing cell inhibition caused by others that have antimicrobial effects.

Practical application

M. oleifera leaves extract could nevertheless be exploited as an alternative to the microbiological treatment of rainwater, mainly against *B. thuringiensis* in particular, for household use in rural areas. However, further work is essential to clarify the effective constituents inactivating bacteria in the extracts, their potential impact on the water organoleptic properties as well as on human health.

Key words: *Bacillus* strains, cells cultivability, extracts antimicrobial compounds, *M. oleifera* leaves extracts, rainwater

1. Introduction

The microbiological treatment of drinking water proceeds by various methods sometimes using panoply of chemical reagents and materials whose acquisition costs are sometimes high. Several products currently used in the bacteriological treatment of drinking water by municipalities or public authorities sometimes leave disinfection by-products which have long-term health risks for water consumers. These by-products derive from the interactions between these products added to water and various chemical compounds present in natural water. If the treatment is ineffective, the eradication of bacteria in the event of an infection generally requires the use of antibiotics. The bacteria are increasingly varied and above all have become more resistant to antibiotics leading an important public health problem (Chen *et al.*, 2022; Wang *et al.*, 2022). This microbial antibiotic resistance varies from one antibiotic to another and from one individual to another. It is sometimes linked to the poverty of the populations in certain regions of the world. The use of the phytotherapy thus seems as an alternative to conventional antibiotics.

In some Asian and African countries, about 80% of the population use medicinal plants to treat various health problems (Salmeron-Manzano *et al.*, 2020). According to Metsopkeng *et al* (2020), medicinal plants are consumed in the form of infusion, decoction or maceration, and solvents are often water or alcohol. These medicinal plants are of various species, genera and families. Species of the genus *Moringa* are widely used throughout the world in the form of decoctions and macerations (aqueous and alcoholic) of the different parts of the plant (Sen & Samanta, 2015). The genus *Moringa* belongs to the superkingdom of Tracheobionta, superdivision of Spermatophyta, division of Magnoliophyta, class of Magnoliopsida, subclass of Dilleniidae, order of Capparales, family of Moringaceae (Mallenakuppe *et al.*, 2019). They includes 13 species (*Moringa oleifera, M. Stenopetala, M. arborea, M. borziana, M. concanensis, M. drouhardi, M. hildebrandtii, M. longituba, M. ovalifolia, M. peregrina, M. pygmaea, M. rivae* and *M. Ruspoliana*), some of which are endemic to Africa (Arora *et al.*, 2013; Laleye *et al.*, 2015). These species produce secondary metabolites which depend on the general physiological properties of the plant (Li *et al.*, 2020).

Several parts of *Moringa* have nutritional benefits. The leaves, for example, are an excellent source of protein. They have several essential amino acids for humans (histidines, arginines, leucines, lysines, phenylalanines, treonine, tryptophanes, tsoleucine) and a relatively high content of metabolizable energy (Laleye *et al.*, 2015; Islam *et al.*, 2021). There are also significant concentrations of vitamins A, B, B1, B2, C, E, ß-carotene and several mineral elements (iron, calcium, zinc, selenium, magnesium, manganese, molybdenum, potassium, sodium, among others) (Adouko *et al.*, 2019; Gonzalez-Burgos *et al.*, 2021). Their seeds rich in oils and proteins have neuroprotective properties against oxidative stress and provide necessary nutrients for a healthy diet (Gonzalez-Burgos *et al.*, 2021). The *M. oleifera* Lam extracts are sometimes used to fight against some microorganisms of health importance including *Escherichia, Aspergillus* and *Candida* cells species (Abd El-Hack *et al.*, 2022; Metsopkeng *et al.*, 2020, 2022).

Many of the bacterial species can be found in the atmosphere in the form of bioaerosols and exhibit pronounced biogeography, driven by a combination of biotic and abiotic factors (Santl-Temkiv *et al.*, 2022). In several regions of the world, the presence of enterococci and other

bacteria of the genera *Escherichia, Clostridium, Aeromonas, Campylobacter, Salmonella* as well as *Giardia lamblia*, has been reported in rainwater (Bitton, 2014). Many *Bacillus* species have also been identified in rainwater samples and their abundance undergoes spatio-temporal variation (Djiala *et al.*, 2022).

Bacteria species, especially the genus *Bacillus* are rod-shaped bacteria. They are gram-positive, aerobic or anaerobic, and are widely found in soil and water. They can form dormant spores under adverse environmental conditions. Some types of *Bacillus* bacteria are harmful to humans. *B. cereus* for example sometimes causes spoilage in canned foods and food poisoning of short duration (Dietrich *et al.*, 2021). *B. subtilis* is a common contaminant of laboratory cultures and is often found on human skin. Others *Bacillus* strains are not pathogenic for humans but may infect humans incidentally (Esmkhani & Shams, 2022; Rollan-Martínez *et al.*, 2022).

In many developing countries around the world, both in rural and urban areas, rainwater with all its microbial and non-microbial pollutants, is used without any prior treatment by a large part of the population to meet water needs. Under natural conditions, warm water vapor rises up through Earth's atmosphere. As this water vapor rises higher and higher, the cool air of the atmosphere causes the water vapor to turn back into liquid water, creating clouds. When a cloud becomes full of liquid water, it falls from the sky as rain or snow, also known as precipitation (MLA, 2023). As a result of precipitation, bioaerosols could reach the soil surface, then the groundwater and surface water through infiltration and surface runoff respectively (Muller *et al.*, 2020). The present study is a contribution to the improvement in general, by use of extracts of natural substances, of the microbiological quality of water that can be used by populations in rural areas. Water and alcohol are often used in traditional therapy to extract the principles of medicinal plants. But little is known about their functional difference or similarity. This study aims to assess the effects of aqueous and ethanolic leaf extracts of *Moringa oleifera* Lam on the cultivability of *Bacillus cereus* and *Bacillus thuringiensis* isolated from rainwater.

2. Materials and Methods

2.1 Harvesting the plant leaves and collection of rain water samples

Moringa oleifera leaves were harvested from a farm in Yaounde, Center Region of Cameroon in May 2021. The plant was identified at the National Herbarium of Cameroon. It was given the code 8241/SRF/Cam. The leaves were dried and then ground.

Rain water samples were collected in the Yaoundé, Center Region of Cameroon in November 2021 using autoclaved glass containers and placed the yard of a specific dwelling. The containers were opened when the rain started falling. The collected water was poured into a sterile 500 mL glass vial. The vials were immediately transported in refrigerated boxes (4±2 °C) to the laboratory for analyses. Bacteriological analyses consisted of the isolation and identification of *Bacillus cereus* and *B. thuringiensis.*

2.2. Isolation of microorganisms

Bacillus cereus was isolated on Mossel agar culture medium (Humeau laboratories) containing Polymyxin and the egg yolk added after autoclaving. *Bacillus thuringiensis* was isolated on Luria Bertani agar culture medium (Sigma-Aldrich) (Pauline, 2013; Nair *et al.*, 2018). For that, 100 mL of water sample was analyzed by

the membrane filtration method (porosity 0.45 µm). Petri dishes were then incubated at 36 ± 1°C for 24 hours. The morphological identification of the colonies considered the size and the outlines the color. *B. cereus* on Mossel agar medium forms colonies of around 5 mm diameter, pink colour (mannitol negative) and typically surrounded by an opaque halo due to egg precipitation (lecethinase positive) (Bouali *et al.*, 2016; Koua Abea *et al.*, 2018). On Luria Bertani agar, the colonies of *B. thuringiensis* are white, with a diameter varying from 0.5 to 1 mm (Hassan *et al.*, 2021). Biochemical identification of strains isolated was further performed using API 20^{E} system (Holt *et al.*, 2000; Bouali *et al.*, 2016; Hassan *et al.*, 2021).

2.3. Preparation of aqueous extract

194 g of leaf powder was mixed with 1940 mL of sterile distilled water for 48h and stirred in the morning and evening. The macerate obtained was filtered successively using cotton wool and Whatman N° 4 filter membrane. The filtrate obtained was dried in an oven at 45 ±2°C until the water is totally evaporated. The amount of extract was 17.05 g. The obtained extract had a paste form and was introduced in sterile glass bottles and kept in the refrigerator/freezer (5± 2°C) for further use.

2.4. Preparation of hydro-ethanolic extract

The macerate obtained was successively filtered using cotton and Whatman No. 4 paper. The filtrate obtained was dried in an oven at 45 ±2°C until the solvent had completely evaporated. 1180 and 760 ml of ethanol (95°) and sterile distilled water respectively and allowed to extract for 48 h with stirring morning and evening. The amount of extract was 158.95 g. The obtained extract had a paste form and was introduced in sterile glass bottles and kept in the refrigerator/freezer (5± 2°C) for further use.

2.5. Evaluation of the effect of extracts on cell cultivability (cells growth)

For aqueous or hydro-ethanolic extract, the solutions were prepared at concentrations 2, 1.5, 1 and 0.5 g/L from physiological sterile water (NaCl, 8.5%). The prepared solutions were first filtered on sterile absorbent cotton then on sterile whatman paper and finally through a filtering membrane of porosity 0.45 µm around a Bunsen burner in order to limit contamination (Metsopkeng *et al.*, 2022). For each type of extract and for each extract concentration, 2 series of sterile tubes encoded A and B were prepared.

Subsequently, the microbial inoculum was prepared from a pure 18-24 hours' culture on standard agar. This microbial suspension was adjusted as standard solution using a densitometer (density 0.5 on the Mc Farland scale). It is composed of 0.5 mL of 0.0048M Bacl2 (1.17% w/v Bacl2. $2H_2O$) and 99.5mL of 0.18M H_2SO_4 (1% v/v). It contains approximately 1.5 x 10^8 CFU/mL and was diluted (to 1/100) for the saline dilutions test (Andrews, 2001).

1 mL of the bacterial suspension was introduced into the 2 series of test tubes A and B containing 9 mL of solutions of aqueous extract of each concentration and 9 mL of physiological water only which was the blank.

The cells concentration in each tube at the initial moment was then approximately $1.5x10^5$ CFU/mL ($1.5x10^4$ CFU/100 µL). The test tubes coded A were incubated at 23°C and those coded B were incubated at 37°C. For each incubation temperature, the incubation times were 3h, 6h, 9h and 24h. For each incubation period, the test tubes were in duplicate. After each incubation period, bacteriological analyses were carried on

agar medium (Mossel agar culture medium for *B. cereus* and Luria Bertani agar culture medium for *B. thuringiensis*) as indicated above.

2.6. Control of the sterility of the extract before the inoculation

To check the absence of *B. cereus* and *B. thuringiensis,* 5 mL of each type of extract was filtered using membrane (0.45 µm of porosity). Mossel agar culture medium and Luria Bertani agar medium were used respectively as indicated above. The sterility of the extracts was controlled by checking the absence of the 2 bacteria considered.

2.7. Preparation of M. oleifera *leaves extracts for chromatographic and mass spectral analyses*

The leaves were cut up and dried in the shade, at laboratory temperature (25 ± 2 ° C) for 30 days. After drying, they were crushed and grinded. Thirty grams (30 g) of powder, was extracted with methanol at room temperature for 24 hours (1 g of powder in 10 mL of methanol). After filtration, the solvent was removed under vacuum to furnish a crude extract (Mellon *et al.*, 2002).

From the crude methanolic extract prepared, 5 mg of crushed leaves were diluted in 5 mL of Methanol (MeOH) HPLC Grade. The solution was then homogenized in an ultrasonic tank. Using a 2 mL syringe, the solution was withdrawn and filtered first through a 0.45 µm porosity membrane, and then through a 0.22 µm porosity membrane. Part of the filtrate was transferred to an HPLC vial.

HPLC analyses were performed on an Agilent 1260 Infinity apparatus, with DAD detector equipped with an Uptisphere C18-3 (250 x 4.6 mm, 5µm) column from Interchim (Montluçon, France). LC-MS analyses were carried out on an UHPLC Ultimate 3000 RSLC chain and an Orbitrap Q-Exactive (Thermo Scientific, Waltham, USA) with the column mentioned above. Source operating conditions were: 3 kV spray voltage; 320 °C heated capillary temperature; 400 °C auxiliary gas temperature; sheath, sweep and auxiliary gas (nitrogen) flow rates were at 50, 10 and 2 arbitrary units, respectively; collision cell was used in stepped nCE mode with ionization voltage between 10 and 50 arbitrary units. Full scan data were obtained at a resolution of 70 000. Data was processed using Xcalibur software (Thermo Fisher Scientific Inc., MA, USA). The identification of all compounds described was carried out using the negative ionization mode.

For both analyses, the mobile phase was a mixture of 0.1% (v:v) formic acid in water (phase A) and 0.1% (v:v) formic acid in acetonitrile (phase B). The gradient of phase A (usable phase) was 100% (0 min), 80% (10 min), 73% (35 min), 0% (40-50 min) and 100% (51-60 min). The flow rate was 0.8 mL/min, and the injection volume was 5 µL.

Mass spectrum data were registered in a standard Excel format. These data included the exact mass of each compound, the mass M+H, the mass M-H, the mass in M+NH4, the mass in M-COOH, the mass M+Na and the experimental mass (Aubert *et al.*, 2019; Gainche *et al.*, 2021). Each compound detected was characterized. Its potential raw formula was then calculated using Xcalibur software (Thermo Fisher Scientific, Inc.), which takes into account its relative abundance, its molar masses and its retention time (Aubert *et al.*, 2019; Gainche *et al.*, 2021). The names of chemical compounds were determined by comparison with analytical standard or according to literature data (Kashiwada *et al.*, 1984; Lalas *et al.*, 2003; Shakeri

et al., 2016; Lin *et al.*, 2019; Xu *et al.*, 2019; Xiong *et al.*, 2022).

2.8. Data presentation and analysis

After each incubation period, the mean values of the cultivable cells abundance (cells that can grow) were assessed and expressed as CFU/100 µL. The decrease in cultivable cells abundance (DCCA) was assessed and expressed in percentage using the following formula:

$$DCCA = \frac{(CAt0 - CAtn)}{CAt0}.100$$

In this formula, CAt0 is the cells abundance at the initial moment, and CAtn is the cells abundance after incubation period tn. This DCCA was assessed only in all cases where CAtn ≤ CAt0. Temporal variations of both data with respect to the type and concentration of extract, as well as the incubation temperature were also illustrated using histograms. Their comparisons were made using the Kruskal-Wallis H-test. All of these analyzes were performed using SPSS version 25.0 software.

3. Results

3.1. Temporal fluctuation of cultivable B. thuringiensis abundance

At 23°C, the lowest abundances of *B. thuringiensis* in aqueous extract at concentrations 0.5, 1, 1.5 and 2 g/L were respectively $0.14x10^4$, $0.14x10^4$, $0.03x10^4$ and $0.05x10^4$ CFU/100µL. All these lowest abundances were recorded after 24 hours of incubation (Figure 1). The highest abundances were respectively $0.28x10^4$, $0.27x10^4$, $0.2x10^4$ and $0.2x10^4$ CFU/100µL. All were recorded after 6 hours of incubation (Figure 1).

In the hydro-ethanolic extracts at temperature 23°C, the lowest abundances of *B. thuringiensis* were respectively $0.005x10^4$, $0.008x10^4$, $0.005x10^4$ and $0.004x10^4$ CFU/100 µL. All were recorded after 3 hours of incubation (Figure 1). The highest abundances were respectively $0.31x10^4$, $0.37x10^4$, $0.14x10^4$ and $0.14x10^4$ CFU/100 µL. They were recorded after 9 hours of incubation in extracts with concentrations 0.5 and 1 g/L, and after 24 hours of incubation in extracts with concentrations 1.5 and 2 g/L (Figure 1).

At 37°C, the lowest abundances of *B. thuringiensis* in the aqueous extracts were all recorded after 24 hours of incubation. They were $0.13x10^4$ CFU/100 µL in the extracts at 0.5 and 1 g/L, and $0.14x10^4$ and $0.07x10^4$ CFU/100 µL respectively in the extracts at 1.5 and 2 g/L. All these lowest cells abundances were recorded after 24 hours of incubation (Figure 1). The highest were $0.24x10^4$, $0.27x10^4$, $0.44x10^4$ and $0.22x10^4$ CFU/100 µL respectively in the extracts at 0.5, 1, 1.5 and 2g/L. They were recorded after 3 h of incubation with extract concentration 0.5, 1 and 2 g/L, and after 9 h of incubation in that 1.5 g/L (Figure 1).

With the hydro-ethanolic extracts at temperature 37°C, a temporal variation in the cultivable cells abundances was also observed. The highest cell abundances were recorded after 9 h of incubation with extract concentrations 0.5, 1.5 and 2 g/L, and after 3 h 1 g/L (Figure 1). The lowest cells abundances were recorded after 3 hours of incubation with extract concentration 1g/L, after 6 hours with extract concentration 2 g/L, and after 24 hours of incubation with extract concentrations 0.5 and 1.5 g/L (Figure 1).

3.2 . Temporal fluctuation of cultivable B. cereus abundance

With the bacteria *Bacillus cereus* at 23°C, the lowest cultivable cells abundances were $0.29x10^4$,

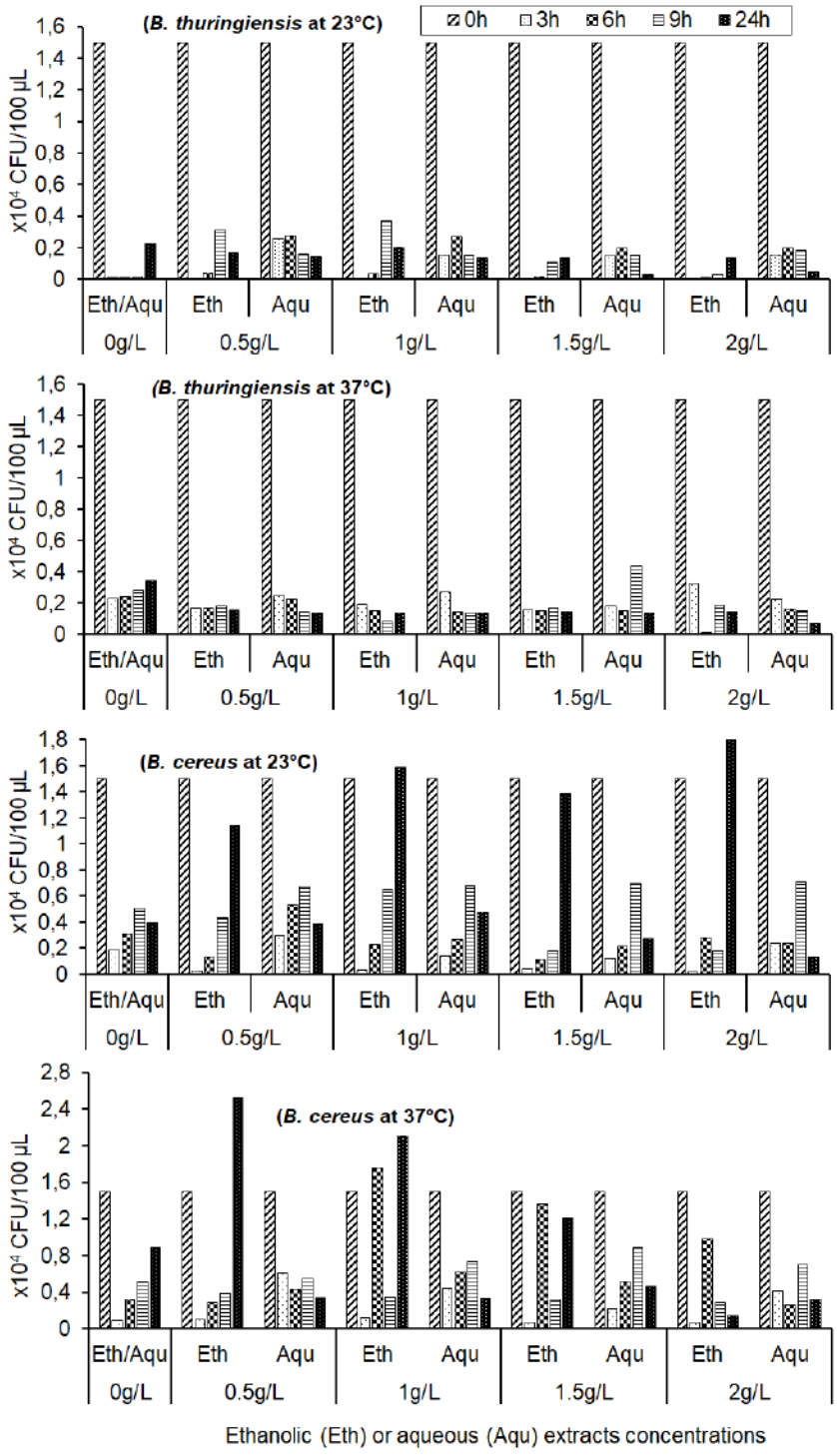

Figure 1. Temporal variation of the abundance of cultivable *B. thuringiensis* and *B. cereus*, at the presence of each concentration of ethanolic (Eth) and aqueous (Aqu) extract, after each incubation duration (0, 3, 6, 9 and 24h) under 23°C and 37°C

$0.14x10^4$, $0.11x10^4$ and $0.13x10^4$ CFU/100 µL in the aqueous extracts with concentrations 0.5, 1, 1.5 and 2 g/L respectively.

These abundances were recorded after 3h of incubation with the extracts 0.5 to 1.5 g/L, and after 24 h of incubation with the extract 2g/L (Figure 1). All the highest cell abundances were recorded after 9 h of incubation. These highest cell abundances were $0.67x10^4$, $0.68x10^4$, $0.69x10^4$ and $0.71x10^4$ CFU/100 µL with extract concentrations 0.5, 1, 1.5 and 2 g/L respectively (Figure 1).

With the hydro-ethanolic extract at 23°C, the highest and lowest cell abundances were $1.13x10^4$ and $0.02x10^4$ CFU/100 µL with extract solution concentration 0.5 g/L, $1.59x10^4$ and $0.03x10^4$ CFU/100 µL with that of 1 g/L, $1.38x10^4$ and $0.04x10^4$ CFU/100 µL with that of 1.5 g/L, and of $1.8x10^4$ and $0.02x10^4$ CFU/100 µL with that of 2 g/L respectively (Figure 1). All the highest abundances were recorded after 24 h of incubation while all the lowest abundances were recorded after 3 h of incubation (Figure 1).

At 37°C, the highest and lowest abundances of *B. cereus* in the aqueous extracts were $0.61x10^4$ and $0.34x10^4$ CFU/100 µL with extract solution concentrations of 0.5 g/L, $0.73x10^4$ and $0.33x10^3$ CFU/100 µL with that of 1g/L, $0.88x10^3$ and $0.21x10^4$ CFU/100 µL with that of 1.5 g/L extract, and $0.70x10^4$ and $0.25x10^4$ CFU/100 µL with that of 2g/L respectively (Figure 1). All the highest cell abundances were recorded after 9h of incubation in the extracts 1, 1.5 and 2 g/L, and after 3h of incubation with extract solution concentration of 0.5 g/L. The lowest cell abundances were recorded after variable incubation periods depending on the extract concentration (Figure 1).

In the presence of hydro-ethanolic extracts, the highest and lowest cell abundances under 37°C were recorded respectively after 24h and 3h of incubation in the extract concentrations of 0.5 and 1 g/L, and after 6 h and 3 h of incubation with those of 1.5 and 2 g/L (Figure 1).

It can thus be noted that there is a clear cell growth in some cases. This was mostly recorded in the ethanoic extracts. The cell abundances at the end of the incubation period were thus sometimes greater than the concentration at the initial instant (Figure 1).

3.3. Temporal variation of decrease in cultivable cells abundance

The abundance of cultivable cells decreased is presented in Figure 2. It can be noted that the decrease in cultivable cells abundance varied with the type and concentration of extract, the incubation temperature and duration, and with the bacterial species. The cultivable *B. thuringiensis* abundance decreased under 23°C varied from 75.09 to 99.68% with the hydro-ethanolic extract, and from 81.55 to 97.94% with aqueous extract. Under 37°C, it varied from 78.34 to 99.61% with hydro-ethanolic extract, and from 70.75 to 95.52% with aqueous extract. The cultivable *B. cereus* abundance decreased in hydro-ethanolic extract reached 98.86% under 23°C and 95.59% under 37°C, and was sometime 0% (Figure 2). In aqueous extract, it varied from 52.55 to 92.20% under 23°C, and from 41.09 to 85.92% under 37°C.

3.4. Comparison of the cells abundances based on the type of extract, the incubation temperatures and the incubation durations

When considering all the temperatures and incubation periods, a comparison of the cell abundances from one type of extract to another was made, and this for each extract's concentration.

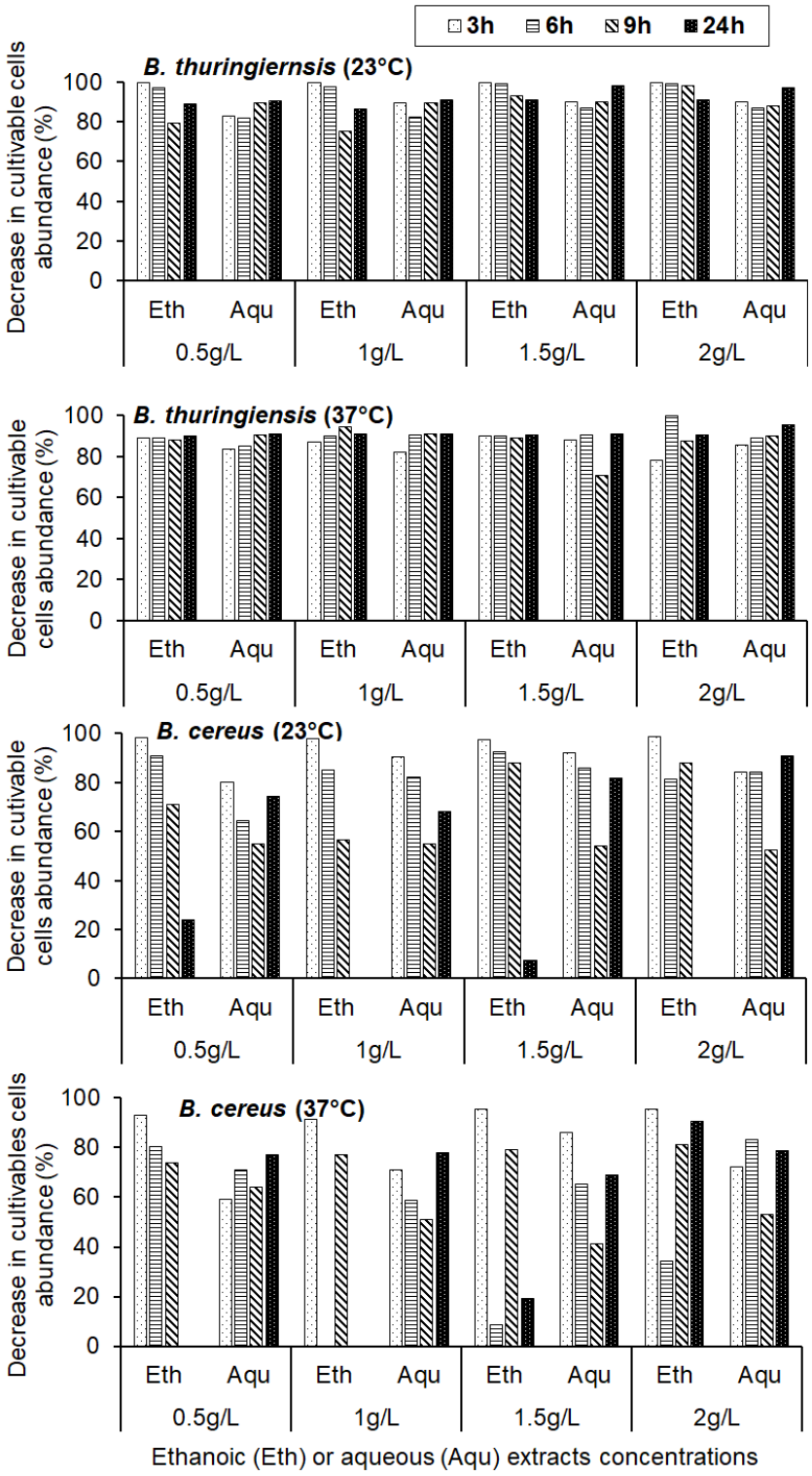

Figure 2. Temporal variation of decrease in cultivable cells abundance at the presence of each concentration of ethanoic (Eth) and aqueous (Aqu) extract, after each incubation duration (3, 6, 9 and 24h) under 23°C and 37°C

It was noted that cell abundances between the aqueous extract and the hydro-ethanolic extract did not significantly differ (P>0.05), except for the concentration 1.5 g/L when the *B. thuringiensis* cells were present (Table 1). Most of the antimicrobial active molecules against these 2 bacterial species would thus be present in each of the 2 types of extracts.

A comparison of the cells abundances between 23 °C and 37°C was also carried out for each type of extract and each extract concentration when considering all the incubation durations. It was noted that cells abundances from one incubation temperature to another are not significantly different (P>0.05), except for hydro-ethanolic extract at concentration 1.5 g/L when the *B. thuringiensis* cells were present (Table 2).

In a third step, a comparison of cell abundances from one incubation period to another was made for each incubation temperature, and for each type and concentration of extract. A significant difference (P<0.05) was noted from one cell abundance to another and from each incubation period to another. The contact duration between the extract and the bacterial cells present thus seems to play an important role, whatever the type of extract and the incubation temperature.

Table 1. "P" values of the comparisons (Kruskal-Wallis test) of the cells abundances between aqueous extract and ethanoic extract at each extract concentration when considering all the incubation durations and all the incubation temperatures

Bacterial species	Extracts concentrations			
	0.5 g/L	**1 g/L**	**1.5 g/L**	**2 g/L**
B. thuringiensis	0.506	0.322	**0.048***	0.062
B. cereus	0.318	0.287	0.690	0.594

*: P≤ 0.05

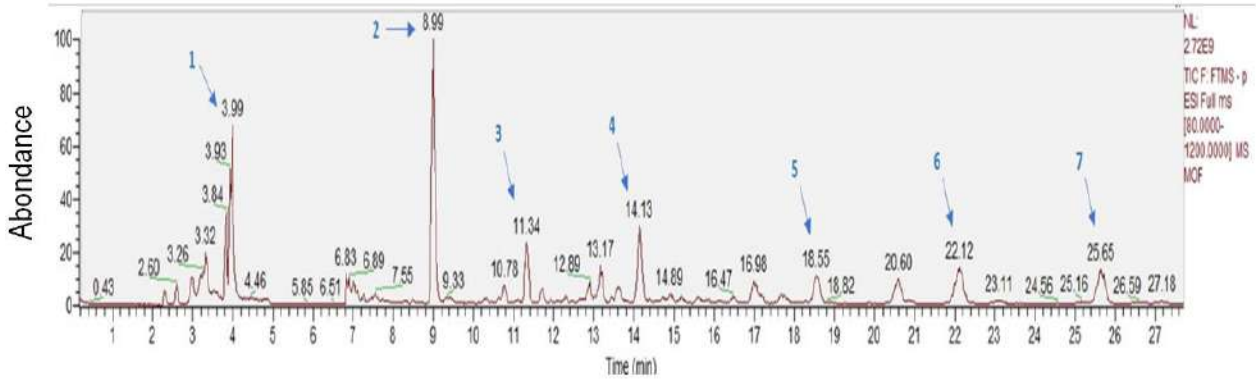

Figure 3. LC/MS profile of the crude methanol extract of *M. oleifera* leaves (spectrum (-))

Table 2. "P" values of the comparison (Kruskal-Wallis test) of the cells abundances between 23°C and 37°C for each type of extract and each extract concentration when considering all the incubation durations

Bacterial species	Type of extract and concentration							
	Aqueous extract				Ethanoic extract			
	0.5 g/L	1 g/L	1.5 g/L	2 g/L	0.5 g/L	1 g/L	1.5 g/L	2 g/L
B. thuringiensis	0.263	0.329	0.709	1	0.707	1	**0.003***	0.062
B. cereus	0.71	0.265	0.213	0.135	0.455	0.136	0.457	0.708

*: P≤ 0.05

Table 3. Phytochemical profile of MeOH leaves extracts of *M. oleifera* (spectrum (-)) (comparison with analytical standard or according literature data (Kashiwada *et al.* 1984; Lalas *et al.* 2003; Shakeri *et al.* 2016; Lin *et al.* 2019; Xu *et al.* 2019; Xiong *et al.* 2022))

N°	Retention time (min)	Compound name	Compound formula	M-H$_{exp}$ (m/z)	Class of molecule
1	3.99	Saccharose	$C_{12}H_{22}O_{11}$	341.1086	Heterosides
2	8.99	Glucomoringinin	$C_{20}H_{29}NO_{14}S_2$	570.0956	Glucosinolates
3	11.34	Neochlorogenic acid	$C_{16}H_{18}O_9$	353.0875	Phenolic acids
4	14.13	O-acetylshanzhiside methyl ester	$C_{19}H_{28}O_{12}$	447.1508	Iridoids
5	18.55	Isoquercetin	$C_{21}H_{20}O_{12}$	463.0883	Flavonoids
6	22.12	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{11}$	447.0931	Flavonoid heterosides
7	26.65	Kaempferol acetyl glucoside	$C_{23}H_{22}O_{12}$	489.1037	Flavonoid heterosides

3.5. Chemical profile of the M. oleifera leaves

Figure 3 shows the mass spectra obtained from leave extract. This profile was obtained by negative ionization. Many chemical compounds have been identified and are presented in Table 3. They include Saccharose (heteroside), Glucomoringinin (glucosinolate), Neochlorogenic acid (phenolic acid), 0-acetylshanzhiside methyl ester (iridoid), Isoquercetin (flavonoid), Kaempferol-3-O-glucoside and Kaempferol acetyl glucoside (flavonoid heteroside) (Table 3).

4. Discussion

It is observed that in the presence of the extracts of the leaves of *M. oleifera*, the cells culture of the 2 *Bacillus* species considered is slowed down. The slowdown seems to be accentuated with the increase in the concentration of the extracts. This would be linked to the chemical compounds present in the extracts. Chemical compounds identified in the extracts include glucomoringinin which belongs to the class of glucosinolates, neochlorogenic acid which belongs to the class of phenolic acids, O-acetylshanzhiside methyl ester, which is of iridoids class, isoquercetin, kaempferol

acetyl glucoside and kaempferol-3-O-glucoside which are of flavonoids and flavonoid heterosides classes respectively. Some of these compounds have been reported to have antibacterial effects on Gram-positive bacteria.

Inhibitory effect of phenolic compounds against the growth of the Gram-positive human pathogens *Bacillus cereus*, *S. aureus*, and *Listeria monocytogenes* have been indicated by Metsamuuronen & Siren (2019). It might be due to their ability to form a complex with bacterial cell wall thus inhibiting the microbial growth (Kozyra *et al.*, 2017). Disruption of metabolic pathways may be the principal mode of action (Erguden & Unver, 2022). The phenolic compounds lipophilicity may be one of the determining factors for their antimicrobial potential; their greater lipophilicity, their greater the inhibitory capacity (Kauffmann & Castro, 2023).

The iridoids health-related significance has been reported. In addition to their their anti-inflammatory, hypoglycemic, antioxidative, antitumor, neuroprotective and hepatoprotective effects, they can inhibit DNA polymerase activity in the bacteria cells (Wang *et al.*, 2020; Przybylska *et al.*, 2022).

Glucosinolates have been reported as expressing a wide range of growth inhibition activity against Gram-positive bacteria as well as against Gram-negative and fungi (Blazevic *et al.* 2010). They are indicated to be more effective against Gram positive bacteria than the Gram negative, and they would specifically increase the oxidation and inhibition of essential proteins/enzymes leading to bacterial cell death (Aires *et al.*, 2009).

Flavonoids exert antibacterial effects by disrupting cell integrity. It has also been suggested that flavonoids can affect bacterial respiration and interfere with energy metabolism by inhibiting the activity of cytochrome *c* reductase in some Gram-positive bacteria (Dzoyem *et al.*, 2013; Li *et al.*, 2022). Their antimicrobial activities would not be related to their special structure, but may be related to their polarities or lipid-water partition coefficients (Yuan *et al.*, 2021). The inhibitory activities of the flavonoids to Gram-positive bacteria are nonlinearly increase in most cases (Yuan *et al.*, 2021).

Kaempferol, although having effects on the central nervous system diseases (Silva dos Santos *et al.*, 2021), also has effects on bacteria cells. It has damaging effect on the cell wall or cell membrane, leading to leakage of contents and cell death finally (Li *et al.*, 2022). It also inhibits the DNA gyrase in some Gram-positive bacteria (Periferakis *et al.*, 2022). It disrupt normal metabolism in bacteria mainly by affecting microbial metabolism in diverse environments, and energy metabolism including carbon metabolism, glyoxylate and dicarboxylate metabolism and fructose and mannose metabolism, and affected bacterial secretion systems and quorum sensing significantly (Li *et al.*, 2022). Quorum sensing is a chemical communication system in which bacterial pathogens express certain virulence genes at high cell densities and communicate with each other by small diffusible signalling molecules called self-inducers (Silva *et al.*, 2016).

Kaempferol may also exert its antibacterial activity by decreasing the ATP content and increasing ATPase activity through a series of intracellular biochemical reactions. This led to metabolic disorders (Li *et al.*, 2022).

Isoquercitin has been reported to inhibit several enzymes in vitro (Valentova *et al.*, 2014). Quercetin could inhibit cell wall synthesis through inhibiting D-alanine-D-alanine ligase (Wu *et al.*,

2008; Li *et al.*, 2022). Isoquercetin, like quercetin, has shown broad-spectrum antiviral activities, for example against severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (Mbikay & Chrétien, 2022). Mixture of kaempferol and quercetin exerts antibacterial effects through cell membrane disruption, followed by activation of apoptosis and DNA fragmentation in some Gram-positive bacteria. They also interact with 3-oxyacyl-[acyl carrier protein] reductase and enoyl-acyl carrier protein reductase therefore inhibiting the biosynthesis of fatty acids in bacteria cell (Periferakis *et al.*, 2022).

Despite all the above information on the antibacterial activities of the chemical compounds identified in the considered extracts, it is noted that all the cells were not inhibited. This would reflect a resistance of a few cells. An *et al* (2022) indicated that kaempferol in some cases could induces bacterial pathogen resistance. In addition, bacteria strains tested were from rainwater. It is indicated that air represents an important active reservoir of diverse antibiotic resistance genes, and their origins may be extensive, including human activity such as wastewater treatment plants, gray water, municipal solid waste landfill, livestock farm, and medical industry and natural phenomena such as volcanic eruptions (Ruiz-Gil *et al.*, 2020; Chen *et al.*, 2022; Wang *et al.*, 2022). *Bacillus* strains antibiotics susceptibility can also undergo temporal variation due to seasonal change (Djiala *et al.*, 2023).

In addition, the decrease in cell abundance in most cases was not always proportional to the incubation durations or to the extract's concentrations. This would probably be linked to the presence of some compounds that could serve as cells growth nutrients, such as saccharose (heteroside) which has been identified. Tian *et al* (2021) showed in fact that sucrose induces robust solid surface motility of *Bacillus* by triggering a signalling cascade, first through extracellular synthesis of polymeric levan, which in turn stimulates strong production of surfactin and hyper-flagellation of the cells, and promotes the cells growth. Furthermore, some authors have indicated that the type of extract for the same plant part varies according to the microorganism to be neutralized in the patient, probably because of the variation in diversity of the antimicrobial molecules which will be diffused (Hadis *et al.*, 2020; Tavares *et al.*, 2021). This would explain a substantial difference between the aqueous extract and the hydro-ethanolic extract, leading to the observed substantial difference between their both activities. The release into the medium of these cell growth ingredients in quantity and quality, as well as other ingredients with antimicrobial properties, would undergo temporal variation, and especially with respect of all of incubation conditions. This could be the cause of no significant difference in cells abundance noted between aqueous extract and hydro-ethanolic extract, as well as between 23°C and 37°C incubation in most cases.

Many ingredients that could promote the bacterial growth have been reported in the extracts by other authors (Adouko *et al.*, 2019; Gonzalez-Burgos *et al.*, 2021; Islam *et al.*, 2021). Those growth cells ingredients would be released much more with the hydro-ethanolic extract and would be more favourable to the growth of *B. cereus.* This would be at the origin of the extremely low percentage of inhibition of cultivable cells of this species (0%) sometimes observed. A relative cryptic growth of cells would counterbalance their inhibition caused by the identified chemical compounds with antimicrobial effects. Furthermore, it is noted that for each incubation temperature, and for each type and concentration of extract, cells abundance significantly varied from one incubation period to

another (P<0.05). The increase in incubation times would allow the molecules with antibacterial effects identified above to reach targets not reached by short periods.

5. Conclusion

The metabolism of *Bacillus* strains contained in rainwater can be relatively slowed down in the presence of aqueous and hydro-ethanolic extracts of *M. oleifera* Lam. This slowing would be the consequence of the presence of some identified antimicrobial compounds belonging to the class of phenolic acids, glucosinates, iridoids and flavonoids. These antimicrobials seem to act on bacterial cells with the same amplitude whether the extract is aqueous or hydro-ethanolic, and whether it is incubated at 37°C or at 23°C. The effects of these compounds on bacteria differ significantly with varying cell-chemical compound contact durations. Bacterial inhibition however may not be complete, probably due to the intrinsic resistance of some cells. Growth cells ingredients and antimicrobial chemicals would be released in the 2 types of extracts and would be much more with the hydro-ethanolic extract. Aqueous *M. oleifera* Lam leaves extracts could nevertheless be exploited as an alternative to the microbiological treatment of rainwater, mostly against *B. thuringiensis,* for household use in rural areas. However, further work is essential to clarify the effective constituents inactivating bacteria in the extracts, their potential impact on the water organoleptic properties as well as on human health.

Conflict of interest

The authors declare that they have no competing interests.

Ethics

This Study does not involve Human or Animal Testing.

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