COMBINED EFFECT OF EUCALYPTUS MICRO CoryS AQUEOUS EXTRACT AND LIGHT ON PATHOGENIC ESCHERICHIA COLI SURVIVAL IN AQUATIC MICRO COSM

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INTRODUCTION

The problems of water supply for households in developing countries constitute nowadays, an important challenge to contribute to a sustainable development while guaranteeing satisfactory sanitary health. In spite of the Millennium Development Goals (MDG), many countries don’t have access to drinking water (Sunda, 2012). 13% of the world’s population, approximately 884 million people, lack access to an improved drinking water source (WHO/UNICEF, 2010). Fresh water is threatened by rising consumption and multiple pollutants. Bacteria responsible for the microbiological water pollution include Salmonella, Escherichia and Vibrio among others (WHO, 2004). The presence of Escherichia coli specie in water is probably a sign of the deterioration of its quality, due probably to contamination by pathogenic strict or opportunistic pathogens (CEAEQ, 2004). For nearly a decade, numerous epidemics attributed to pathogenic Escherichia coli strains are regularly reported in the world (Germani and Le Bouguen├®ec, 2008). Ingestion of contaminated water due to lack of hygiene and unsanitary water, sanitation and hygiene, contributes to about 1.5 million child deaths per year and around 88% of them from diarrhoea (WHO, 2002). In many countries of the world, the need for disinfected drinking water already constitutes a huge problem (Bergmann et al., 2002). Traditionally, chlorine is the most widely used chemical compound for water disinfection in many water treatment plants. However, the production of by-products of chlorination such as halogenated organic compounds, has been shown to be associated with various ailments in humans (Hwang et al., 2008). The combined effect of sunlight and temperature on bacterial cells is responsible for their inhibition in the solar irradiation process (Reed, 2004). Although the methods of disinfecting water by boiling, by adding chemicals or sunlight have their merits, but the slightest negligence in this area can lead to severe gastrointestinal illness and even death in children or immunodeficient people. According to literature, Medicinal plants are used by more than 80% of African and Asian households for therapeutical purposes. Indigenous people use hundreds of plants to treat themselves (Weathers et al., 2014). Recently, studies have been focused on treatment of water with plant extracts. Studies have shown that some aqueous extracts of Hibiscus rosa-sinensis, Cymbogon citratus and Lantana Camara, have a bactericidal effect in the aquatic environment (Sunda et al., 2008). They also showed that the combined action of light and Artemisia annua extract inhibit growth of Enterococcus faecalis coll (Mohil et al., 2015). However, the use of aqueous plant extract in the process of water disinfection, destined to drinking, remains low. Little information is available on the synergistic effect of light intensity and aqueous extract of Eucalyptus microcorys on planktonic cells of pathogenic Escherichia coli in aquatic microcosms. Few information is also available on the impact of various extract concentrations of Eucalyptus microcorys on bacteria, likewise few information relates the influence of the aqueous extract of E. microcorys on bacteria with respect to the duration of exposition under light intensity. This study aims to evaluate in aquatic microcosm, the combined impact of light intensity and the duration of exposure to photons on the inactivation of pathogenic Escherichia coli under different concentration of aqueous extract solution of Eucalyptus microcorys.

MATERIAL AND METHODS

Preparation of E. microcorys Leaf Crude Extract

Fresh leaf samples of E. microcorys were harvested in Yaound├®, Center Region (Cameroon) and dried up at room temperature (23±2 °C) in the laboratory for 30 days (Tamsa Arfao, 2017). Dried samples were ground (for 2 mins) to fine powder (~200 μm) using a laboratory grinder at maximum speed. Fifty grams of the powder obtained, were mixed with 100 ml of hot distilled water. This mixture was boiled for thirty minutes in a conical flask. The extract was filtered through Whatman filter paper number 1. The filtrate obtained constituting the decoction, was dried in an oven at 45-50 °C (Tamsa Arfao et al., 2013). The obtained crystals were used to prepare the crude extract. Six extract concentrations 0.05%, 0.1%, 0.5%, 1%, 1.5% and 2% were prepared thus while using sterile physiological water. Every concentration was filtered with nitrate cellulose membrane of 0.45 μm porosity.

Qualitative phytochemical screenings were done according to the standard protocols. Alkaloids test was done according to Dragendorff’s and Meyer's
tests, whereas Barfoed’s and Fehling tests were used for carbohydrates. The reduction of FeCl3, the frothing and Salkowski tests allowed to screen flavonoids, tannins, saponins and terpenoids respectively (Treonse and Evans, 1983; Sofowora, 1993; Mobil, 2015).

Isolation and identification of pathogenic Escherichia coli

This bacterium was isolated from the urban stream in the Center Region of Cameroon. It was isolated on Endo medium, using membrane filtration technique at 44°C for 24 hours (Rompré et al., 2002). Bacterial colonies with metallic green sheen observed on Endo medium were identified according to standard method (Holt et al., 2000). These biochemical tests are performed on conventional galleries consisting of media introduced in tubes. The identification of the bacterial strain is obtained by comparing its biochemical profile with those pre-established in the API20E Biochemical Test. These tests are grouped into basic and orientation tests for the confirmation diagnosis of Escherichia coli strains. Characterization of E. coli was done according to Nougang et al. (2011) protocols.

Preparation of pathogenic Escherichia coli culture

For the preparation of bacterial stocks, a colony forming unit (CFU) from Endo medium was inoculated into 100 mL of nutrient broth (Oxford) for 24 h at 37°C. After this period, cells were harvested by centrifugation at 8000 rev/min for 10 min at 10°C and washed twice with NaCl (0.85%) solution. Each pellet was re-suspended in 50 mL of NaCl solution. After homogenization, 1 mL of the obtained solution was then transferred into 500 mL of sterile NaCl solution (0.85%) in Erlenmeyer flask and stocked.

Experimental setup

For this experimentation, 35 flasks of 350 mL were used. They were organized into seven series of five flasks: A, B, C, D, E, F and G. 200 mL of physiological water at 0.85% of NaCl concentration were used for control tests and were considered as the A series flasks. The six other series names B, C, D, E, F and G contained 200 mL of the extract at different concentrations 0.05%, 0.1%, 0.5%, 1%, 1.5% and 2% respectively. The concentrations chosen were based on our previous work (Tamsa Arfao et al., 2013) and according to Nicolas (2012) recommendations. At initial time, 1 mL of the inoculum was transferred in to each flask. At T0, the concentration cell was 27x10^6 CFU/mL. The contact times were 3, 6, 9, 12 and 24 hours. The light intensity was that of the order of 0 lx (darkness condition), 1000 lx, 2000 lx and 3000 lx (light condition). Our experimentation microcosms were subjected to illumination provided by 100 W Tungsten filament lamps (TESLA HOLE-SOVICE) (Nola et al., 2010). A lamp was placed at 25 cm above each water solution. The 4 lamps were connected in series on a single cable. A luxmeter (PHYWE) was used to measure the light intensity reaching the bottom of each container. The electrode of the luxmeter was immersed in the container. Figure 1 depicts the experimental setup used for this study. Bacteriological analysis was performed for each incubation duration.

![Figure 1](image)

Figure 1 Experimental setup showing the process used (Nola et al., 2010).

Data analysis

The variation patterns of pathogenic E. coli cell abundances at different concentrations of Eucalyptus microcorys aqueous extract (0.05%, 0.1%, 0.5%, 1%, 1.5% and 2%) and different experimental conditions (darkness condition, intensity light at 1000 lx, 2000 lx and 3000 lx) were displayed using the Self-Organizing Map (SOM) by means of the toolbox developed for Matlab (Alhoniemi et al., 2003). After living in the extract solution of Eucalyptus microcorys at different concentration and incubation duration, the cell abundances were arranged as a matrix of 5 rows (corresponding to the different hours of incubation of cells in the extract solution: 3, 6, 9, 12 and 24 hours) and 24 columns. The Self-Organizing Map algorithm was then applied to classify samples in each group according to their similarity in terms of cell abundances. Self-Organizing Map preserves neighborhood so that samples with similar abundance values are grouped together on the map, whereas samples with very different abundances are far from each other.

The percentage of inhibition (PI) was calculated using the following formulae as described by Tamsa Arfao et al. (2013).

\[
PI = \left( \frac{N_0 - N_t}{N_0} \right) \times 100
\]

where \(N_0\) = cell abundance in control (NaCl: 0.85%); \(N_t\) = cell abundance after the action of extract solution.

According to the first 12 hours of contact time with extract concentration, the straight Ln of the form \(y = ax + b\) were plotted for each considered parameters. 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 \(x = 0\)) (Tofallis, 2009). The slope of the straight line was then considered as the dark inhibition coefficient \(K_D\) in dark condition and light inhibition coefficient \(K_L\) in light condition.

The rate of disinfection as described by the Chick-Watson equation was used to compare the data obtained after combined effect of the extract concentration and the reaction time. Generally, disinfection models are established with Ct values derived from Chick-Watson kinetics based on the values obtained in laboratory inhibition studies (Sunil and Nitin, 2012). This Watson function was done using Excel program of the following form: log \((N/N_0) = - K C^t\) (Chick 1980; Watson 1908).

In many cases, the \(n\) value for Chick-Watson law is close to 1.0 and hence a fixed value of the product of concentration and time (Ct product) results in affixed degree of inactivation (AWWA, 1999). In this formulae, \(N\) represent cell abundances at incubation time \(t\) exposure in the extract solution, \(N_o\) a number of cells bacteria in control (NaCl: 0.85%), \(t\) is contact time, \(k\) is the disinfection rate constant, \(C\) extract concentration and log is logarithm to base 10.

RESULTS AND DISCUSSION

Phytochemical screening of extract plant

The phytochemical screening of Eucalyptus microcorys revealed the presence of polyphenols, sterols, triterpenoids, flavonoids, gallic tannins, anthraquinons, anthocyanins, alkaloids and saponins. The different chemical constituents of Eucalyptus microcorys leaves extract and their relative abundances are summarized in the Table 1. Some studies revealed the presence of these compounds in other plants of the Myrtaceae family to which Eucalyptus microcorys belongs (Pamplona-Roger, 1999). The presence of hydroxyls tannins has also been reported in Eucalyptus microcorys extract (Moore et al., 2004).

Table 1 Chemical constituents of Eucalyptus microcorys leaves extract

| Chemical compounds tested | Appreciation of relative abundance |
|---------------------------|-------------------------------------|
| Sterol and Triterpenoids   | +                                   |
| Polyphenols                | +                                   |
| Flavonoids                 | ++                                 |
| Catechics                  |                                     |
| Gallics Tannins            | +                                   |
| Anthraquinons              | +++                                |
| Anthocyanins               | +                                   |
| Alkaloids                  | +++                                |
| Saponins                   | ++                                 |
| Lipids                     | -                                   |

Legend: +++ : Abundant; ++ : quite abundant; + : scanty; - : Non detected
Inhibition patterns and percentage of inhibition

Figure 2(a) presents the SOM map of the cell abundances of pathogenic *E. coli* obtained after different incubation period at different concentrations of *Eucalyptus microcorys* aqueous extract solution. The Self-Organizing Map obtained was classified in the 12 output nodes, so that each node included samples with similar abundances. The Self-Organizing Map of displayed three ranges (I, II and III) of samples at different levels of the Euclidean distance. Results revealed that grouping of bacteria abundances was related to the incubation duration according to the Log ratio (G-test). Cluster I in the upper-left part of the SOM map is composed of cells abundances registered after 24 hours in the extract solution. The second Cluster in upper-right of the SOM map is made up of all cell abundances registered after 9 and 12 hours in the extract solution. Cluster III in the lower part of the SOM map is constituted by cells abundances obtained after 3 and 6 hours in the extract solution. Figure 2(b) depicts the inhibition patterns of pathogenic *E. coli* for each incubation period at different light intensity and extract concentration. Dark areas represent high cell abundances, whereas pale or bright areas represent low values of bacteria abundances. Overall, cell abundances are very low in clusters I and II at the extract concentrations 0.5%, 1%, 1.5%, and 2%, for 2000 lx and 3000 lx light intensity. However, there are cases where abundances remain high even after 24 hours of incubation in the extract solution in dark conditions.

The rate of cells remained after exposure to extract solution were calculated in different condition. In dark condition, the percentage of inhibition ranged between 17 and 99%. The augmentation of contact time with light impact the percentage of inhibition which increase significantly. They also increased relatively with high concentration of aqueous extract of *Eucalyptus microcorys*. The percentage of inhibition fluctuated between 16 to 100% under 1000 lx, and between 38 and 100% under 2000 lx. The highest rate of inhibition was observed with extract concentration 0.05% after 12 hours of incubation under 3000 lx. The presence of different bioactive compounds in extract solution could explain the variation of inhibition rate at different light intensity and incubation duration (Franco et al., 2005). In fact, compounds like flavonoids change their configuration or properties over time (Tamsa Arfao et al., 2013). This observed variation of inhibition rate could also be explained by the rapid reactivation of *Escherichia coli* cells in some cases which could be the result of a photo adaptation. According to Ben Sai et al. (2011), photo adaptation is able to increase tolerance to bactericidal radiation by reducing the accumulation of photon products generated at the genetic support and maintain there after cell survival.

Dark (K<sub>D</sub>) and light (K<sub>L</sub>) inactivation and temperature effects

Figure 3 depicts the variation of the hourly inhibitory rate of *Escherichia coli* cells with respect to each concentration of the *E. microcorys* leaves extract at different light intensity.

After exposure to aqueous extract, dark inhibition coefficient of pathogenic *Escherichia coli* was respectively 0.102 h⁻¹, 0.116 h⁻¹, 0.111 h⁻¹, 0.123 h⁻¹, 0.136 h⁻¹ and 0.146 h⁻¹ with 0.05%, 0.1%, 0.5%, 1%, 1.5% and 2% of extract concentration in the dark condition. In the light condition, we noted a relative increase of K<sub>L</sub>. Under 1000 lx, we registered the highest value of K<sub>L</sub> (0.639 h⁻¹) at extract concentration 1.5%. Under 2000 lx, the highest K<sub>L</sub> (0.366 h⁻¹) was recorded at extract concentration 2%. The lowest K<sub>L</sub> (0.260 h⁻¹) was recorded at extract concentration 0.1%. Under 3000 lx, the K<sub>L</sub> was respectively 0.343 h⁻¹, 0.361 h⁻¹, 0.394 h⁻¹, 0.300 h⁻¹, 0.314 h⁻¹, 0.453 h⁻¹ with 0.05%, 0.1%, 0.5%, 1%, 1.5% and 2% of extract concentration. The K<sub>L</sub> varied with the bacteria present in the aqueous extract solution, increasing with light intensity and aqueous extract concentration. Many studies showed that *Eucalyptus* species have antimicrobial activity against many bacteria (Franco et al, 2005). The essential oils of *Eucalyptus camaldulensis* and *Eucalyptus globulus* were indicated as a bactericidal or bacteriostatic agent against *Escherichia coli* (Ghaem and Mohamed, 2008).
plants (Sunda et al., 2008). Singlet oxygen is the product resulting from the action of combined photosensitizer, light and oxygen, as follows (with ISC being the Intersystem Crossing):

\[
\text{Sens} + h\nu \rightarrow \text{Sens}^* \rightarrow \text{Sens} + O_2
\]

It is a form of oxygen, with a higher energy than conventional oxygen, formed by the excitation of the latter. The basic oxygen is an important participant in the photochemical processes because it contains a high chemical potential. The formation of singlet oxygen from a photosensitizing molecule begins with the excitation of the sensitizer by absorption of a photon. After intersystem crossing (ISC), the photosensitizer is in the triplet state. Triplet-triplet annihilation from oxygen in the ground state and the photosensitizer in its triplet state provides the singlet oxygen (\(O_2^\cdot\)) and leaves the photosensitizer in its initial state (Towers, 1985). The singlet oxygen formation and its reaction are as follows:

\[
O_2 + e^- \rightarrow O_2^\cdot
\]

The triplet state reacts directly with the biological substrates (phospholipids, proteins, steroids...) by a transfer of electrons or hydrogen atoms which leads to the formation of free radical (Raven et al., 2007). These radicals can react with oxygen in the ground state to form the superoxide ion. If this case is not particularly reactive, its protonated form can however be transformed into hydrogen peroxide. The triplet state can also react directly with oxygen by transferring its excess energy to the oxygen in the ground state, moving it to triplet (\(O_2\)) in its singlet state (\(O_2^\cdot\)). This singlet oxygen also reacts with the nitrogenous bases of the DNA, mainly thymine and guanine. Due to its electrophilic nature, singlet oxygen reacts with the unsaturated lipids, including cholesterol (Sunda, 2012).  

During the study, we noted a change in temperature registered during the experiment. In dark condition, the temperature fluctuated between 22 and 24°C. Under 1000 lx, it ranged from 38 to 42°C. Under 2000 lx and 3000 lx, respectively it changed from 39 to 50°C and 40 to 54°C. The increase of temperature with light intensity could also impact respectively the survival of these bacteria. Studies showed that temperature is generally involved on variation of the abundances of bacteria (Davey, 1989). Calculating the sums of square of the percentage factors, the cells inhibition of pathogenic E. coli is controlled by the temperature recorded during the experiment (52.03%), followed by extract concentration (18.79%), themselves followed by the incubation duration (18.79%) and in turn followed by the light intensity (0.21%). It appears clearly that the temperature (52.03%) has a considerable effect on the inactivation of bacteria compared to the light intensity (0.21%). Based on the data presented in the study of Tamsa Arfao et al. (2013), the temperature is a factor of great importance. It acts on biochemical reactions of microbial metabolism (Regnault, 2002). It indirectly influences the productivity of bacteria by modifying the chemical and physical characteristics of the medium (Mauguin et al., 2004). The augmentation in reaction kinetic is accompanied by an increase in metabolic wastes. However, several research projects have shown that at high temperature, there are synergistic effects between solar radiation and thermal inactivation. The temperature was merely a kinetic effect because the maximum values reached (54°C) are below lethal values for fecal coliforms (55°C), but in equal to the required values (45°C) for the effectiveness of the synergistic effects with radiation (McGuigan et al., 1998). 

The degree of connection between parameters showed a significant and negative correlation (P<0.05) between cell bacteria and incubation time at each extract concentration. When considering the whole incubation duration, at each experimental condition, a high significant negative correlation has been noted. The corresponding correlation matrix is presented in Tables 2 and 3.

**Chick-Watson model for Kinetics of Disinfection**

Chick-Watson model for different extract concentration is presented in Table 4. Globally, for extract concentration 1, 1.5 and 2%, Chick-Watson model obtained for *Eucalyptus microcorys* was log(N/No) = - 0.1 Ct. Globally, for extract concentration 1, 1.5 and 2%, Chick-Watson model obtained for

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**Figure 3 Variation of the hourly inhibitory rate of pathogenic *E. coli* cells after exposure after exposure to aqueous extract of *E. microcorys***

Previous study showed that aqueous extract of *Eucalyptus microcorys* has antibacterial activities which vary according to the type of bacteria cell and the environmental conditions (Tamsa Arfao et al., 2013). The progressive decrease of cells was noted during the contact period with extract solution, indicating a progressive increase in the cell inhibition coefficient for fixed experimental conditions. Some authors observed that bacterial inactivation after irradiation is significantly correlated to the intensity of light (Sinton et al., 2002).

The combined effect of light action and extract of *Eucalyptus microcorys* influence considerably the variation of the hourly inhibitory coefficient of cells with respect to each extract concentration (Tamsa Arfao, 2017). In fact, we noticed that the hourly inhibition coefficient is very low in dark conditions and increase with the augmentation of light intensity. Bacteria have photosensitive sites P, which in the presence of light, are converted into reactive forms P*. These activated forms P* convert oxygen molecules into state singlet oxygen (\(O_2^\cdot\)) which is a powerful oxidant and that destroys the cells (Stanier et al., 1990). The toxicity is due to superoxide radicals and hydrogen peroxide which are produced during oxidation reactions (Nola et al., 2010). According to Maiga et al. (2009), the two major pathways involved in this process of inactivation by sunlight would be photobiological reactions (DNA alteration and oxidation of cellular elements). The presence of oxygen during exposure to light is necessary for the formation of reactive oxygen species toxic to bacteria (Jeffrey and Mitchell, 1997). The dimerization between adjacent pyrimidine bases is the most probable reaction resulting from the direct action of UV on DNA. The two major photoproducts formed are those of the nitrogenous bases is the most probable reaction resulting from the direct action of UV on DNA. The two major photoproducts formed are those of nitrogenous bases

\[
\begin{align*}
\text{Sens} + h\nu &\rightarrow \text{Sens}^* \\
\text{Sens} + O_2 &\rightarrow \text{Sens} + O_2^\cdot
\end{align*}
\]

These activated forms P* convert oxygen molecules into state singlet oxygen (\(O_2^\cdot\)) which is a powerful oxidant and that destroys the cells (Stanier et al., 1990). The toxicity is due to superoxide radicals and hydrogen peroxide which are produced during oxidation reactions (Nola et al., 2010). According to Maiga et al. (2009), the two major pathways involved in this process of inactivation by sunlight would be photobiological reactions (DNA alteration and oxidation of cellular elements). The presence of oxygen during exposure to light is necessary for the formation of reactive oxygen species toxic to bacteria (Jeffrey and Mitchell, 1997). The dimerization between adjacent pyrimidine bases is the most probable reaction resulting from the direct action of UV on DNA. The two major photoproducts formed are those of the nitrogenous bases

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\text{Sens} + O_2 &\rightarrow \text{Sens} + O_2^\cdot
\end{align*}
\]
Eucalyptus microcorys was log(N/No) = -0.1 Ct. This value is very close to chloride disinfectant log(N/No) = -0.16 Ct (Yoon Jin Lee et al., 2002). Sunil and Nitin (2012) obtained similar result with the plant Anjan: log (N/No) = -0.17 Ct.

### Table 2
Correlation coefficients between cell bacteria and contact time at each extract concentration

| Extract concentration and cell abundances | 0.05% | 0.1% | 0.5% | 1% | 1.5% | 2% |
|-------------------------------------------|-------|------|------|----|------|----|
| Pathogenic E. coli                        | -0.460*| -0.478*| -0.472*| -0.371| -0.472*| -0.454*|

N: 20; *: P < 0.05

### Table 3
Correlation coefficients between cell bacteria and extract concentration at each experimental condition

| Cells | Light intensity |
|-------|-----------------|
|       | 0 lx | 1000 lx | 2000 lx | 3000 lx |
| Pathogenic E. coli | -0.741** | -0.734** | -0.730** | -0.271 |

Number of observations: 30; **: P<0.01

### Table 4
Chick-Watson model for different extract concentration

| light intensity | Value of log(N/No) (Ct) for each extract concentration (%) |
|-----------------|------------------------------------------------------------|
| 0 lx            | 0.05 | 0.1 | 0.5 | 1 | 1.5 | 2 |
| 1000 lx         | -1.1 Ct | -0.7 | -0.5 | -0.1 | -0.1 | -0.1 |
| 2000 lx         | -1.2 Ct | -0.7 | -0.5 | -0.1 | -0.1 | -0.1 |
| 3000 lx         | -5.2 Ct | -1.5 | -0.3 | -0.1 | -0.1 | -0.1 |

CONCLUSION

The photo-inhibition of Pathogenic Escherichia coli in the aquatic microcosm increases with Eucalyptus microcorys extract in water. The photosensitivity reaction generating singlet oxygen, can improve in the presence of molecules like quinones and anthraquinones. The capture of a photon of light by a photosensitizer causes excitation of the latter. The stored energy can be transferred to the primary oxygen to generate singlet oxygen. The harmful effects of singlet oxygen on microorganisms are known. However, temperatures have a more considerable effect on the inactivation of bacteria compared to the light intensity. During the process of treatment bacterial contaminated water, it seems primordial to consider the influence of the synergistic effects between solar radiation and thermal inactivation with aqueous extract of Eucalyptus microcorys. Considering Chick-Watson model obtained, extract of Eucalyptus microcorys seems effective for inactivation of cell bacteria during water treatment.

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