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Article

Additive effect of the probiotics *Lactobacillus* exopolysaccharides and the *Satureja calamintha* extracts on enteropathogenic *Escherichia coli* adhesion

Hamida Benfreha[1], Emanuella Chiara Valença Pereira[2], Larissa Araújo Rolim[2], Nadia Chelli[1], Jackson Roberto Guedes da Silva Almeida[2,3,*], Aicha Tirtouil1, Boumediène Meddah[1]

[1]Laboratory of Bioconversion, Microbiological Engineering and Sanitary Safety (LBMESS), Faculty of Nature and Life Sciences, University Mustapha Stambouli of Mascara, Algeria. [2]Central of Analysis of Drugs, Medicines and Food, Federal University of Vale do São Francisco, Petrolina, Pernambuco, Brazil. [3]Center for Studies and Research of Medicinal Plants, Federal University of Vale do São Francisco, Petrolina, Pernambuco, Brazil

This study assessed the inhibitory potential of the probiotics *Lactobacillus* (LB) exopolysaccharides (EPS) with or without extracts of *Satureja calamintha* on enteropathogenic *Escherichia coli* (EPEc) responsible for gastroenteritis. Methanolic and hydromethanolic extracts were prepared by cold maceration and subjected to phytochemical screening. The compounds of the extracts were determined with the colorimetric assays and identified using high-performance liquid chromatography coupled with diode array detector (HPLC-DAD). Antioxidant activities of the extracts were also evaluated by using 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging. Antibacterial effect on EPEc was evaluated by using both agar disc diffusion and microdilution methods. The *in vitro* test of auto-aggregation was investigated. Microbiological analysis showed that 63% of the isolated LB were producing EPS, with the amount ranging from 8.21 to 43.13 mg/L. Chemical analysis of the extracts revealed the presence of polyphenols and flavonoids, more abundant in the hydromethanolic extract, which presented the highest content with 2.11 mg EGA/g of polyphenol and 1.64 mg EC/g of flavonoids and 1.71 mg EGA/g of polyphenol and 1.15 mg EC/g of flavonoids in the methanolic extract. Hydromethanolic extracts and EPS exhibited a more important activity than did the methanolic extract against EPEc. The combined action of EPS and extracts reduced the aggregation ability of EPEc and decreased the rate of their adhesion.

Keywords: *Satureja calamintha subsp. Nepeta*. Extract. EPEc. Probiotics. Exopolysaccharides.

INTRODUCTION

Infectious gastroenteritis accounts for 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 the world, leading to four million of deaths. Both the colonization of mucosa and the competition with commensal bacterial flora are often the first step in most intestinal infections (Da Re *et al.*, 2013). The main germ responsible for digestive infection is very well known: *Escherichia coli*. Many of these strains are harmless and live in the human and animal intestines. Hundreds of strains of *E. coli* caused gastrointestinal problems. However, one of them is pathogenic and can cause severe gastric problems. The symptoms of an enteropathogenic *Escherichia coli* (EPEc) infection are gastric cramps, diarrhea and fever (Mariani, Bonacorsi, Bingen, 2016). In order to deal with this infection, the use of an antibiotic treatment has been widely requested. However, the intense and irrational use of antibiotics, even biocides, favored

***Correspondence:** J. R. G. da S. Almeida. Núcleo de Estudos e Pesquisas de Plantas Medicinais (NEPLAME). Universidade Federal do Vale do São Francisco (UNIVASF). 56.304-205, Petrolina, Pernambuco, Brasil. Phone: (+55) 87 988050771. E-mail address: jackson.guedes@univasf.edu.br. ORCID: http://orcid.org/0000-0002-0867-1357

the selection, the persistence and the emergence of the resistant bacteria for antibiotics, standing out as a problem of public health worldwide (Pulcini *et al*., 2010). With the increase in bacterial multiresistance, the appearance of new infectious agents and the therapeutic failures due to the available antibacterial agents, the search for new natural active substances is necessary.

The selection has always been the essential way for the alternative treatment of new families of antimicrobial and antifungal molecules. The use of the probiotics lactic acid bacteria has been shown to be both an effective and inexpensive approach to fight against enteric infections in susceptible populations (Mkrtchyan *et al*., 2010).

In recent years, several health benefits have been attributed to exopolysaccharides (EPS) from LAB (Ruas-Madiedo *et al*., 2002). EPS contributed to human health as a prebiotics or due to their antitumor, antiulcer, immunomodulatory or cholesterol-lowering activity (Ismail, Nampoothiri, 2010). In this regard, it has been proposed that EPS produced by intestinal bacteria could be involved in the adherence to intestinal mucus and also in the interaction with enteropathogens (Ruas-Madiedo *et al*., 2006). In addition, one of the applied strategies is to explore plants used in traditional medicine (Vanden, Vlietinck, 1991). Medicinal plants deserve more attention due to their numerous health related benefits (Akerele, 1988).

These plants contain many chemical bioactive compounds with a wide range of biological activities. The genus *Satureja*, which belongs to the Lamiaceae family, is represented by about 200 species of herbs and shrubs, often aromatic, widely distributed in the Mediterranean area, Asia and boreal America (Soodabeh *et al*., 2016). *Satureja calamintha* species is used in folk medicine like mints, mainly as stimulant, digestive, tonic and antiseptic (Baytop, 1999). Investigations showed that leaves and flowers of *Calamintha* species are effective as an antiseptic, antispasmodic and tonic (Radi *et al*., 2019).

The aim of this study was to investigate *in vitro* the antibacterial and anti-aggregation abilities of two extracts (methanolic and hydromethanolic) of *Satureja calamintha* collected in the South West of Algeria (city of Saida - Ain el Hdjar), with or without Lactobacilli EPS against isolated multiresistant enteropathogenic *Escherichia coli* (EPEc) responsible for gastroenteritis.

MATERIAL AND METHODS

Isolation, identification and purification of strains

Lactobacillus

Lactobacillus strains were isolated from feces samples (n= 31), from normal, breastfed, new-born babies, aged between 1 day and 29 months. The isolation and the identification of the *Lactobacillus* was made in a culture in MRS agar followed by Gram coloration and biochemical test using automate microbiological system identification (API 50CH), according to Bergey's Handbook recommendations (1986).

Determination of the probiotics Lactobacillus

A preliminary study of the selection criteria for the probiotics *Lactobacillus* was carried out by antibiotic resistance, resistance to acidic pH and bile salts and the capacity to produce antimicrobial substances.

Enteropathogenic bacteria E. coli (EPEc)

Strains of EPEc were isolated from children with gastroenteritis and provided by the Laboratory of Medical Analysis of the city of Mascara. The strain was isolated on medium EMB followed by Gram coloration and identified by biochemical test using automate microbiological system identification (API 20E). The antibiogram was tested by the standard discs diffusion method, on Mueller-Hinton agar according to the recommendations of Antibiogram Committee for French Society of Microbiology (2014). The following antibiotics were tested: cephalexin (30 µg), chloramphenicol (30 µg), aztreonam (30 µg), gentamycin (15 µg), trimethoprim-sulfamethoxazol (1.3/24 µg), oxacillin (5 µg), amoxicillin (25 µg), penicillin (6 µg) and tetracyclin (30 UI).

Exopolysaccharides (EPS) content

In order to optimize the production of EPS, three media were tested: MRS, M17 and hypersaccharosis medium.

EPS were extracted from LB and tested according to Ricciardi *et al.* (2002). *Lactobacilli* culture was incubated for 24 h at 37 °C. The immobilization of bacteria was carried out by exposing the bacterial suspensions to ultrasounds (52 khz /for 10 minutes). The cells were pelleted down by centrifugation at 5,000 g for 15 min after boiling at 80 °C for 15 min.

The supernatant was collected in a sterilized container at +4 °C and three volumes of cold ethanol were added, followed by centrifugation at 10,000 g for 20 min at +4 °C to precipitate EPS. Finally, the pellet was dissolved in 100 ml of distilled water and precipitated twice (Ricciardi *et al.*, 2002). The quantification of EPS was performed by the total sugar assay (Dubois *et al.*, 1956). After vortex, the absorbance (A) of the mixture was measured at 490 nm and compared to the control (without extract).

Plant material

Fresh *Satureja calamintha* L. leaves were collected during the flowering phase from March to April 2015 in Ain El Hdjar in the region of Saida (Northwestern Algeria). This plant was identified according to the African Flowering Plants Database. The plant material was identified by a local expert and a voucher specimen (LA00005) was deposited in the Herbarium Center of the Laboratory of Bioconversion, Genie Microbiology and Health Security of the Faculty of Sciences of the Nature and the Life of the University of Mascara (Northwestern Algeria) for future reference. Fresh aerial parts (leaves) were washed and dried at room temperature for 2 weeks according to the standard procedures. The powder was obtained using the ball mill.

Preparation of the methanolic extract

The extraction was nade by cold maceration of fine powder (20 g) in 200 ml of methanol. The mixture was agitated for 30 min (Mau, Chao, Wu, 2001), and then maintained at rest for 24 h. The solvent was completely removed using a rotary evaporator. The resulting extract was sterilized by filtration and stored at +4 °C until further use. Before testing, the methanolic extract was freshly reconstituted in methanol at a final concentration of 200 mg/ml, which was used for the further preparation of serial dilutions from 200 mg/ml to 25 mg/ml. The yield was calculated according to this formula: $R(\%) = M / M_o \times 100$. Where:

R(%): yield expressed in %.

M: Mass in grams of the resulting dry extract
M_o: Mass in grams of plant material to be treated

Preparation of the hydromethanolic extract

The extraction was made by cold maceration of fine powder (20 g) in 160 ml of methanol and 40 ml of distilled water, homogenized and shaken for 24 h, at room temperature. The extracts were filtered through Whatman N° 1 filter paper and evaporated using a rotary evaporator and freeze dryer, respectively, to yield the crude dried extract. The sterile dried extracts were stored at +4 °C until the use (Diallo *et al.*, 2004). The yield was also calculated.

Phytochemical screening

Total phenolic content

The amount of total polyphenols was determined using the Folin-Ciocalteu's method. Briefly, 1 ml of the methanolic and hydromethanolic extracts was mixed with 1 ml of 1/10th Folin-Ciocalteu reagent. After 5 min, 10 ml of aqueous Na_2CO_3 (7%, w/v) was added. The mixture was allowed to stand for 90 min at 23 °C and then the absorbance was read at 750 nm (Jenway IC 6400 UV/visible equipment). A standard curve was prepared using gallic acid over a range of 0 to 1 mg/ml. Total polyphenolic values were expressed in gallic acid equivalents (GAE) per gram of dry weight (mg GAE/gDW) (Dewanto *et al.*, 2002).

Total flavonoid content

The total flavonoid content was determined by using the colorimetric assay according to Yi *et al.* (2007). A

calibration curve was prepared with catechin and the results were expressed as mg of catechin equivalent to (CE)/g dried plant. Briefly, an aliquot of 1 ml of sample was added to an equal volume of solution of 2% $AlCl_3.6H_2O$, mixed evenly and allowed to stand at room temperature for 10 min. The absorbance was then read at 430 nm.

HPLC-DAD analysis

The solutions of the study (methanolic, hydromethanolic extracts and standards) were selected and individually analyzed by high-performance liquid chromatography coupled to diode array detector (HPLC-DAD). The chromatographic analyses were performed on a HPLC from Shimadzu® with a diode array detector (DAD) and a C18 column with dimensions of 250 x 4.6 mm, 5 µm (Luna®, Phenomenex®). Two solutions were used as mobile phase: Solution A consisted of ultrapurified water + trifluoroacetic acid 0.1 % (v/v) and B, acetonitrile solution, with a flow of 0.6 ml/min. A gradient between ultrapurified water + trifluoroacetic acid 0.1% (v/v) (A) and acetonitrile (B) according to Table I was used as the mobile phase, at a flow rate of 0.6 ml/min. The temperature was kept stable at 30 °C throughout the analysis. The analytical standards and samples were injected in the volume of 20 µl and the detection was performed in DAD at wavelengths of 190 to 800 nm. Data were treated with the aid of the software LC Solution (Shimadzu) by CAFMA (Central of Analysis of Drugs, Medicines and Food) Laboratory team at UNIVASF (Federal University of the São Francisco Valley), Petrolina-PE, Brazil.

TABLE I - Gradient system used in the analysis through HPLC-DAD

Time (min)	Solvent A (%)	Solvent B (%)
0.00	100	0
2.00-5.00	80	20
60.00-62.00	60	40
65	100	0

For qualitative determination of compounds, the following chemicals were analyzed: caffeic acid, chlorogenic acid, gallic acid, *p*-coumaric acid, protocatechuic acid, tannic acid, apigenin, borneol, catechin, chrysin, epicatechin, fisetin, galocatechin, hesperedin, lupeol, miricetin, narigenin, quercetin, isoquercetin, resveratrol, rutin, scopoletin, cirsiliol, harman, hesperetin and vitexin at the concentrations of 200 µg/ml.

For the quantitative analysis of rutin in the samples, a calibration curve was constructed at concentrations of 10, 20, 40, 80 and 160 µg/ml. The calibration curve was obtained under the same chromatographic conditions of the samples and the injection volume was of 20 µl.

Determination of antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method

In order to measure the antioxidant activity, DPPH free radical scavenging assay was used. The method was carried out as described by Mansouri *et al.* (2005). DPPH solution was prepared by solubilization of DPPH (2.4 mg) in methanol (100 ml). Next, 50 ml of each extract was removed and mixed with the DPPH solution (1.95 ml) in a test tube. After 30 min, the absorbance of these solutions was read at 517 nm. Ascorbic acid was used as positive control. IC_{50} values were determined graphically from the sigmoid-shaped curve of antioxidant concentration (mg/ml) versus % inhibition. For comparison purposes, the reciprocal $1/IC_{50}$ values were used (Vinson *et al.*, 2005).

***In vitro* evaluation of the antibacterial activities of *Satureja calamintha* extracts with or without EPS against EPEc**

Diffusion agar method

Antibacterial activity was determined by the agar disc diffusion assay (NCCLS document, 2005)**.** The extracts were dissolved in dimethyl sulfoxide (DMSO). Petri plates were prepared with 20 ml of sterile Mueller Hinton agar (Sigma, Paris, France) surface inoculate by the suspension of cell (200 µl) adjusted by McFarland 0.5

method (10^8 CFU/ml). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. The tests were conducted at different concentrations of the sterile exopolysaccharides (5, 2.5, 1.25 and 0.62 mg/ml) methanolic and hydromethanolic extracts of *Satureja calamintha* (200, 100, 50 and 25 mg/ml) in sterile filter paper discs (6 mm). The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. The plates were incubated at 37 °C for 24 h. Gentamicin (15 µg) and cefotaxime (30 µg) were used as positive controls. Negative control was performed using paper discs loaded with 20 µl of the aqueous DMSO. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. The inhibition zones were measured in millimeters by Vernier calipers. An inhibition zone of 14 mm or greater (including the diameter of the disc) was considered as high antibacterial activity.

Microdilution method

This method was assessed for the determination of Minimum Inhibitory Concentration (MIC) by a serial dilution technique using 96-well microtiter plates (Elof, 1998; Shanmugapriya *et al*., 2012)**.** The amount of substance used in MIC determination was calculated after evaporating the solvent of 1 ml of extract and then solubilizing the dry extract in 20% v/v DMSO. The solution was subsequently diluted 10-fold with Mueller Hinton broth. One hundred microliters from broth bacterium (10^6 FCU/ml) and dilutions were transferred into microtitration plates and incubated for 24 h at 37 °C. The positive control contained 100 µl of bacterium solution plus 100 µl Mueller Hinton broth. Negative control containing only 100 µl dilute plus 100 µl of the extract without bacteria was evaluated according to turbidity after 24 h by comparing to the control well. MIC values were recorded as the lowest concentration of the extract that completely inhibited bacterial growth, which is well clear. Growth was estimated by measuring well optical density at 620 nm using a Microplate Absorbance Reader Sunrise (Tecan Austria GmbH RC/TS/TS) comparatively to control wells (nutrient both inoculum). All experiments were made in duplicates.

Anti-aggregation effect (auto-aggregation)

Auto-aggregation assays were assessed with some modifications (Kos *et al*., 2003). 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*-glucose 1.0 g, distilled water 1 L). The cells were harvested by centrifugation at 5,000 g for 15 min, washed twice and resuspended in phosphate-buffered saline (PBS) to yield viable counts of 10^8 CFU/ml, by diluting fresh cultures and comparing to McFarland standards (OD 650 nm= 0.7) (Al-Bayati, Sulaiman,2008). *Satureja calamintha* extracts and exopolysaccharides were added in various amounts (25, 50, 100 and 200 µl/ml) for plant extracts and (0.62, 1.25, 2.5 and 5 µl/ml) for EPS.

Cell suspensions (4 ml) were mixed by vortex for 10 s. Auto-aggregation was determined after 1, 2 and 3 h of incubation at room temperature. At each time point, 0.1 ml of the upper suspension was transferred to another tube with 3.9 ml of PBS and the absorbance (A) was measured at 600 nm. The auto-aggregation percentage was calculated as follows: $\% = (1\text{-}A_t/A_0) \times 100$, where A_t represents the absorbance at either time t= 1, 2 or 3 h and A_0 the absorbance at t= 0.

Statistical analysis

All experimentations were conducted in duplicate and all results are represented as arithmetic means ± standard error of the mean. Data were statistically analyzed by using Student's *t*-test (paired data) and ANOVA test (STAVIEW version 5.0, Abacus Concepts, Berkeley, CA) (Core Team, 2020). Quantification of extract compounds and EPS were expressed as %. For *in vitro* antimicrobial activity, we consider Log CFU ≤ Log1 as significant (Molly, Vande Woestyne, Verstaete, 1993). A p values ≤ 0.05 were considered as significant.

RESULTS AND DISCUSSION

Characterization of *Lactobacillus* as probiotics

46 isolates of lactic acid bacteria (LAB), of which 26 belong to the genus *Lactobacillus*, were isolated and

identified. The analysis demonstrated that all *Lactobacillus* isolated have criteria for their selection as probiotics.

Quantification of EPS from selected *Lactobacillus* (LB)

Hypersaccharosis mediums were used in order to optimize the production of the *Lactobacillus* EPS. Our results showed that 63% of isolated and identified LB produced EPS. The amount of exopolysaccharides (EPS) produced varied from 8.21 to 43.13 mg/L (Figure 1).

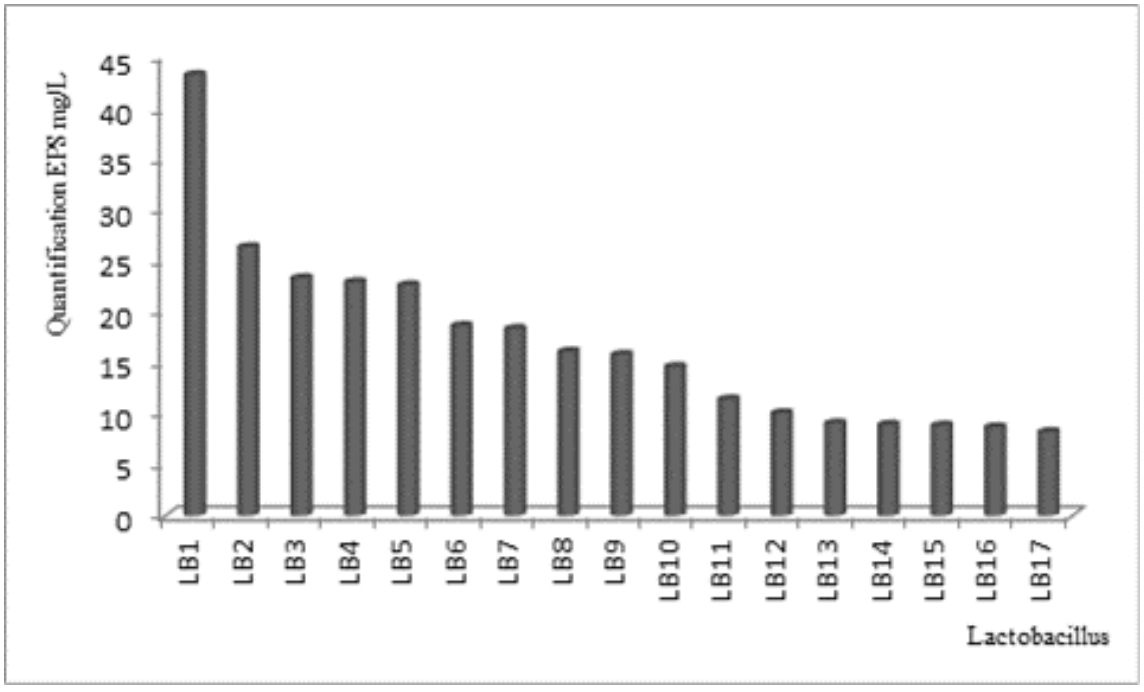

FIGURE 1 - Amount of exopolysaccharides EPS (mg/L) produced from *Lactobacillus*.

Looijesteijn *et al.* (2001) reported that within the same species of lactic acid bacterium, the results may be different. We have been able to identify strains producing EPS (very strongly) and non-producing strains without this character causing growth disparities. For the following tests, the LB1 has been selected.

Antibiogram profile of EPEc strains

The antibiotic susceptibility of the studied strains was estimated as diameter of inhibition zone in mm (Table II) according to the recommendations of the Antibiogram Committee of the French Microbiology Society (2010). Results show that *E. coli* is resistant to major antibiotics, a multi-resistant bacteria responsible for gastroenteritis.

TABLE II - Antibiogram test of EPEC strain (diameter of inhibition zone in mm)

	CN (15 µg)	OX (5 µg)	C (30 µg)	AX (30 µg)	ATM (30 µg)	P (6 µg)	GEN (30 µg)	TE (30 µg)	STX (1,25/23,75)
	DC : S≥ 10-15		R<10			D Diameter (mm)			
Strain	D	D	D	D	D	D	D	D	D
E.coli	19(S)	6(R)	25(S)	11(I)	20(S)	8(R)	22(S)	7(R)	27(S)

R: **Resistant**; S: **Sensitive**; I: I**ntermediate**; DC: **Critical Diameter**; D: **Diameterread**; CN: **Cefalexin**; TE: **Tetracyclin**; AX: **Amoxicillin**; C: **Chloramphenicol**; OX: **Oxacillin**; ATM: **Aztreonam**; P: **Penicillin** ; GEN: **Gentamycin**; CT: **Colistin**; STX: **Trimethoprim-sulfamethoxazole**

Phytochemical screening of *Satureja calamintha* extracts

Methanolic and hydromethanolic extracts revealed yields about 8.58 and 12.3%, respectively. Polyphenol and flavonoid contents of the dry extracts were determined as equivalents of gallic acid and catechin. Values obtained for the hydromethanolic extract (HME) showed that these extracts had the highest polyphenol and flavonoid contents 2.11 ± 0.6 mg EGA/g and 1.64 ± 0.04 mg EC/g, respectively, and for the methanolic extracts (ME) the values showed 1.71 ± 0.51 mg EGA/g of polyphenols and 1.15 ± 0.02 mg EC/g of flavonoids (Figure 2).

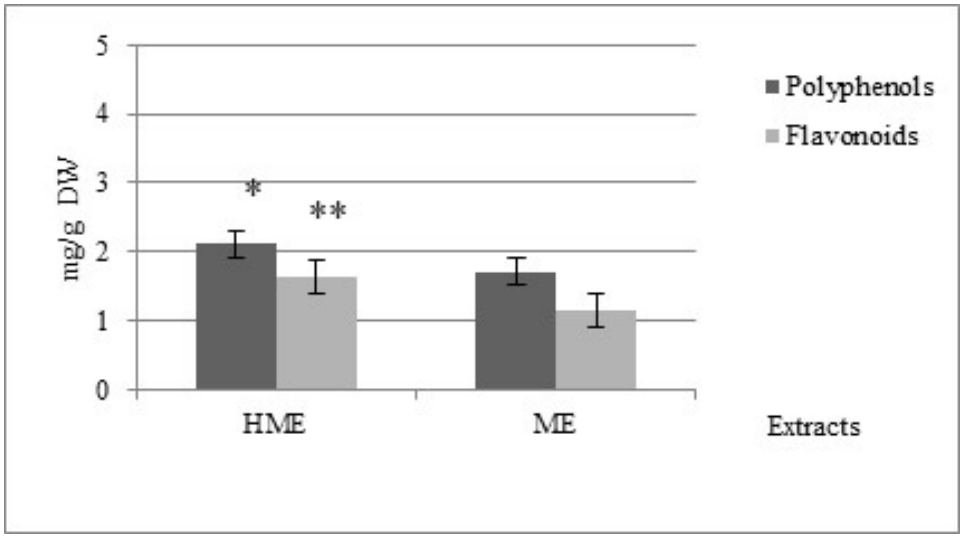

FIGURE 2 - Content of polyphenols and flavonoids of *Satureja calamintha* extracts. HME: hydromethanolic extract, ME: methanolic extract. *p < 0.01, HME vs ME. **p < 0.01, HME vs ME.

This result corroborates those reported by Bougandoura and Bendimerad (2012), who estimate polyphenols and flavonoids in the methanolic extract at 2.96 ± 0.80 mg EGA/g and 1.28 ± 0.07 mg EC/g, respectively, and in the aqueous extract at 12.6 ± 0.77 mg EGA/g of polyphenols and 3.13 ± 0.15 mg EC/g of flavonoids.

The polyphenolic profile of plant extracts may vary under the influence of various factors including variety, climate, geographical location (Ryan, Muller, Pfanner, 1999)**,** temperature and extraction solvent (Sousa, Dias, Antunes, 2006; Conde *et al.*, 2009)**.**

HPLC/DAD

Among the standards analyzed, the only one that could be identified in the sample was the flavonoid rutin. This identification was performed by comparing the retention time observed in the chromatogram (Figure 3A) and the UV spectrum (Figure 3B) between the sample peaks and the standard rutin solution.

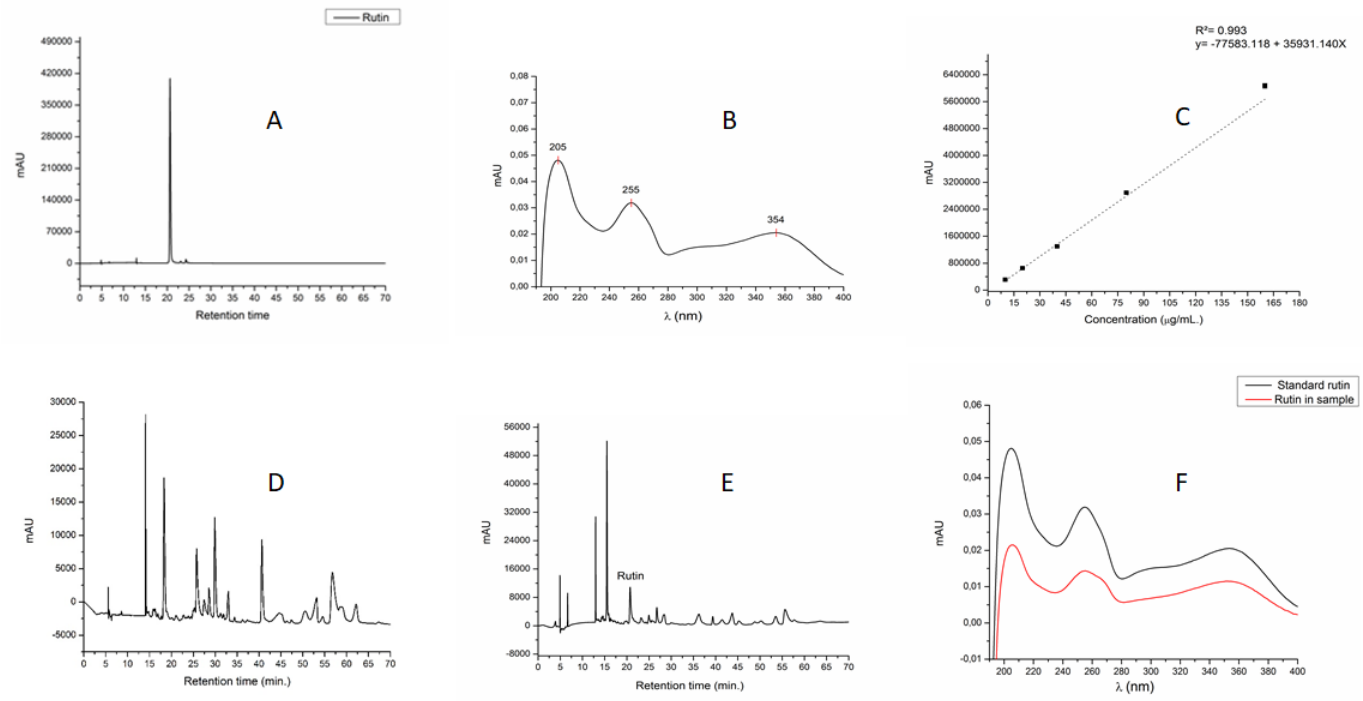

FIGURE 3 - A: Chromatogram of the flavonoid rutin with retention time at 20.7 min. B: UV spectrum of the flavonoid rutin. C: Calibration curve of the flavonoid rutin. D: Chromatographic profile of the methanolic extract at 340 nm. E: Chromatographic profile of the hydromethanolic extract at 340 nm. F: Comparation between the UV spectra of the standard rutin (in black) and rutin in the hydromethanolic extract (in red).

The calibration curve ($R^2 > 0.99$) obtained is shown in Figure 3C. It provided the equation of the line (y = -77583.118 + 35931.140 X; where y is the peak area and X a sample concentration in µg/ml.) used to calculate the rutin concentration in the samples.

In this study, we developed a method based on HPLC-DAD in order to obtain a chromatographic system that was able to elute and provide good resolution in the separation of compounds in the methanolic extract, as it can be seen in Figure 3D. It was not possible to identify the substances present in the methanolic extract through the comparison of retention time and maximum absorption spectra at the analytical standards.

The chromatographic profile of the hydromethanolic extract can be observed in Figure 3E. It was possible to identify the flavonoid rutin, since the retention time and the UV absorption profile observed in the sample were compatible with the one of the standard (Figure 3F).

A previous phytochemical study of *Satureja calamintha* extracts collected from the Ouzzane region in Morocco has shown the presence of flavonoids, gallic and catechic tannins, cyanidin, sterols and triterpenes (Hayani *et al.*, 2020). In another study, the three most abundant compounds identified in the essential oils of this species were l-menthone, neo-menthol and pulegone. The oils had significant antimicrobial activities against

bacterial and fungal strains, except for *Bacillus cereus* and *Candida albicans* (Boudjema *et al*., 2018).

Antioxidant activity

The percent of inhibition (%I) for each extract was calculated and the inhibition of the radical DPPH was evaluated for each extract of the plant. The results are illustrated in Figure 4. A low value of IC_{50} indicates a strong antioxidant activity (Hebi, Eddouks, 2016). The value of IC_{50} was calculated by linear regression of the percentages of inhibition calculated according to various concentrations of the extracts prepared (Table III).

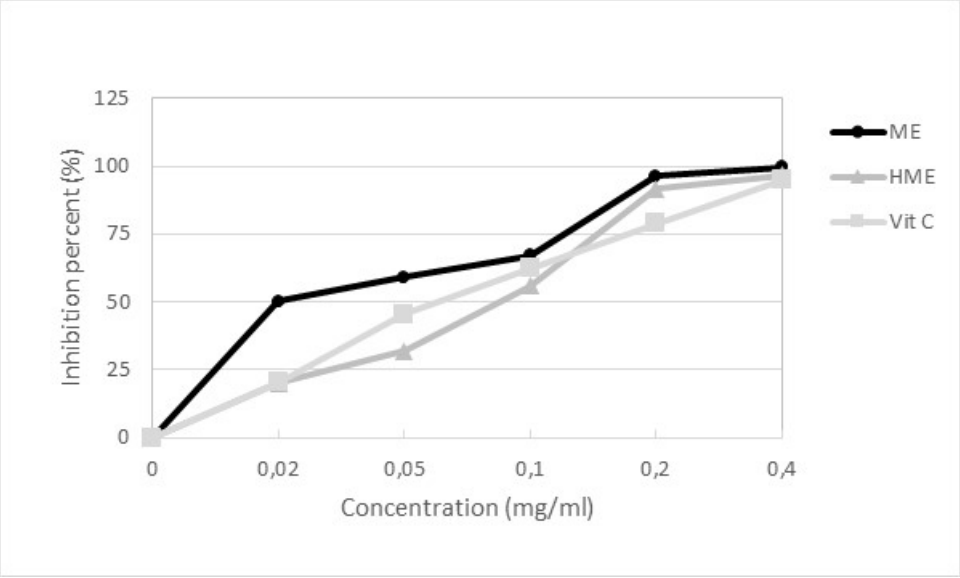

FIGURE 4 - Percent inhibition of the radical DPPH• of methanolic (ME) and hydromethanolic (HME) extracts, and Vitamin C (ascorbic acid).

TABLE III - IC_{50} values of methanolic (ME), hydromethanolic (HME) extracts and Vitamin C

Sample	IC_{50} (mg/ml)
ME	5.06
HME	7.78
Vit C	1.02

Our results showed an IC_{50} of 7.78 ± 0.18 µg/ml and 5.06 ± 0.04 µg/ml for methanolic and hydromethanolic extracts, respectively. These values were more important than those of Bougandoura and Bendimrad (2012), who found the IC_{50} values of the order of 1.876 mg/ml for the aqueous extract and 2.075 mg/ml for the methanolic extract, which were relatively low compared to that of ascorbic acid, which was 0.134 mg/ml.

This antioxidant activity is due to the presence of antioxidant molecules such as ascorbic acid, tocopherol, flavonoids and tannins that reduce and discolor DPPH because of their ability to yield hydrogen. The polyphenols contained in extracts of *Satureja calamintha* are probably responsible for the antioxidant activity of these extracts.

In vitro evaluation of the antibacterial activities of *Satureja calamintha* extracts with or without EPS against EPEc

Diffusion agar method

The results of the antimicrobial activity of EPS *Lactobacillus* extract, methanolic and hydromethanolic extracts of *Satureja calamintha* leaves are given in Figure 5.

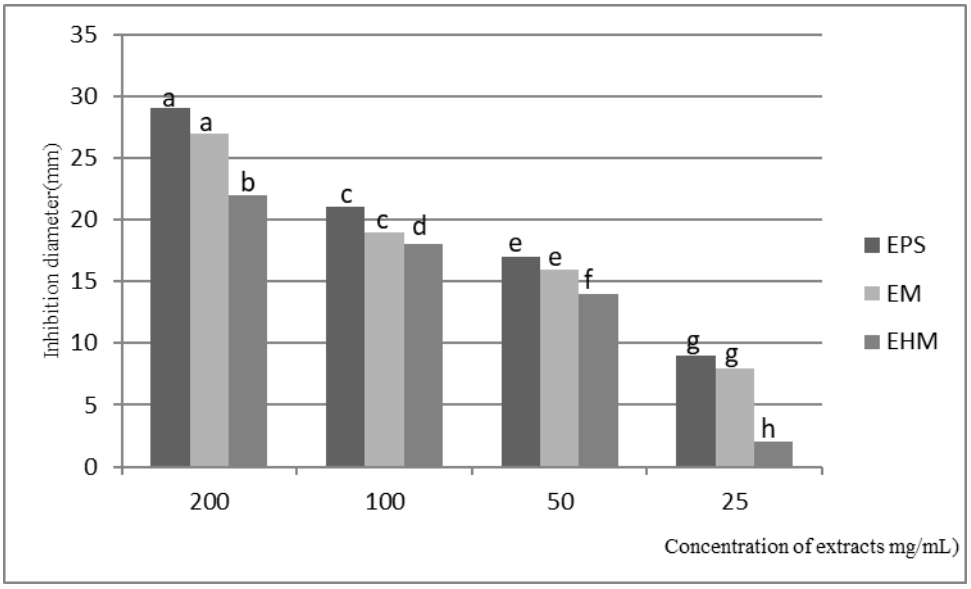

FIGURE 5 - Antibacterial activity profile of EPS *Lactobacillus* extracts and extracts of *Satureja calamintha* leaves (expressed as diameter of inhibition zone in mm). EPS: exopolysaccharides; HME: hydromethanolic extract; ME: methanolic extract. a,b: $p < 0.005$ (EPS, ME vs HEM). c,d: $p < 0.005$ (EPS, ME vs HEM). e,f: $p < 0.005$ (EPS, ME vs HEM. g,h: $p < 0.005$ (EPS, ME vs HEM).

It is noted that different concentrations of all compounds have a remarkable effect on the growth of EPEc. The effect increases with rising concentration of extracts. At 25 mg/ml, the inhibition diameter is between 14-17 mm; at 50 mg/ml, 18-21 mm; at 100 mg/ml, 22-29 mm and at 200 mg/ml, XX-XX mm.

Extracts of EPS are the most active on the growth of EPEc (9-29 mm) followed by the methanolic extract (8-27 mm) and the hydromethanolic extract (2-22 mm) in any the concentration.

The mechanism of action of probiotics is to inhibit the growth of pathogenic bacteria through antimicrobial compounds (Cotter, Hill, Ross, 2005). The result of the antagonism test allowed us to search for inhibitory agents in the genus *Lactobacillus* (Servin, 2003), such as exopolysaccharides that are active *in vitro* and *in vivo* against the pathogenic microorganisms involved in diarrhea cases (Servin, 2004).

Al-Bayati and Sulaiman (2008) specify that the antibacterial effect can also be due to various chemical substances contained in the extract. According to Kanyonga *et al.* (2011), the methanolic extract of *Satureja calamintha* was rather effective against *E. coli*. Generally, the mechanism of plant extract activity is probably due to their ability to complex with extracellular and soluble proteins and then to complex with bacterial cell walls.

Microdilution method

The results of the experiments assessing the bacteriostatic effects of solvent extract compounds and EPS prebiotics on enteropathogenic EPEc demonstrated that hydromethanolic extracts and EPS exhibited an activity that is more important than the methanolic one against EPEc. The MIC of methanolic extract of *Satureja calamintha* is equal to 100 mg/ml. That of the hydromethanolic extract is equal to 50 mg/ml (Figure 6).

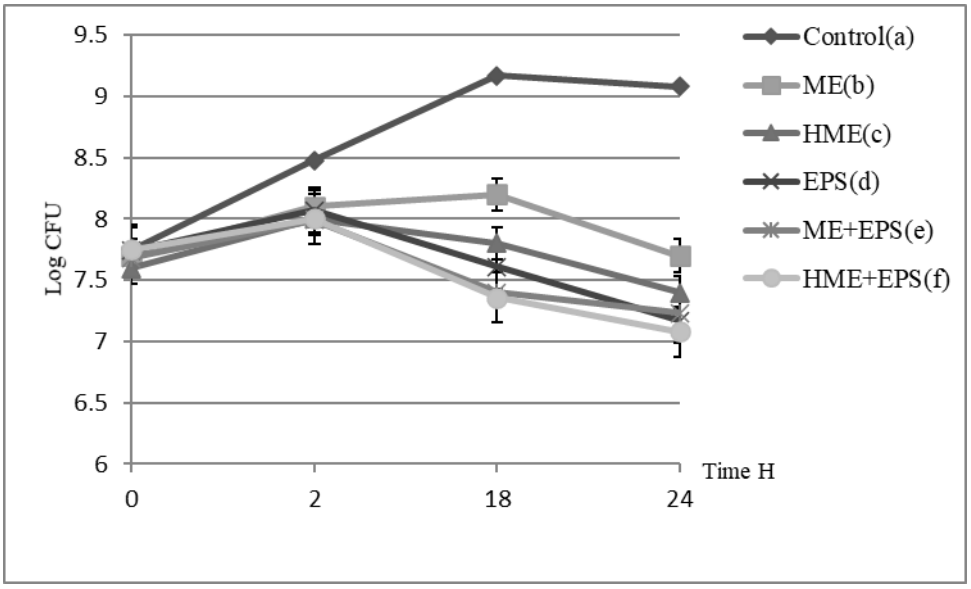

FIGURE 6 - Antibacterial effect of methanolic (ME) and hydromethanolic (HME) extracts of *Satureja calamintha,* with or without EPS on EPEc. a: $p < 0.05$ Control vs all. b: $p < 0.05$ ME vs HME, EPS. c: $p < 0.05$ HME vs HME + EPS. d: $p < 0.05$ EPS vs c. e: $p < 0.05$ ME + EPS vs c. f: $p < 0.05$ HME + EPS vs c, ME.

EPS and methanolic and hydromethanolic extracts of *S. calamintha* have an effect on the growth kinetics of *E. coli*. These results indicate that the extract from *S. calamintha* presents an important therapeutic alternative.

Studies by Bernet-Camarad *et al.* (1997) have shown that strains of lactic acid bacteria strongly adhering to intestinal cells inhibit the adhesion of pathogenic microorganisms such as *E. coli*. Complete inhibition of *E. coli* is noticed by the prior addition of these probiotic strains (Coconnier *et al.*, 1993, Bernet *et al.*, 1994, Mack *et al.*, 1999).

These authors proposed that the prior adhesion of probiotics to intestinal cells would help to limit pathogen access to enterocytes and increase mucus secretion, which could also prevent the adhesion of pathogens to intestinal cells. These probiotic strains were able to exclude and compete with pathogens significantly on mucus (Lee *et al.*, 2003).

Anti-adhesion effect (auto-aggregation)

Aggregation is a character related to cell adherence properties (Pelletier *et al.*, 1997). Our strains showed a strong auto-aggregating character. Strains with values lower than 10% are designed as non-auto-aggregating (Del Re *et al.*, 2000). Generally, the presence of EPS and extracts of EPS and *Satureja calamintha* reduced the capacity of aggregation of the studied bacteria and led to a decrease in their adhesion rate (Figure 7). The pathogenicity of bacteria is related to the phenomenon of adhesion to the intestinal mucosa, which is the beginning of the process of the gastroenteritis. For the enterobacteria, adhesion is usually mediated by different types of pilli and fimbriae (Struve, Bojer, Krogfelt, 2008). Finally, we can say that the EPS of LB associated with the methanolic extract of *S. calamintha* affects the auto-aggregation of EPEc and leads to a decrease in their adhesion rate.

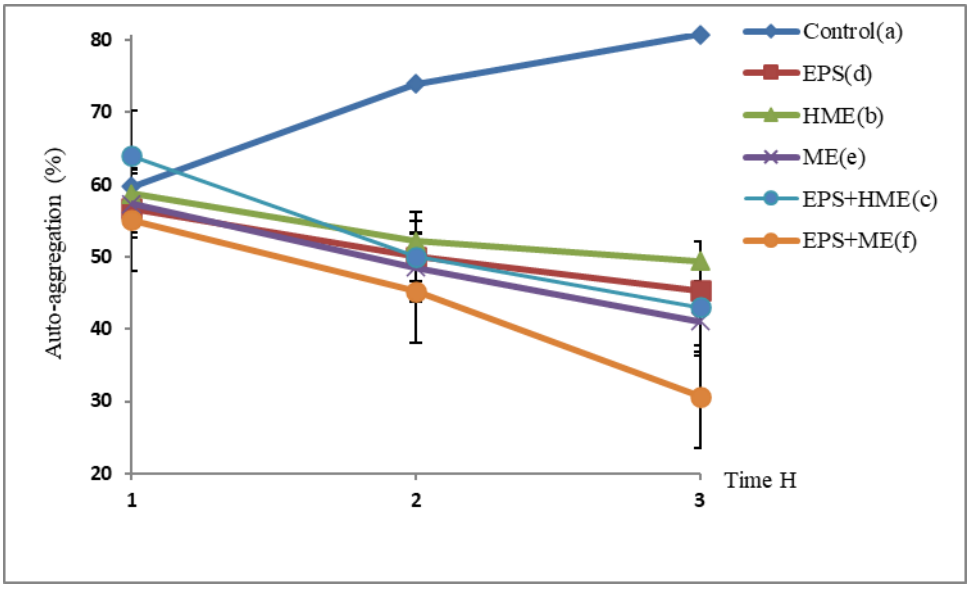

FIGURE 7 - Auto-aggregation of EPEc strains treated with EPS and *Satureja calamintha* extracts. (ME) Methanolic extract, (HME) Hydromethanolic extract, with and without EPS. a: $p < 0.05$ Control vs all. b: $p < 0.05$ HME vs EPS + ME. c: $p < 0.05$ EPS + HME vs EPS + ME. d: $p < 0.05$ EPS vs EPS + ME. e: $p < 0.05$ ME vs EPS + ME. f: $p < 0.05$ EPS + ME vs all.

The results of our study show a remarkable increase in the aggregation rate over time at EPEc, which reached 80.74%. However, after the addition of EPS and extracts of *S. calamintha* alone or associated (EPS + ME) and (EPS + HME), the aggregation capacity of EPEc usually decreases. For a concentration of 100 mg/ml of extract the rate of aggregation dropped from 56.71 to 45.3% after the addition of EPS, from 58.73 to 49.43% for HME and from 57.36 to 41.08% for ME.

Adhesion is an action that is characterized by all the physicochemical and biological phenomena allowing bacteria to adhere to a surface in a sustainable way (Nordman, Naas, Poirel, 2011).

The adhesion phenomena depend on the germs, the pilli or fimbriea, adhesins (adhesion antigens encoded by plasmids or glycocalix (long polysaccharide fibers) (Banin, Vasil, Greenberg, 2005).

Our results are very promising and represent a contribution to a better valorization of *Satureja calamintha* and EPS extracts. Furthermore, it is still necessary to characterize active principles and investigate *in vivo* bioactivity and cytotoxicity of the extracts to explore their potential beneficial use in gastroenteritis caused by EPEc.

CONCLUSION

The results of the present research demonstrated that the association of the methanolic extract of *Satureja calamintha* leaves with the probiotics *Lactobacillus* EPS can affect the growth and the adhesion of EPEc. We suggest that these extracts may be a promising alternative for the treatment of enteric infections. However, other *in vivo* and clinical studies will be required.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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