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Effect of *Mentha viridis* L. extracts on Pathogenic Bacteria Adhesion

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Abstract

Micro-organisms do not always exist in planctonic forms (single cells or small groups). To survive, especially in limiting media, they may adhere to inert or living surfaces. This enables them to multiply within a community protected by an extracellular matrix, thus forming a biofilm which protects them from antimicrobials agent especially antibiotics. In this paper, the aim of this study was to investigate *in vitro* the autoaggregation and coaggregation of resistant pathogenic bacteria responsible of intestinal infections (on young children), the surface hydrophobicity and the exopolysaccharides production treated with methanolic, hydro-methanolic and aqueous extract of *Mentha viridis* collected in South West of Algeria(wilaya of El Bayadh). The qualitative analysis revealed the presence of polyphenols and flavonoids, which were confirmed-by-quantitative-analysis. All extracts showed an antibacterial activity (anti-adhesiveness).The percentage of auto aggregation, co aggregation decreased in presence of extract comparing to control. *E.coli* was hydrophobic while the others hydrophilic. The amount of exopolysaccharides (EPS) produces by bacteria decreased considerably with all extracts comparatively to control (without extracts).

Keywords: anti-adhesiveness activity, extracts, *Mentha viridis,* multi-resistant bacteria

1. Introduction

The mucosal surface of the intestinal tract is a complex ecosystem composed of the gastrointestinal epithelium, immune cells and the resident bacterial micro flora. In this environment, bacteria are either in contact with intestinal surfaces or embedded in host- produced mucus [1]. In the absence of an intact microbiota, the susceptibility to pathogens drastically increases, underlining the fact that colonization of mucosa and competition with commensal bacterial is often the first step in most intestinal infections [2]. Bacterial infections are orchestrated by virulence factors that facilitate various aspects of their pathophysiology critical for disease in the host. These include adhesions and membrane proteins that mediate bacterial attachment, colonization, and invasion of host cell [3]. Thus, infectious gastroenteritis constitutes one of the main causes of morbidity and mortality among children under 5 years old. Each year, 1.3 billion of gastroenteritis episodes are observed in children in the world and four million die [4]. This infection is especially problematic among infants, young children, and immune compromised persons. The colonization of mucosa and competition with commensal bacterial flora is often the first step in most intestinal infections [1]. The microbial consortia compact often confer certain advantages on the microbial population, such as antibiotic resistance, immune evasion, shear resistance and general persistence in changing and often hostile environments. Although drugs exist to treat these diseases are not always effective because of the appearance of bacteria resistant to one or more antibiotics (multiresistance) and toxicity of the

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products [5]. The emergence of antibiotic resistance in pathogenic bacteria has led to renewed interest in exploring the potential of plant derived antimicrobials as an alternative therapeutic strategy to combat microbial infections. Humanity has used various plants found in its environment, to treat all kinds of diseases. These plants contain many chemical bioactive compounds responsible of a wide range of biological activities. The search for new therapeutic molecules with antibacterial activity proves consequently necessary; the return towards the naturalness became more than essential [6]. In this present research, the most important aim was to assess *in vitro* the antibacterial activity (anti-adhesiveness) of various extracts (methanolic, hydromethanolic and aqueous extract) of *Mentha viridis*, a plant largely used throughout the world against multiresistant bacteria responsible of gastroenteritis and food poisoning.More than a thousand two hundred varieties of this plant are cultivated out of the five continents. It belongs to the *Lamiaceae* family, known for its antimicrobial, antispasmodic and anti-inflammatory properties [7]. *Mentha viridis* was collected from region of El Ghassoul (situated in the Atlas Sahara), El Bayadh city, where this medicinal plant is badly exploited.

2. Material and Methods

2.1 Plant Material

Mentha viridis L. collected in the commune of El-Ghassoul, Daira de Brezina (Wilaya d' El-Bayadh) (South West of Algeria characterized by a semi-arid climate) was identified according to African Flowering Plants Database. It was identified by a local expert and a voucher specimen (#1546) was deposited at the herbarium center of the laboratory of Bioconversion, Genie Microbiology and healthy security of the Faculty of Science of the Nature and the Life of the University of Mascara (North West of Algeria) for future reference. The collected plant was allowed to dry at room temperature for 2 weeks according to standard procedures. They were then grounded to powder in a ball mill.

2.2 Bacterial Strains

Pathogenic bacteria frequently implied in infantile gastroenteritis: *Clostridium difficile* isolated from beef meat and the cow's milk, along with *Staphylococcus aureus*, *Escherichia coli, Klebsiella oxytoca* , strains isolated from ill children's stools were provided by the Laboratory of Medical Analysis of the Wilaya of Mascara. Strains were maintained on nutrient agar slopes at +4°C. Before experimental use, cultures were sub cultured twice in nutrient broth (peptone 15.0 g, yeast extract 3.0 g, sodium chloride 6.0 g, D (+) glucose 1.0 g, distilled water 1 L)

2.3 Antibiogram (Agar Diffusion Method)

A suspension of each strain containing 10^6CFU/mL was shown on the agar surface and discs containing the antibiotics mentioned below were added to the Petri dishes, after a 24-h incubation at 37°C, diameters of inhibition zones were measured [8]. The antibiotics used were: colistine (CT), aztréonam (ATM), Gentamicin (CN), oxacillin (OX), Cefazolin (CZ) and the spiramycin (SP).

2.4 Preparation of the Extracts

First, for preparation of methanolic extract, we need 20g of pulverized plant material with 200 mL of pur methanol (cold maceration). Second, for hydromethanolic extract we add hydromethanol (80/20) (v/v) to 20 g of the same powder. Third, the aqueous extract was prepared by mixing 20g of fine powder in 200mL of water [9]. These extracts were then filtered through Whatman's No. 1 filter paper and the methanol filtrate was separately concentrated to dryness under vacuum using a rotary evaporator (at 60°C) to remove methanol. The aqueous extract was lyophilized [10].

2.5 Total Phenolic Content

The amount of total polyphenols was determined using the Folin–Ciocalteu's method. Briefly, 1mL of the methanolic extract was mixed with 1 mL of 1/10th Folin–Ciocalteu reagent. After 5 min, 10 mL of aqueous Na_2CO_3 (7%, w/v) were added. The mixture was allowed to stand for 90 min at 23°C and then absorbance was read at 750 nm (JENWAY IC 6400 UV/ visible). A standard curve was prepared using gallic acid over a range of 0 to 1 mg/ml. Total polyphenolics values are expressed in gallic acid equivalents (GAE) per gram of dry weight (mg GAE g^{-1} DW) [11].

2.6 Total Flavonoid Content

The method of the aluminium trichloride ($AlCl_3$) [12] was employed to determine the content of total flavonoid in

the various extracts. A calibration curve was established by the catechin (0-40 µg/mL), under the same operating conditions as the samples and served for the quantification of the total flavonoids expressed in microgram of equivalent of catechin per milligram of extracts [12].

2.7 In vitro Evaluation of the Antibacterial Activities of *Mentha viridis*

2.7.1 Auto Aggregation and Co Aggregation

Auto aggregation assays were assessed with some modifications [13]. Bacteria were grown for 18 h at 37°C in sterile nutritive agar or broth (peptone 15.0 g, yeast extract 3.0 g, sodium chloride 6.0 g, D (þ) glucose 1.0 g, distilled water 1 L). The cells were harvested by centrifugation at 5000 g for 15 min, washed twice and resuspended in their culture supernatant fluid or in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU /mL, by diluting fresh cultures and comparison to Mac Farland standards (OD_{650nm}=0.7) [14]. The *Mentha viridis* extracts were added in various amounts (50µL/mL, 100µL/mL, 200µL/mL and 300µL/mL). Cell suspensions (4mL) were mixed by vortexing for 10 s. Auto aggregation was determined after 1, 2 and 3 h of incubation at room temperature. At each time point, 0.1mL of the upper suspension was transferred to another tube with 3.9mL of PBS and the absorbance (A) measured at 600 nm. The autoaggregation percentage was calculated as follows: 1-(A_t/A_0)· 100, where A_t represents the absorbance either time t =1, 2 or 3h and A_0 the absorbance at t = 0.

The method for preparing the cell suspensions for co aggregation was the same as that for auto aggregation assay. Different co-aggregates were prepared as follows: (*E. coli/S. aureus)*, (E.*coli/ K.oxytoca*) and (*S.aureus/ C.difficile*). Equal volumes (2mL) of each cell suspension were mixed together in pairs by vortexing for 10s. Control tubes were set up at the same time, containing 4mL of each bacterial suspension on its own. The absorbance (A) at 600 nm of the suspensions was measured after mixing and after 3h incubation at room temperature. Samples were taken in the same way as in the auto aggregation assay. The percentage of co aggregation was calculated using the equation: Co aggregation (%) = ((A_x+ A_y) /2-A(x+y)) / A_x+ A_y /2 .100 Where x and y each represents one of the two strains in the control tubes, and (x + y) the mixture.

2.7.2 Microbial Adhesion to Solvents

Microbial adhesion to solvents (MATS) was measured according to the method of Kos et al. [15] with some modifications [16, 17]. Bacteria were harvested in the stationary phase by centrifugation at 5000 g for 15 min, washed twice, and resuspended in 0,1mol/L KNO_3 (pH 6,2) to reach approximately 10^8 CFU/mL. The absorbance of the cell suspension was measured at 600 nm (A_0). One millilitre of solvent was added to 3 mL of cell suspension. After a 10-min pre-incubation at room temperature, the two phase system was mixed by vortexing for 2min. The aqueous phase was then removed after 20min of incubation at room temperature, and its absorbance at 600 nm (A_1) read. The percentage of bacterial adhesion to solvent was calculated as (1-A_1/A_0). 100. Three different solvents were tested in this study: xylene, which is an apolar solvent; distilled water, a monopolar and acidic solvent; and ethyl acetate, a monopolar and basic solvent. Only bacterial adhesion to xylene reflects the cell surface hydrophobicity or hydrophilicity. The values of MATS obtained with the two other solvents were considered as a measure of electron donor (basic) and electron acceptor (acidic) characteristics of bacteria, respectively [13].

2.7.3 Exopolysaccharides (EPS) Content

The immobilization of bacteria was carried out by exposing the bacterial suspensions to ultrasounds (52 khz /for 10 minutes). 1mL of extracts were added. After incubation at 37°C for 24h, the extraction of EPS was carried out according to [18]. The cells were harvested by centrifugation at 5000g /for 15min after boiling at 80°C for15 min to precipitate the EPS. The supernatant was filtered at +4°C. The EPS was precipitated by the addition of three volumes of cold ethanol, followed by centrifugation at 10 000 g for 20 min at +4°C.The pellet was redissolved in 100 mL distilled water and reprecipitated twice. The quantification of EPS was performed by the total sugar essay [19]. After vortexing, the absorbance (A) of the mixture was measured at 490nm and compared to that of control (without extract).

2.8 Statistical Analysis

All experiments were made in duplicate. All data are presented as means ±SD.

3. Results and Discussion

3.1 Extraction Yield

The greatest yield was observed with the aqueous extract (10%), followed by the methanolic extract (9%) then the hydro-methanolic extract (6%) respectively.

3.2 Total polyphenol Content

Values obtained for the methanolic and aqueous extracts showed that these extracts had the highest polyphenol contents (16.72 ± 6.10 mg EGA/g DW and 16.61 ± 0.51 mg EGA/g DW, respectively) followed by the hydro-methanolic extract (10.40 ± 2.22 mg EAG/g DW).A study by showed that freeze drying produced dried spearmint had a high total phenolics (34.6 ± 1.9 mg/g) content [20]. Orphanides et al. added that drying was a very useful technic to extend the shelf-life of spearmint and to produce dried spearmint with a high phenolic content. Also, the distribution of secondary metabolites can change during the development of the plant. This can be related to the hard climatic conditions (the high temperature, solar exposure, drought, salinity), which stimulate the biosynthesis of the secondary metabolites such as the polyphenols [21]. Indeed, the phenolic content of a plant depends on a certain number of intrinsic factors (genetic) and extrinsic ones (climatic conditions, cultural practices, maturity with harvest and conditions of storage) [21]. The solvent for extraction used in this work could carry on non-phenolic substances like sugars, proteins and dyes which can interfere during any phenolic evaluation [22].

3.3 Total Flavonoid Content

Amount of flavonoid in the methanol extract was found to be significant as 2.2 ± 0.06 mg EC/g of extract followed by the aqueous extract with 1.35 ± 0.04 mg EC/g and the hydro-methanolic extract with 0.98 ± 0.02 mg EC/g of extract . A study of *Mentha pulegium* of southern Spain, obtained values of either 28± 0.06 mg EC/g for the alcoholic extract or 24 ± 0.01mg EC/g for the aqueous extracts [23]. Flavonoid can be produced in abundance for parasitized plants, to attack insects by their unpleasant tastes [24]. However, it is difficult to compare our results with those of the literature because various methods of extraction were used and differences in climatic and storage conditions existed, hence reducing the reliability of a comparison between these studies [24].

3.4 Results of in vitro Antibacterial Activities of *Mentha viridis*

3.4.1 Antibiogram

The antibiotic susceptibility of the studied strains are estimated as diameter of inhibition zone (in mm) according to the recommendations committee of the antibiogram of the French Microbiology Society [25]

3.4.2 Capacity of Adhesion of Studied Strains

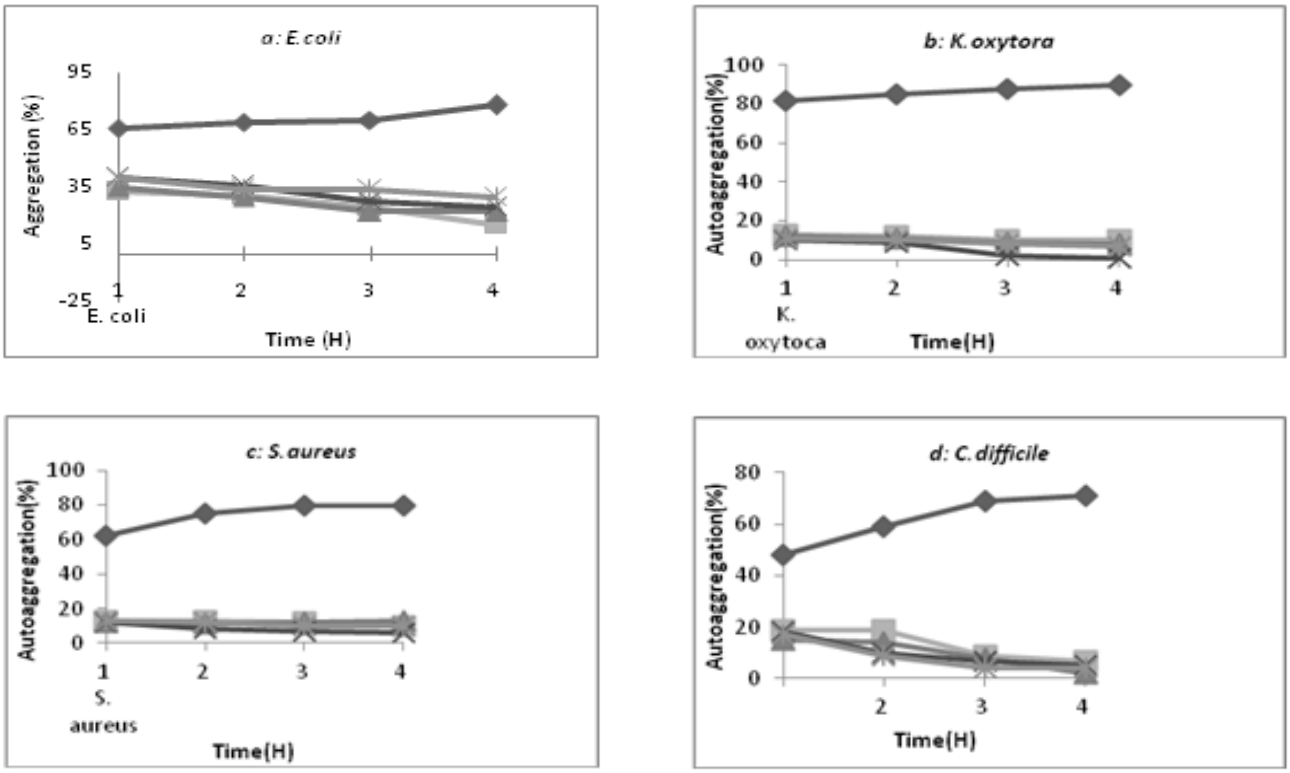
3.4.2.1 Autoaggregation of Tested Strains

Aggregation is a phenotype related to cell adherence properties [13, 26]. Our strains showed a strong autoaggregating phenotype. Strains with values lower than 10% are designed as non-autoaggregating [27]. Without extracts of *Mentha viridis*, the capacity of adhesion of the adhesines which are structures present at surface of the bacterial cell [28]. Generally, the presence of the extracts of *Mentha viridis* reduces the capacity of aggregation of the studied bacteria (Fig. 1A, 1B, 1C). At the molecular level, the adhesiveness of strains to the intestinal tissue is an obligatory stage for the pathogenic

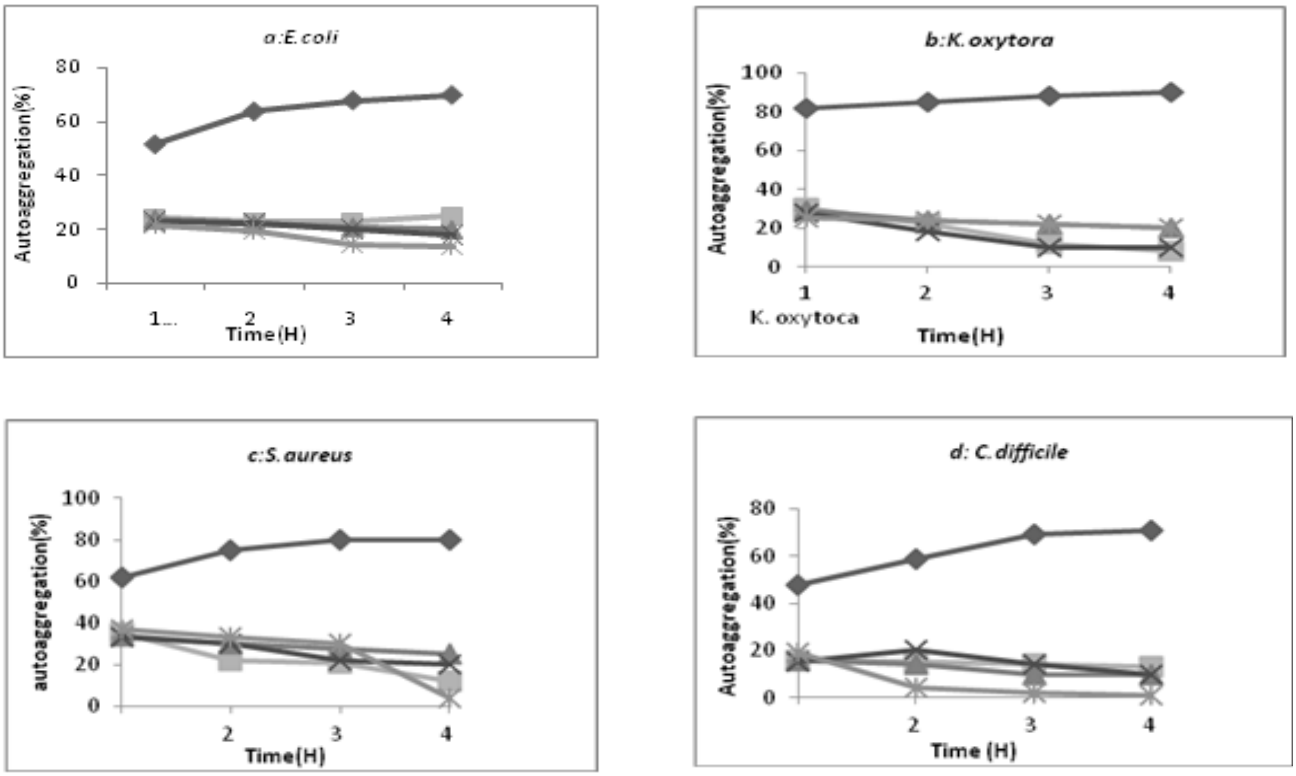
Table 1: Antimicrobial activity of selected antibiotics on tested strains (expressed as diameter of inhibition zone in mm)

Bacteria \ antibiotic	CT	ATM	CN	OX	CZ	SP
Escherichia coli	S(24)	S(45)	S(35)	R(6)	R(5)	R(5)
Klesbsiella oxytoca	S(15)	R(8)	R(14)	R(8)	R(5)	R(6)
Staphylococcus aureus	R(5)	R(15)	S(20)	S(24)	S(15)	I(20)
Clostridium difficile	S(12)	R(10)	S(15)	R(7)	R(8)	R(8)

R: resistant, S: susceptible, CT: colistin, ATM: aztreonam, CN: gentamycin, OX:oxacillin, CZ: cefazolin, SP: spiramycin


a: E.coli
Aggregation (%)
95
65
35
5
-25
1
2
3
4
E. coli
Time (H)
b: K.oxytora
Autoaggregation(%)
100
80
60
40
20
0
1
2
3
4
K. oxytoca
Time(H)
c: S.aureus
Autoaggregation(%)
100
80
60
40
20
0
1
2
3
4
S. aureus
Time(H)
d: C.difficile
Autoaggregation(%)
80
60
40
20
0
2
3
4
Time(H)

(A)


a:E.coli
Autoaggregation(%)
80
60
40
20
0
1...
2
3
4
Time(H)
b:K.oxytora
Autoaggregation(%)
100
80
60
40
20
0
1
2
3
4
K. oxytoca
Time(H)
c:S.aureus
autoaggregation(%)
100
80
60
40
20
0
2
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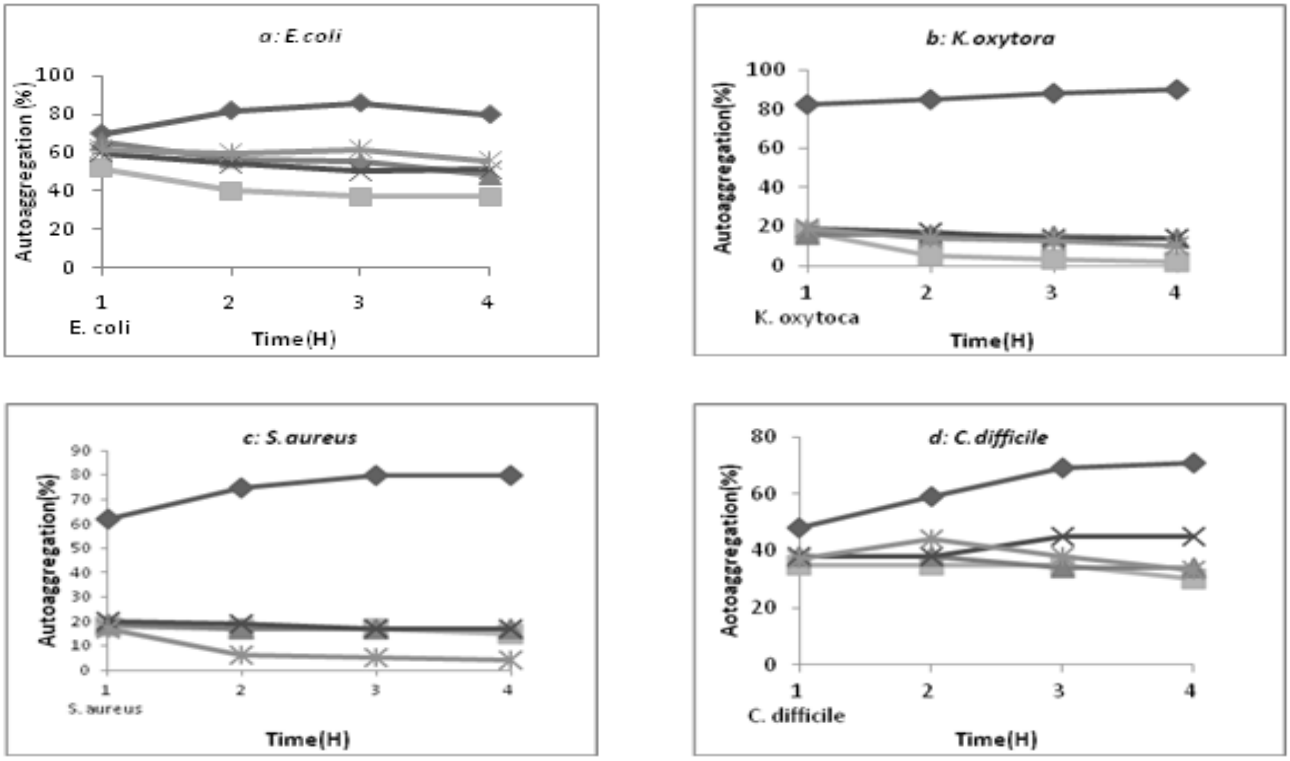
4
Time(H)
d: C.difficile
Autoaggregation(%)
80
60
40
20
0
2
3
4
Time (H)

(B)



(C)

Fig. 1. Autoaggregation of studied strains treated with *Mentha viridis* extracts.
(A) Methanolic extract, (B) Hydromethanolic extract, (C) Aqueous extract

bacteria: it is the beginning of the process of release of the gastroenteritis. Before adhering, the cell must be transported towards surface; this transport can be carried out either thanks to the mobility of the bacteria or thanks to the movement of the fluid (Brownian movement, sedimentation, flow) in which bacteria is in suspension. Fixing starts with a molecular recognition of receiver-ligand type; the systems of recognition are specific: the put molecules concerned are adhesines and receivers membrane of proteinic or saccharidic type. The Gram negative bacteria have common pili which are proteinic structures which their make it possible to stick to mucous surfaces along the intestine [28]. It is important to note, that *E.coli* possess an additional pili which increases adhesion with the cells of the small intestine; moreover, the bacteria secrete a toxin which is responsible for the symptoms of the gastroenteritis. The adhesion of the bacteria on the surface of the mucous membrane, not only supports colonization, but potentiates also the action of the toxin (e.g. S. *aureus*), can be by allowing its attachment effective the level as of receivers located on the mucous membranes [28]. Without this property of adhesion, no bacteria could multiply or be at the origin of pathological phenomena. Our extracts were found to contain a more or less large quantity of flavonoid, therefore one can say that the inhibiting activity of the bacterial adhesion can be explained by the presence of active chemical compounds in their composition [29]. The phenolic compounds contained in the cranberries (*Vaccinium sp*.) would be able to be fixed on certain bacteria (*Escherichia coli* and *Klebsiella sp*.) responsible for the gastroenteritis and cystitis and to prevent them from adhesiveness to the intestine or bladder [30]. Not being able more to adhere, these strains was naturally eliminated by the natural ways. Besides the antiadhesive action, the mechanisms of the antimicrobial effects of polyphenols are very complex; among the advanced assumptions, one can quote; the inhibition of the microbial extracellular enzymes, the sequestration of substrate necessary to the microbial growth and the inhibition of microbial metabolism [29]. It is known also that the flavonoids are

powerful inhibitors *in vitro* of the DNA gyrase [29]. It is important, to specify that a result observed during the evaluation of a pure extract or an enriched fraction is the component of two parameters: the intrinsic activity of the active products and their relative quantity in the extract. For example, a proven activity of an extract can as well be the reflection of a small quantity of very active components as of a great quantity of not very active components, or with certain components such as hydrocarbons and alcohols which show a synergism [31].

3.4.2.2 Coaggregation of Tested Strains With and Without Extracts

Coaggregation property is strain-specific and the degree gradually increases over time [32]. According to Fig. 2, in the absence of extracts of *Mentha viridis*, the level of coaggregation increased with time. This indicates that the bacteria organized themselves in biofilms which does not represent a simple assembly of microbial cells attached on a surface. As it is a biological matrix of origin, containing various chemical elements [33]. In such communities as biofilms, the horizontal transfer of genes is very frequent. The conditions which allow the formation of a mixed biofilm are not clearly defined. The mixture of the bacterial populations is partly dependent on nutrients. Thus, in the presence of chloro-biphenyl as source of carbon, *Pseudomonas putida* and some enterobacteria can nourish itself from metabolites produced by *Burkholderia* and formed mixed biofilms [34]. On one hand, in the presence of citrate, usable by the two species, each one will form its biofilm with a radically different structure. In the other hand, a low anticoaggregation activity was carried on by the extracts of *Mentha viridis* against studied strains (Fig. 2).

Within the biofilms, a complex arrangement of cell and relations are established between bacteria, leading to a cellular answer and an integrated metabolic cooperation. Moreover, physicochemical and environmental characteristics (pH, oxygenation, metabolites, antibiotics…) are harmful with the good bacterial development and constitute stressing conditions: The bacteria set up answers of stress (adaptation to these adverse conditions). The response of stress makes the bacteria more resistant to any form of destruction [28]. The attack of a biofilm can be done by: (i) inhibition or blocking of adhesion, (ii) inhibition or blocking of the communications "quorum-sensing", (iii) inhibition of the production of the exopolysaccharides, (iv)inhibition of the formation or the structuring (architecture) of the biofilm, (iiv) release or the dispersion of the planktonic micro-organisms (free) while degradation or by disorganizing the biofilm [35].

3.4.3 Bacterial Adhesion to Solvents

The MATS method was used to evaluate the hydrophobic/hydrophilic cell surface properties of bacteria. If the percentage is higher than 50%, bacteria is hydrophobic, under 20 % it is hydrophilic, between these two values bacteria is fairly hydrophobic [36].

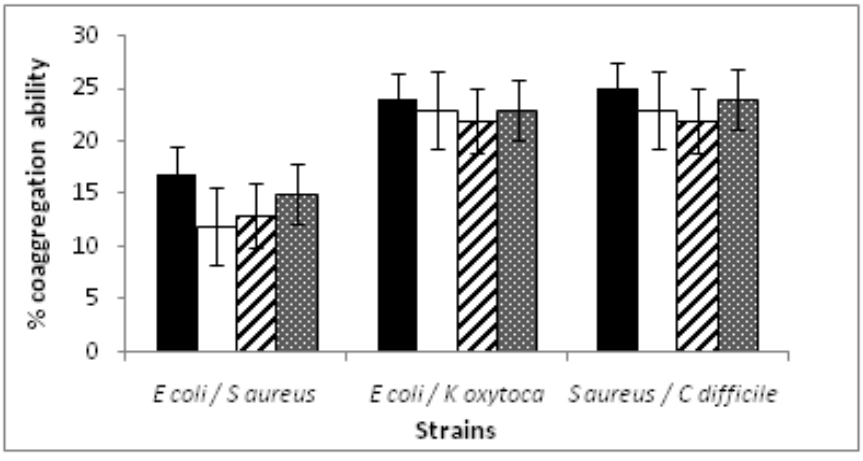


Fig. 2. Coaggregation ability between strains after 3h of incubation in presence of extracts (%) at room temperature in PBS (pH 7, 2). ME: methanolic extract, HME: hydromethanolic extract, AE: aqueous extract

The results indicated that *K. oxytoca, S. aureus*, and *C. difficile* were fully hydrophilic (Fig. 3). They showed an affinity to distilled water and ethyl acetate. This hydrophilic character can be related to a Lewis acid–base characteristic of the bacterial cell surfaces. The differences of the physicochemical properties of surface could be related to protein modifications of surface implied in the mechanism of resistance to antibiotics for example, resistance to the methicillin generally related on changes of one or more linked proteins to penicillin (PLP) which are made proteins of surface [37]. *E.coli* was slightly hydrophobic (Fig. 5). Giaouris *et al.* said that if the affinity to an apolar solvent above 40%, hydrophobic characteristics will be elevated [38]. Several studies suggested that the hydrophobicity results from the presence of glycoproteins on the bacterial surface, whereas the hydrophilic character can be associated to polysaccharids [39]. Moreover, the lipotecoïc acid (ALT) can confer this hydrophobicity on the surface of the Gram positive bacteria; *in vitro* hydrophobic *Staphylococcus* adheres better than the other strains [40]. Also, the MATS test showed a considerable impact of the physical status (solid or liquid) of the culture broth on the physicochemical properties of surface of the Gram positive and negative bacteria [38].

3.4.4 Quantification of the EPS With and Without Mentha viridis Treatment

The amount of exopolysaccharids (EPS) varied from 0.8 to 3.2 mg/L (Fig. 4). *In vivo*, the EPS production is strongly competing; they give to the bacteria a competitive advantage. This matrix could physically prevent the entry of some antimicrobial agents inside the biofilm, while acting like an ion exchange for example, and thus reducing the diffusion of compounds in biofilm [41].

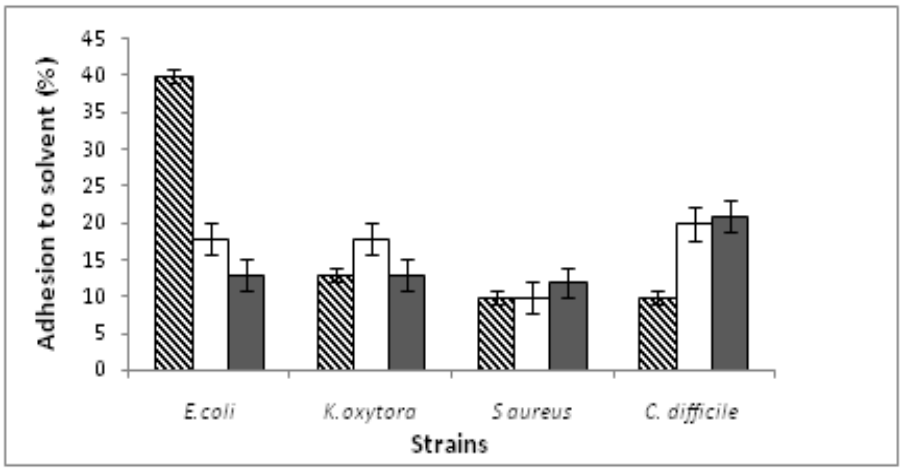


Fig. 3. Percent (%) of adhesion of tested strains to solvents: xylene, ethyl acetate and distilled water.

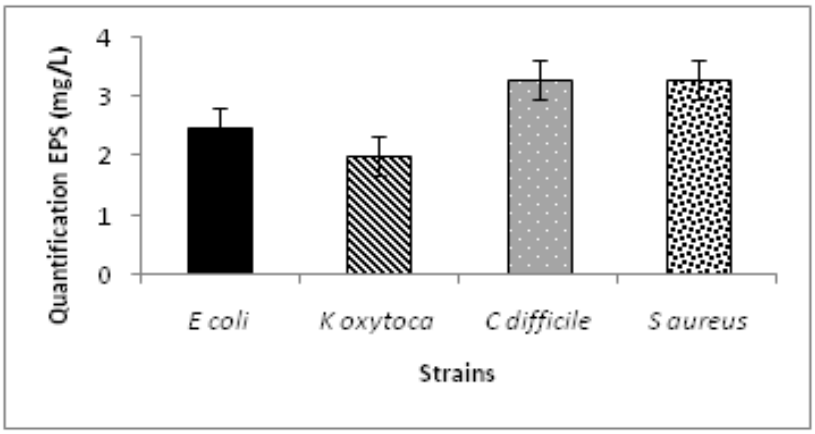


Fig. 4. Amount of exopolysaccharids EPS (mg/L) of tested strains without *M.viridis*.

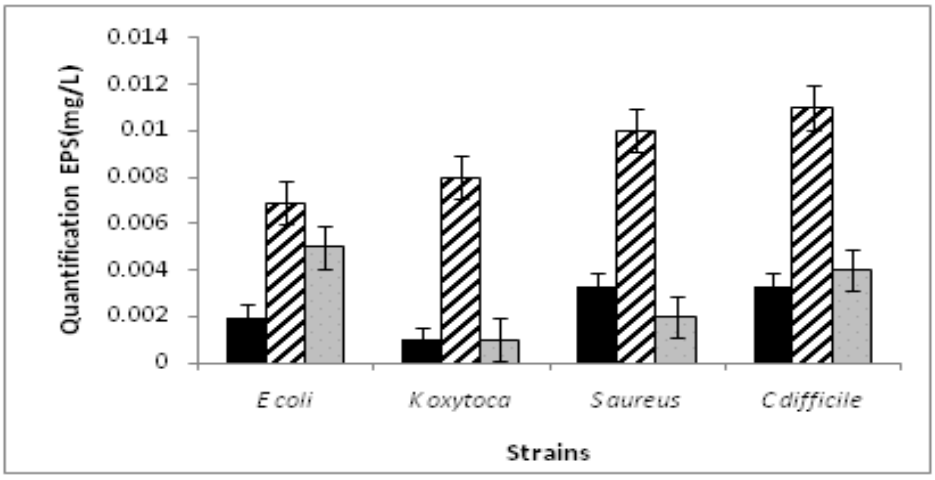


Fig. 5. Exopolysaccharides content of tested strains treated with *Mentha viridis*.
EPS: exopolysaccharides, ME: methanolic extract, HME: hydromethanolic extract, AE: aqueous extract

With *Mentha viridis* extracts (Fig. 5), an important anti-production activity of the EPS was observed for the tested strains. Various factors can affect this production such as the concentration in divalent cations, the ratio carbon-azotes and the availability in substrates. Indeed, the production of EPS required much energy and it thus appears improbable that bacteria used as much energy and substrate without an advantage gains [42].

4. Conclusion

The natural substances occupy an important place into therapeutic more and more. Indeed, the medicinal herbs constitute true chemical plants from which it is necessary to gain the maximum of profit. *M.viridis* extracts presented an important yield (6, 9 and 10% for the hydro-methanolic, methanolic and aqueous extract successively). The autoaggregation and coaggregation value exhibited a power antiadhesion effect of the three extracts on studied strains responsible of infantile gastroenteritis (*E. coli, Klebsiella oxytoca*) and food poisoning (*Staphylococcus aureus* and *Clostridium difficile*). *Mentha viridis* may be useful for the treatment of gastroenteritis in young children. Complementary tests will be necessary and have to be able to confirm the performances put in obviousness's. We think of showing through this work which plants can constitute a tank very interest for research in the future.

5. References

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