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Research Paper

Cultivability of *Salmonella typhi, Vibrio cholerae* and enteropathogenic *Escherichia coli* in water microcosm in the presence of *Eucalyptus microcorys* leaves extract: Effect of the concentration of leaves extract and incubation temperature

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Abstract

This study aimed to assess the effect of the concentration of an aqueous extract of a Myrtaceae (*Eucalyptus microcorys*) on bacterial survival under various temperature conditions. At 7°C, the highest concentration of cultivable cells were 21.30, 21.32 and 21.08 (Ln (CFU/100 ml)) respectively at the concentration of extract solution 1, 1.5 and 2% for enteropathogenic *E. coli*, 18.12, 16.27 and 15.31 (Ln (CFU/100 ml)) at 1, 1.5 and 2% for *S. typhi*, and 22.32, 22.23 and 19.99 (Ln (CFU/100 ml)) at 1, 1.5 and 2% for *V. cholerae.* At 23 and 37 °C, the highest concentrations of cultivable enteropathogenic *E. coli, S. typhi* and *V. cholera* were all noted at the extract concentration 1%. The hourly cell inhibition rate of enteropathogenic *E. coli* varied from 0.35 to 0.81, from 0.42 to 1.07, and from 0.44 to 1.05 h^{-1} respectively at the extract concentration 1, 1.5 and 2%. That of *S. typhi* varied respectively from 0.55 to 0.65, from 0.62 to 0.69, and from 0.67 to 0.76 h^{-1}. It varied respectively from 0.29 to 0.40, from 0.32 to 0.48 and from 0.43 to 0.86 h^{-1} for *V. cholera*. Secondary metabolites found in the plant extract would have an impact on the variation of the CFUs abundance noted.

Keywords: bacteria cultivability, *E. microcorys* extract, concentration, incubation temperature

Introduction

Water, despite being vital to our lives, is a vehicle for viral, bacterial, fungal, protozoa and helminthes. Water borne diseases possess a relatively high mortality rate in developing countries [1, 2]. Diarrheic diseases are caused by a large range of infectious agents and rank among the most deathly in developing countries, especially in countries between the tropics [3]. The world has witnessed since 2000 an increase in the number of populations who have access to potable water. This process progresses remains, however, minute given the demographic growth of most of these countries. In order to solve their need for potable water, many populations now use underground water.

There are several simple methods used to render water free from microbes. Basically, it involves the use of chlorinated product and boiling. These methods alter the organoleptic properties of water. By solar radiation method or Solar Water Disinfection (SODIS), simple and accessible has been proposed for the destruction of microbes present in water fetched from wells or other point for drinking [4]. Unfortunately, this method poses a restriction because the water treated with this method can only be conserved during 24 hours at most. Beyond this period, chances for bacteria to regenerate are high [5]. The adhesion of bacteria-contaminants on rocks immerged has been suggested for reducing planktonic bacteria [6]. This method, interesting however, remains limited.

To allow the populations to reach a water of good quality, in addition to the means above quoted, a method of water decontamination by the extract of plants has also been proposed like new alternative of water treatment to the level of the households. The use of plant for therapeutic purposes began thousands of years ago. According to the WHO more than 80% of the world's population relies on traditional medicine for primary healthcare needs [7]. Natural compounds have the advantages of having grand chemical structure diversity with sometime a huge range of biological properties [8]. In fact some crude extracts of *Lantana camara*, *Cymbogon citratus* and *Hibiscus rosa-sinensis* have been proven to possess bactericide properties in aqueous medium [9]. The infusion of *Artemisia annua* causes the destruction of bacteria and does not lead to an irreversible sublethale alteration in others [10]. Lutgen and Michels have reported the bactericide or bacteriostatic effect of *Artemisia annua* linked to the peroxide group of Artemisinin [11].

Many plant of *Eucalyptus* genus (Myrtaceae) are used to control several diseases derived from microbial infections [12]. Leaf extracts of *Eucalyptus* have been approved as food additives, and the extracts are also currently used in cosmetic formulations. Recently, attention has been focused on the medicinal properties of these extracts. Research data has demonstrated that the extracts exhibited various biological effects, such as antibacterial, antihyperglycemic and antioxidant activities [13, 14]. It has been reported that macrocarpals from *Eucalyptus macrocarpa* and grandinol from *Eucalyptus perriniana* were effective against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) [15, 16]. However the use of plant extracts in the process of water purification, destined to drinking, remains low. Few studies have been carried out on the properties of *Eucalyptus microcorys.* This plant belongs to the *Magnoliophyta* division, *Magnoliopsida* Class, *Myrtales* Order, *Myrtaceae* Family and *Eucalyptus* Genus. we have few information on the impact of varying concentration on bacteria, likewise few information relate the influence of the aqueous extract on bacteria with respect to the duration of exposition and to temperature.

The main objective of the present study is to investigate on the effects of extract of *Eucalyptus microcorys* on cultivability of planktonic bacteria *Salmonella typhi, Vibrio cholerae* and enteropathogenic *E. coli* in aquatic microcosm and to evaluate the impact of extract concentration, incubation temperature and period on this effect.

Materials and Methods

Harvest of *E. microcorys* and crude extract obtaining

Fresh leaves of *Eucalyptus microcorys* were harvest in Yaoundé, center region (Cameroon) and dried up at room temperature (23±2 °C) in the laboratory for 30 days. Thereafter, leaves were ground into powder. Fifty grams of the obtained powder were mixed with 100 ml of warm distilled water and heated immediately up to boiling temperature, thirty minutes after the mixture is left to settle, the supernatant is removed by filtration. The filtrate obtained constitutes the decoction. The latter is dried in an oven at 45-50 °C [17, 18].

The method used in this experiment was selected for it closeness to that of the herbalists. The obtained crystals were used to prepare the crude extract. The output of the extraction was 22.43% (±0.5%). The output of the extraction is the report of the mass of crystals on the mass of the plant powder (50g) used. Three ranges of extract concentration 1%, 1.5%, 2% have been prepared thus while using sterile physiological water. Every range has been filtered then first with the help of the sterile cotton, then on Whatman membrane, and finally on membrane in nitrate of cellulose of 0.45 µm porosity.

Qualitative phytochemical screening

The phytochemical sifting was done according to the usual protocols [19, 20]. It ascertained the presence of polyphenol, triterpenoids, sterols, alkaloids, saponins, gallic tannins, Flavonoids, Anthraquinones and Anthocyanines.

Bacterial isolation and storage

The bacteria used were *Salmonella typhi*, *Vibrio cholerae* and enteropathogenic *Escherichia coli*. These bacteria have been chosen because of their importance in hygiene and in public health [21, 22]. They were isolated from the urban stream in the equatorial region of Cameroon.

Enteropathogenic *E. coli*, *S. typhi* and *V. cholerae* were isolated on Endo (Bio-Rad), Wilson Blair (Bio-Rad) and TCBS (Bio-Rad) agar medium respectively, using membrane filtration technique [23, 24]. Their identification was made according to standard method [25]. For the preparation of bacterial stocks, a colony forming unit (CFU) of each strain from standard agar medium was inoculated into 100 ml of nutriment broth (Oxford) for 24h at 37ºC. After cells were harvested by centrifugation at 8000 rev/min for 10 min at 10ºC and washed twice with NaCl (8.5 g/l) solution. Each pellet was re-suspended in 50 ml of NaCl solution. After homogenization, 1ml of this was then transferred into 500 ml of sterile NaCl solution (0.85%) contained in the Erlenmeyer flash and the stocked.

Experimental protocol

A number of 60 flasks of 250 ml were used for this study. The latter were organized in four series: A, B, C and D, each series of flask is divided into three subgroups of 5 flasks each. All 15 flasks of series A contained 200 ml of physiological water (NaCl: 0.85%) each and were used as control. The three other series contained 200ml of the extract at different concentrations. The B, C and D series contained 1%, 1.5% and 2% of the extract respectively. One ml of the stock of bacteria was then transferred into each flask. T_0, the initial time, corresponds to the point of time when the transfer of stock is done. At T_0 the cell concentration was $27x10^8$ CFU/ml. The first subgroup of each group A, B, C and D were incubated at 7±1 °C. The second and the third subgroups of each group were respectively incubated at 23±1 °C and 37±1 °C. The counting of bacteria was done in each flask through the surface spreading method on agar medium, this at the end of each incubation period. The Endo (Bio-Rad), Wilson Blair (Bio-Rad) and TCBS (Bio-Rad) agar culture medium were used for bacteria Enteropathogenic *E. coli*, *S. typhi* and *V. cholerae* respectively. Results were expressed in the number of units that constitute a colony in 100ml of physiological water sampled.

Data analysis

The temporal variation of bacterial abundances is illustrated using histograms. The percentage of inhibition (PI) was calculated using the following formulae as described by Namour and Tamsa Arfao et al [18, 26]:

$$PI = \left(\frac{No - Nn}{No}\right).100$$

Where N_0 = number of bacteria in control (NaCl: 0.85%), N_n = remaining bacteria after the action of *Eucalyptus microcorys* extract. Correlation coefficients among considered parameters were assessed using Spearman correlation test. The comparisons of the abundances of planktonic bacteria among the extract concentrations, incubation durations and incubation temperatures have been carried out using the H-test of Kruskal-Wallis on statistical software package (SPSS 16.0).

The straight Ln (number of CFUs) lines against incubation duration of the form $y = ax+b$ were plotted for each of the extract concentrations and incubation temperatures. In this equation, x is the explanatory variable and y is the dependent variable, *a* is the slope of the regression line, and b is the intercept point of the regression line on the y axis (the value of y when x = 0) [27, 28]. The slope of the straight line was then considered as the cell inhibition rate (CIR).

Results and Discussion

Qualitative phytochemical screening

The phytochemical screening of *Eucalyptus microcorys* revealed the presence of Polyphenols, Sterols, Triterpenoids, Flavonoids, Gallic tannins, Anthraquinones, Anthocyanines, Alkaloids and Saponins. Catechics tannins and the lipids are absents.

Temporal variation of cells abundances

Considering temperature 7°C, it was noted that the value abundances of cells in the NaCl solution (0.85%) ranged from 21.19 to 21.40 (Ln (CFU/100 ml)) for enteropathogenic *E. coli*, and ranged from 19.84 to 20.98 (Ln (CFU/100 ml) for *S. typhi* and finally ranged from 24.54 to 24.78 (Ln (CFU/100 ml)) for *V. cholerae* (Figure 1). In the same condition, in the presence of different concentration extract solution (1%, 1.5% and 2%) of *Eucalyptus microcorys*, the abundance of cells varied from 21.13 to 21.30, from 19.53 to 21.32 and from 19.30 to 21.08 (Ln (CFU/100 ml)) respectively at 1%, 1.5% and 2% for enteropathogenic *E. coli*. It ranged respectively from 16.19 to 18.12, 14.23 to 16.27, and 12.79 to 15.31 (Ln (CFU/100ml)) at 1%, 1.5% and 2% for *S. typhi*. For *V. cholerae* it fluctuated between 19.94 and 22.32 (Ln (CFU/100 ml) at 1%, between 19.65 and 22.23 (Ln (CFU/100 ml)) at 1.5%, and between 18.92 to 19.99 (Ln (CFU/100ml) at 2% (Figure 1).

For incubation temperature of 23°C, in the NaCl solution (0.85%), the highest abundance of enteropathogenic *E. coli, S. typhi and V. cholerae* was recorded respectively 21.71, 20.26 and 24.76 (Ln (CFU/100ml)) (Figure 2). It was observed that, in the extract solution, the density of cells bacteria ranged respectively from 18.84 to 21.15, 14.75 to 20.68 and 13.14 to 20.58 (Ln (CFU/100 ml)) at 1%, 1.5% and 2% for enteropathogenic *E. coli*, it ranged respectively from 16.75 to 18.42, 16.01 to 17.23, and 14.17 to 16.27 (Ln (CFU/100 ml)) at 1%, 1.5% and 2% for *S. typhi*. For *V. cholerae* it ranged from 21.22 to 22.47, from 20.02 to 22.32 and from 18.92 to 21.84 (Ln (CFU/100 ml)) respectively at 1%, 1.5% and 2% (Figure 2).

The highest cell concentrations cell in the NaCl solution (0.85%) were respectively 21.68, 20.37, and 25.08 (Ln (CFU/100 ml)) for enteropathogenic *E. coli, S. typhi* and *V. cholerae* at incubation temperature 37°C. In the same condition, the concentration cell, in different concentration extract solution of *Eucalyptus microcorys*, was fluctuated between 15.49 and 21.55 (Ln (CFU/100 ml)) to 1%, between 13.54 and 21.48 (Ln (CFU/100 ml)) to 1.5% and between 12.44 and 17.46 (Ln (CFU/100 ml)) to 2% for enteropathogenic *E. coli* (Figure 3). The cellular density of *S. typhi* is between 13.70 and 16.75 (Ln (CFU/100 ml)) to 1%, between 13.70 and 16.58 (Ln (CFU/100 ml)) to 1.5% and between 12.79 and 14.40 (Ln (CFU/100 ml)) to 2%. For *V. cholerae* the cell abundance ranged from 19.39 to 21.56, from 18.81 to 21.12 and from 13.75 to 18.42 (Ln (CFU/100ml)) respectively at 1%, 1.5% and 2% (Figure 3).

In most cases, the low cellular densities have been observed after 24 h of incubation except enteropathogenic *E. coli* with recorded after 6 h to 23°C [13.14(Ln (CFU/100 ml))] and after 9 h to 37°C [12.44 (Ln (CFU/100 ml))]. The abundance of *S. typhi* was relatively lower after 3 h to 37°C [12.79 (Ln (CFU/100 ml))] (Figure 1 and 3).

Percentage of inhibition

The percentage of inhibition was calculated to evaluate the direct impact of aqueous extract on Culturability of tree bacteria species studied and asses the influence of incubation temperature and duration. The (Table 2) showed that cultivability of enteropathogenic *E. coli* with extract concentration 1%, percentage of inhibition ranged from 3 to 24%, 8 to 91% and 3 to 100% at 7, 23 and 37°C respectively (Table 1). With 1.5% of extract concentration, we observed 100% of inhibition at 37°C after 9 h. The percentage of inhibition attainted the maximum value with extract concentration 2% after 1 day and ranged from 86 to 100 % for all incubation temperature and extract concentration. Concerning *S. typhi*, the percentage of inhibition ranged from 10 to 100 %. The lower percentage (9.83%) was noted after 9 h at 7°C at the extract concentration 1%. For *V. cholerae* the inhibition percentage ranged from 25 to 100%. In most cases the inhibition percentage was higher than 80%.

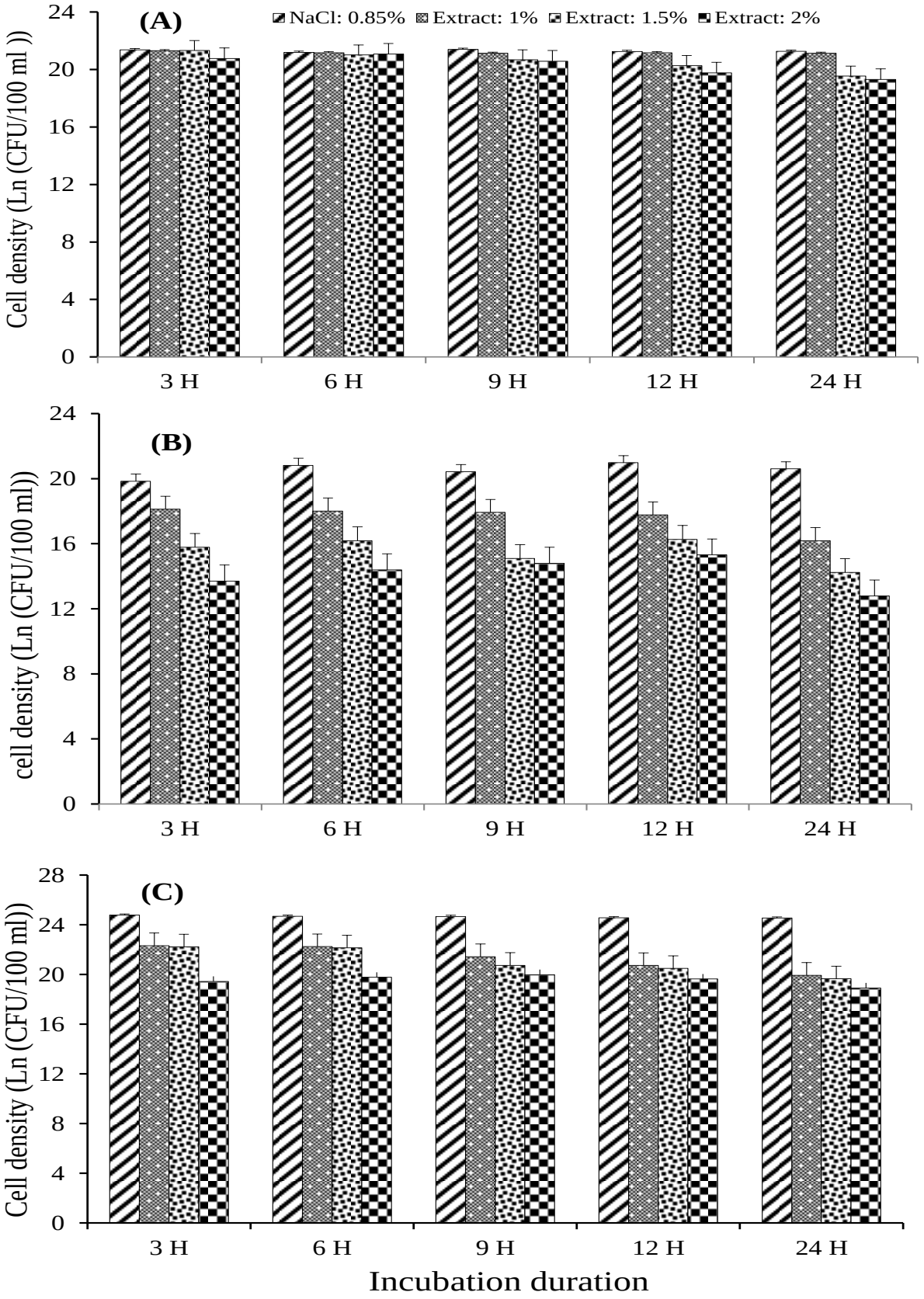

Figure 1: Temporal evolution of planktonic cells abundance in presence of *Eucalyptus* extract at temperature of 7°C ((A): Enteropathogenic *E. coli*, (B): *S. typhi* , (C): *V. cholera*)

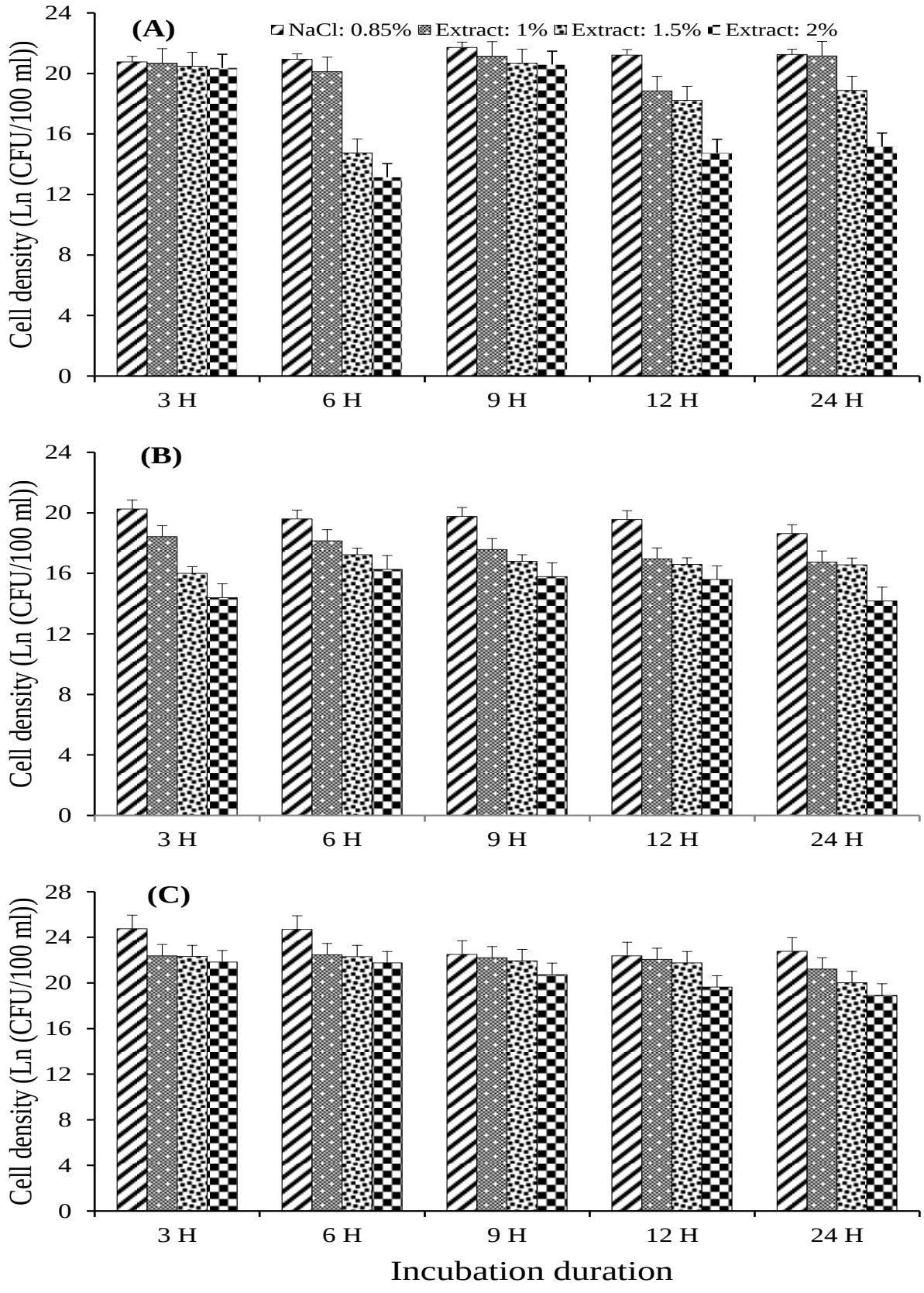

Figure 2: Temporal evolution of planktonic cells abundance in presence of *Eucalyptus* extract at temperature of 23°C ((A): Enteropathogenic *E. coli* , (B): *S. typhi* , (C): *V. cholera*)

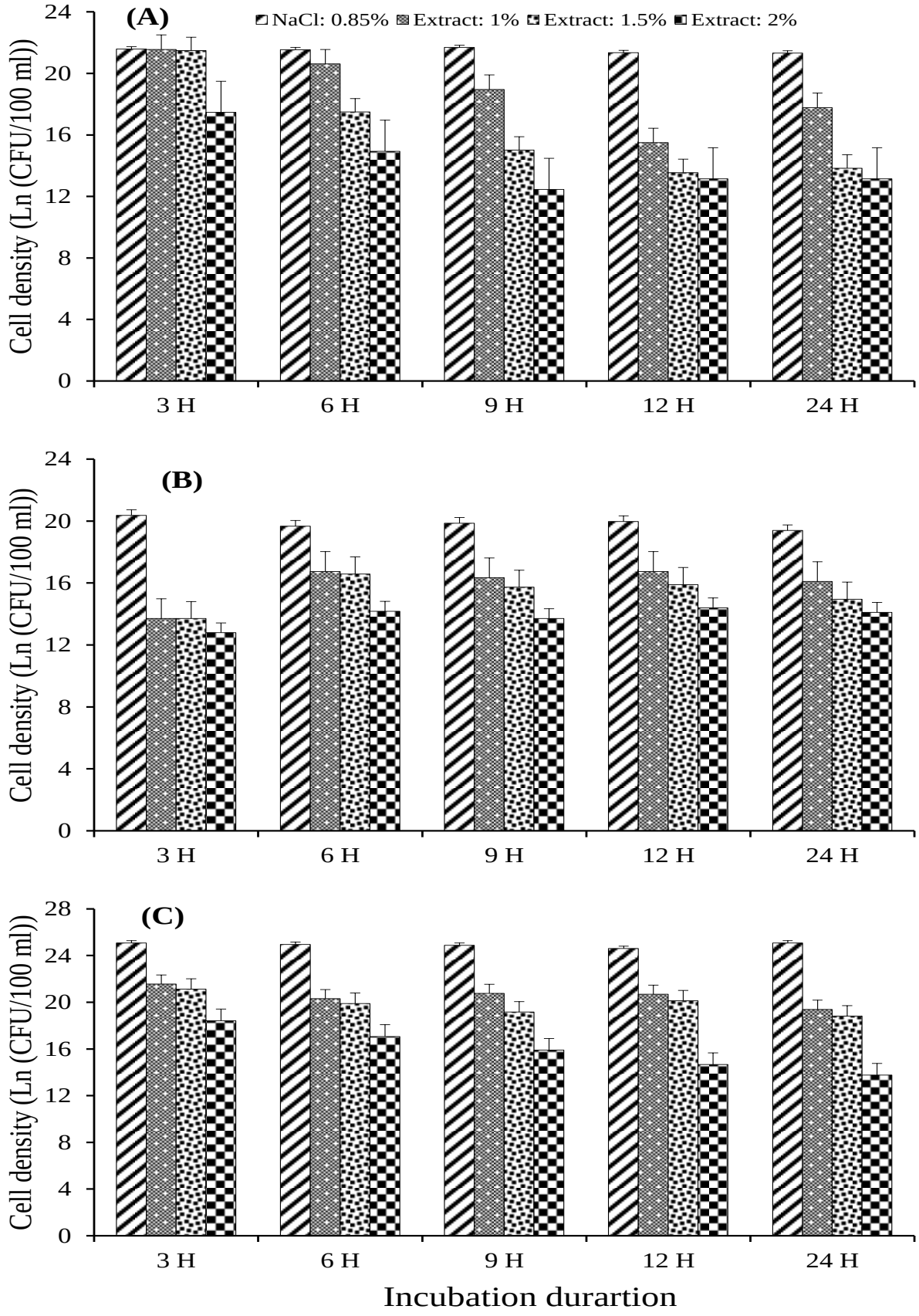

Figure 3: Temporal evolution of planktonic cells abundance in presence of *Eucalyptus* extract at temperature of 37°C ((A): Enteropathogenic *E. coli,* (B): *S. typhi* , (C): *V. cholera*)

Table 1: Percentage of inhibition (PI) of each bacterial species at each incubation temperature and after each incubation period

Bacterial species and incubation temperature with different extracts concentrations			Values of PI (%) after each incubation period				
Bacterial species	**Incubation temperature**	**Extract concentration**	**3 h**	**6 h**	**9 h**	**12 h**	**24 h**
Enteropa-thogenic *E. coli*	7°C	1%	6.52	3.17	23.97	8.96	12.50
		1.5%	5.25	16.67	51.44	62.69	82.35
		2%	45.48	11.11	56.19	77.61	86.03
	23°C	1%	8.45	55.82	43.63	90.70	9.29
		1.5%	25.54	99.79	64.45	95.04	90.65
		2%	32.86	99.96	67.92	99.84	99.78
	37°C	1%	2.54	60.36	93.45	99.71	97.12
		1.5%	9.04	98.25	99.87	99.96	99.94
		2%	98.36	99.86	99.99	99.97	99.97
S. typhi	7°C	1%	82.11	93.97	9.83	95.97	98.79
		1.5%	98.28	99.02	99.52	99.10	99.83
		2%	99.78	99.84	99.64	99.65	99.96
	23°C	1%	84.09	76.37	88.79	92.53	84.78
		1.5%	98.58	90.66	94.86	94.83	87.25
		2%	99.72	96.43	98.13	98.10	98.84
	37°C	1%	99.87	94.64	97.03	96.02	96.28
		1.5%	99.87	95.46	98.39	98.30	98.82
		2%	99.95	99.56	99.79	99.62	99.49
V. cholerae	7°C	1%	91.43	91.31	96.04	97.82	99.00
		1.5%	92.18	92.16	98.03	98.30	99.25
		2%	99.52	99.27	99.07	99.27	99.64
	23°C	1%	90.69	89.20	25.90	27.28	78.92
		1.5%	91.28	90.80	43.08	45.89	93.67
		2%	94.59	94.68	83.00	93.56	97.89
	37°C	1%	97.05	99.04	98.40	98.01	99.66
		1.5%	98.11	99.37	99.67	98.87	99.81
		2%	99.87	99.96	99.99	100.00	100.00

Estimation of Cell Inhibition Rate (CIR)

The straight Ln (number of CFUs) lines against incubation duration were plotted for each of the extract concentrations and incubation temperatures. The slope of each straight line was then considered as the cell inhibition rate (CIR) in each experimental condition. The CIR values are presented in Table 1. Considering enteropathogenic *E.coli* and all concentration extract, the CIR ranged respectively from 0.35 h^{-1} to 0.44 h^{-1}, from 0.48 h^{-1} to 0.76 h^{-1} and from 0.81 h^{-1} to 1.07 h^{-1} when water was incubated at 7°C, 23°C and 37°C respectively. At each extract concentration, the highest CIR of enteropathogenic *E. coli* was noted at the highest incubation temperature (37°C). For *S. typhi* at the same incubation temperature, it varied from 0.58 h^{-1} to 0.70 $h^{-1,}$ from 0.65 h^{-1} to 0.67 h^{-1}, and from 0.55 h^{-1} to 0.76 h^{-1} respectively. We registered the highest value of CIR (0.76 h^{-1}) at the highest incubation temperature (37°C). Those of *V. cholera* ranged from 0.40 h^{-1} to 0.44 h^{-1}, from 0.29 h^{-1} to 0.48 h^{-1}, and from 0.40 h^{-1} to 0.86 h^{-1} respectively (Table 2). In most cases, the highest values of the CIR were registered at the highest extract concentrations for a given incubation temperature.

The CIR values thus increased with the increasing of the extract concentration and the incubation temperature (Table 2, Figure 4).

Table 2: Values of CIR (and regression coefficient) of cells at each experimental condition

Bacterial species and incubation temperature		CIR value (h^{-1}) at each extract concentration and in the NaCl (0.85%) solution			
		NaCl : 0.85%	Extract : 1%	Extract : 1,5%	Extract : 2%
Enteropathogenic *E. coli*	7°C	0.34 (0.51)	0.35 (0.52)	0.42 (0.65)	0.44 (0.64)
	23°C	0.31 (0.39)	0.48 (0.64)	0.53 (0.36)	0.76 (0.48)
	37°C	0.33 (0.53)	0.81 (0.94)	1.07 (0.95)	1.05 (0.78)
S. typhi	7°C	0.34 (0.37)	0.58 (0.53)	0.69 (0.49)	0.70 (0.39)
	23°C	0.47 (0.57)	0.65 (0.64)	0.62 (0.46)	0.67 (0.42)
	37°C	0.44 (0.54)	0.55 (0.29)	0.63 (0.36)	0.76 (0.41)
V. cholerae	7°C	0.12 (0.60)	0.40 (0.77)	0.44 (0.79)	0.43 (0.47)
	23°C	0.34 (0.92)	0.29 (0.57)	0.32 (0.62)	0.48 (0.81)
	37°C	0.12 (0.74)	0.40 (0.58)	0.48 (0.62)	0.86 (0.79)

Table 3: Spearman correlation coefficients between evolution rate values of planktonic cell abundance and extract concentration, incubation period

Parameters considered	Bacteria species and incubation temperatures								
	Enteropathogenic *E. coli*			*S. typhi*			*V. cholerae*		
	7°C	23°C	37°C	7°C	23°C	37°C	7°C	23°C	37°C
Extract concentration	-0.66**	-0.52*	-0.72**	-0.84**	-0.87**	-0.63*	-0.69**	-0.64**	-0.83**
Incubation period	-0.59*	-0.09	-0.58*	-0.22	-0.22	0.35	-0.54*	-0.72**	-0.42

Ddl: 14, **: Most Significant correlation (p<0.01), *: Significant correlation (p<0.05)

Table 4: Risk values of probability related to the comparison between extract concentration, incubation temperature and the abundance mean of cells bacteria

Parameter considered	Bacteria species		
	Enteropathogenic *E. coli*	*S. typhi*	*V. cholerae*
Extract concentration (ddl: 2)	0.008**	0.000**	0.001**
Incubation temperature (ddl : 2)	0.001**	0.026*	0.001**
Incubation period (ddl : 4)	0.058	0.20	0.031*

**: Most Significant difference (P<0.01), *: Significant difference (P<0.05)

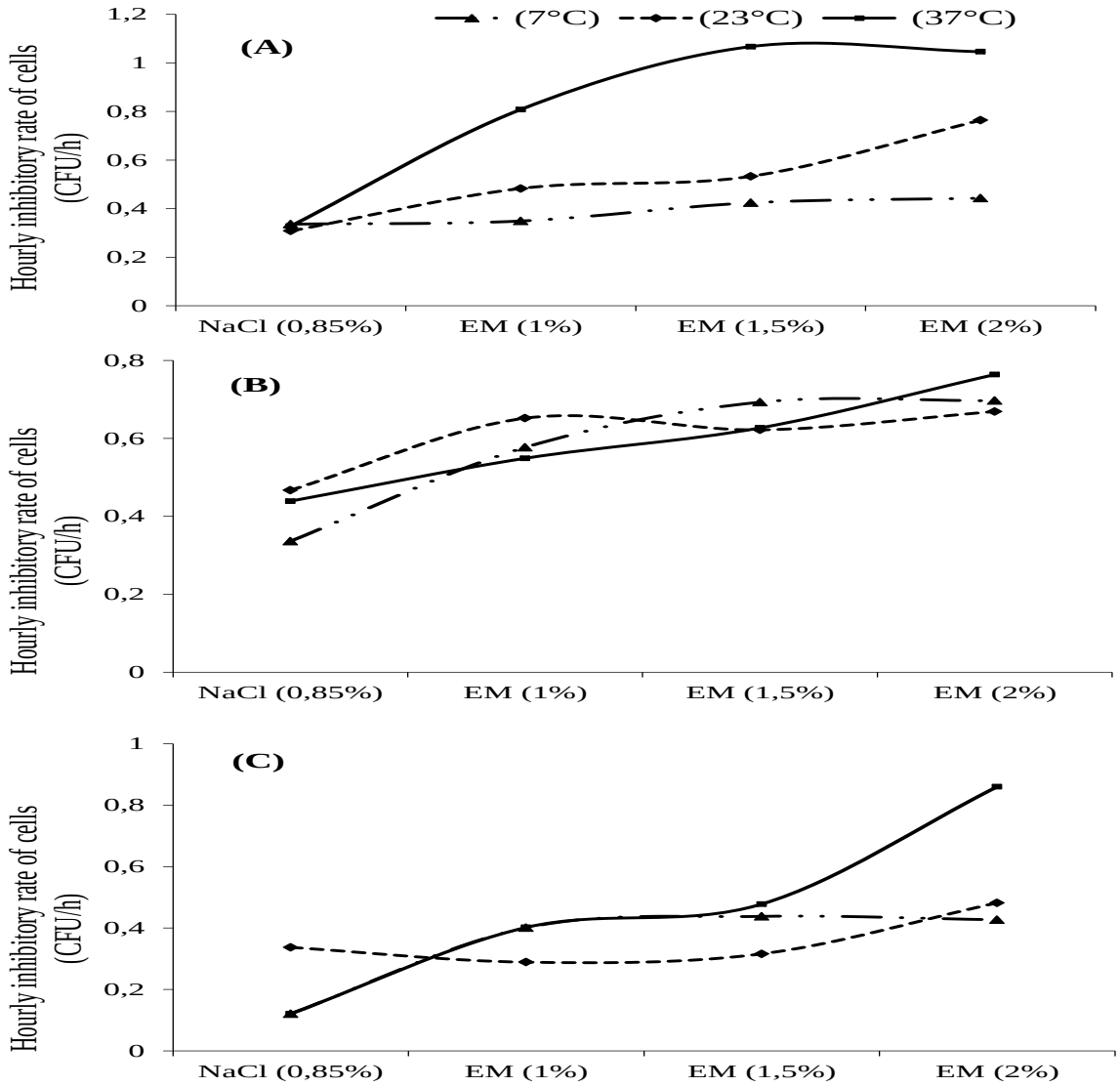

Figure 4: Evolution of the hourly inhibitory rate of cells with respect to each concentration of the *E. microcorys* leaves extract at different incubation temperature ((A): Enteropathogenic *E. coli*, (B): *S. typhi*, (C): *V. cholera*)

Relationship among the considered parameters

The relationships between the cells abundance and the extract concentration of *Eucalyptus microcorys* used at each experimental condition were evaluated. In most cases, it is noted abundance of cells at each incubation temperature significantly decreased ($P<0.05$) with an increasing of the extract concentration (Table 3). An increasing in the extract concentration would significantly decrease the concentration of the cultivable cells. An overall correlation between bacterial abundances and incubation duration has been carried out considering the concentrations of extract used. It noted that incubation duration do not significantly influence the cultivability of *S. typhi* at each temperature in the presence of the *Eucalyptus microcorys* extract ($P<0.05$) (Table 3). However, in the presence of this extract, incubation duration significantly impacted the cultivability of *V. cholera* and Enteropathogenic *E. coli* at the most of incubation temperatures used ($P>0.05$).

Using the Kruskal-Wallis test, the comparison of the cell concentrations of each of bacteria species for the whole extract concentrations and incubation temperatures. The risk values of probability are presented on (Table 4). It shows that the abundance of cultivable cells of each bacteria species

significantly differ from one extract concentration to another and from one incubation temperature to another ($P<0.05$). No significantly difference was noted with incubation period except with *V. cholerae*.

Our study has showed evidence that extract of *Eucalyptus microcorys* has a bactericidal properties whose scope vary relatively according to the type of cell and the environmental conditions. For each of the species of bacteria used, the abundances of cultivable cells varied with respect to the incubation duration and to the concentration of extract. Cultivable cells happen to be relatively less abundant at temperatures 23°C and 37°C than 7°C throughout the study length that stretched from 9 h to 24 h (Figures 1-3). Planktonic cells of Enteropathogenic *E. coli*, *S. typhi* and *V. cholerae* have proven to be more resistant to bactericide properties of the *Eucalyptus microcorys* extract in psychrophilic conditions. The temperature seems to be the cause of the variation of bacterial abundances [29]. It indirectly influences the productivity of bacteria by modifying the physical and chemical properties of the medium [30].

An increase in temperature leads to an increase of enzymatic activity, leading to an increase in the speed of metabolic and biochemical reactions and so, impact on the bacteria productivity. The increase in reaction speed is inevitably accompanied by an increase in metabolic wastes, some of them relatively toxic. This may be the reason of the high inhibition rates observed at 23 and 37°C (Table 1). Lessard and Sieburth suggested that with low temperatures bacteria may have better living conditions since their metabolism is slowed down as well as the production of toxic wastes [31].

It was observed that the concentration of extract increases alongside that the rate of inhibition cell, this with values ranging from 0.29 h^{-1} to 1.07 h^{-1}. This variation of cell abundances could be due to the accumulation of certain chemical products which may turn to be toxic for the bacteria [32]. The apparent inhibition may as well be due to the presence of several classes of phenolic products such as flavonoids, triterpenoids, alkaloids, tannins, Saponins present in our extract. Indeed, *Eucalyptus* species is known to produce numerous volatile compounds in large amounts, especially isoprenoids (referred as terpenes) which are accumulated in glands abundantly distributed throughout the leaf parenchyma and bark [33, 34].

The variations in the percentages of inhibition with respect to incubation temperatures and incubation period have been noted (Table 1). This could be due to the variation of the antibacterial activities of bioactive compounds present in the extract of *Eucalyptus microcorys*. The plant extract contain bioactive compounds whose configuration or properties change with time, like flavonoids. In addition, the complexing properties (reversible and irreversible) of flavonoids may also explain the variations in the percentage of inhibition observed during the incubation period. The stability and the reactivity of secondary metabolites like flavonoids are commonly linked to their molecular structures [35]. The reactivity of flavonoids dissolved in water leads to the molecular instability and this may vary with changes in environmental properties. Parameters such as light, pH, temperature, nature of the solvent, the presence of enzyme and oxidant can act on flavonoids [35]. The fluctuation of the percentage inhibition seems to vary with respect to the bacterial species as well as the concentration of the extract, temperature and incubation time. The bactericidal activity of an antibacterial substance or antibiotic may be time-dependent (time-dependent bactericidal antibiotic) or concentration-dependent (dependent bactericidal antibiotic concentration) [36]. It has also been suggested that antimicrobial agent is differentiated according to the selective toxicity, and bacteriostatic agent is often as inhibitors of protein synthesis and acts by binding to ribosomes. If its concentration is reduced, it would be released from the ribosome and the bacterial growth starts [1].

The mechanism of the microbial resistance against antibacterial agents may also explain the lower inhibition percentages sometimes observed. Microorganisms can alter the chemical structure of the antibacterial agent in an inactive form and then change the target of the antibacterial agent [1, 37]. The variation of inhibition percentages may also depends of the potential secondary metabolites which are present. Each of them may act solely or in synergetic with others. The impact magnitude of this may relatively vary with respect to the incubation duration and temperature.

Polyphenolic products, notably flavonoids and tannins are known for their toxicity against microorganisms. The mechanism by which these molecules act is certainly complex and may involve many action modes. On the enzymatic ground, the toxicity mechanism may be associated with the

inhibition of hydrolytic enzymes (the proteases and the carbohydrolases), or other interaction for the inactivation of microbial adhesines, transport and envelop proteins [38]. These molecules equally inhibit extracellular microbial enzymes, the sequestration of necessary substrates for microbial growth or the neutralization of elements such as iron [39]. Moreover, les flavonoids devoid of free hydroxide group have a higher bactericide action as compare to those that possess this group, with a hydroxide group there is an increase in chemical affinity to lipid membrane. The targets of flavonoids are cell membrane [38]. The antimicrobial activity depends on the presence of phenolic compound and probably on that of many secondary metabolites, the position and the number of hydroxide groups that it contains [40, 41].

Tannins can give complex with proteins leading to the inactivation of enzymes, either directly by binding to the active site or indirectly by sterique congestion [42]. Given their structural configuration, polyphenolic molecules may play a role in the degradation of cellular wall, destabilization of cytoplasmic membrane leading to the release of cellular components. The have a role in DNA and RNA synthesis [43], as well as of protein and lipid and in mitochondrial function, the cell wall creation is also influence by these elements [44, 45].

Flavonoids and alkaloids from *Eucalyptus sp* are known to possess antimicrobial activity [46, 47]. The toxicity of flavonoids against micro organisms results from a non specific interaction such as hydrogen bonds with cellular wall proteins or enzyme, neutralization of metallic ions, inhibition of the bacterial metabolism, sequestration of substances necessary for the cell growth [48]. Flavonoids such as myricetine impede the growth of multi-resistance bacteria *Burkholderia cepacia*, *Enterococcus* vancomycine-resistante, *Klebsiella pneumoniae* and *Staphylococcus epidermidis* [49].

It has also been noted that for each of the considered bacteria, the concentration of cultivable cells varied most significantly with respect to the extract concentration ($P<0.01$). The same observation was made with incubation temperature ($P<0.05$), (Table 4). Globally, this study showed that extract aqueous of *Eucalyptus microcorys* has a real effect on cultivability of each bacteria studied, with the highest percentage inhibition observed at extract concentration 2% in the most cases. The bactericidal effect in aquatic environment was noted using extract aqueous of *Lantana camara*, *Cymbogon citratus* and *Hibiscus rosasinensis* [9]. The same observations were noted with *E. microcorys* aqueous leaves extracts which can be used in the microbial treatment of water [18]. Lutgen and Michels showed that the addition of *Artemisia annua* tea to contaminated water drastically reduces the bacterial load and levels lower than those obtained by boiling treatment method [11].

Conclusion

In the aquatic environment containing *Eucalyptus microcorys* extract, cells cultivability is impacted. The CIR observed may be due to the presence of some chemical compounds contained in *E. microcorys* such as flavonoids, terpenoids, phenolics and alkaloids. Each of these secondary metabolites may act solely or in synergetic with others. The impact magnitude relatively varied with respect to the incubation temperature.

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