Zizyphus lotus (L.) Extracts as Prebiotics in the Aggregation and Adhesion of Probiotic and Inhibition of Pathologic Bacteria from Patients with Colorectal Cancer

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Abstract

The mucosal surfaces of the intestinal tract harbor a complex microbiota. Bacteria enter in contact with intestinal cells and bestow important nutritional, metabolic and protective functions which benefit the host. Various factors are able to alter the balances between different intestinal bacteria. Dysbiosis has been described in various pathologies and metabolic diseases in humans, such as cancer. Colorectal cancer pathology can benefit from gut microbial imbalance. Its overgrowth may lead to acute symptoms. An alternative strategy to restore this balance is the use of plant extracts which exhibit a prebiotic activity by stimulating probiotic bacteria and antimicrobial activity against pathogenic bacteria colonization. The present study aims to evaluate the *in vitro* antioxidant and antibacterial activity of phenolic extracts (Aqueous extract "AE" and Methanolic extract "ME") from *Zizyphus lotus*. It aims also to investigate the effect of this extract as a prebiotic on the aggregation of probiotic and inhibitory effects of pathogenic bacteria isolated from faeces of patients with colorectal cancer. Phytochemical analysis of different extracts from *Zizyphus lotus* by HPLC showed that all are rich in phenolic compounds (225.40 mgAGE/gDW for AE and 63.04 mgAGE/gDW for ME extracts). Moreover, all extracts showed an important antioxidant activity (227 μg/ml for AE and 195 μg/ml for ME). These extracts also exhibited a significant prebiotic effect and antibacterial activity. Characterization of the *in vitro* effect of the aqueous extract showed that the percentage of autoaggregation and adhesion of probiotic and inhibitory effects of pathogenic bacteria increased in the presence of aqueous extracts.

Keywords: *Zizyphus lotus(L.)*; Extracts; Colorectal cancer; Aggregation; Adhesion; Antimicrobial activity

1 Introduction

The human gut microbiota can be considered as a separate organ that has co-evolved with humans to achieve a symbiotic relationship leading to physiological homeostasis (Backhed, Ley, L Sonnenburg, Peterson & I Gordon, 2005). A number of gastrointestinal disease states or disorders have been proposed to be associated with changes in the composition or function of gut microbiota. However, opportunistic and pathogenic infections can benefit from gut microbial imbalance. Their overgrowth may lead to acute symptoms. This is implicated in many digestive diseases (van Nimwegen et al., 2011) like inflammatory bowel diseases and colorectal cancer. Gut microbiota are an important factor associated with colorectal cancer (CRC), one of the

most common malignant tumor types in the world (Azcarate-Peril, Sikes & Bruno-Barcena, 2011). Each year, nearly one million new CRC cases are diagnosed worldwide and more than 500,000 deaths are reported (Jemal et al., 2011). It is the third most-diagnosed malignancy in the world according to the GLOBOCAN study (Parkin, Bray, Ferlay & Pisani, 2001) and the second deadliest cancer after lung cancer in western countries. There is also emerging evidence that intestinal bacteria may play a role in the initiation of colon cancer through the production of carcinogenic chemicals (Rowland, 2009).

Recently, the use of probiotics, which are beneficial bacteria present in the intestine, is an alternative strategy to maintain the balance of the intestinal microbiota by the reduction of its pathogenic members and by increasing the potentially beneficial ones (Gomes, Pintado, Freitas & Silva, 2014). At the present time, some plant products have attracted the attention of researchers to exploit some phytochemicals as antimicrobials and anticancer drugs. Such plant products would be biodegradable and safe for human health (Kumar, Shukla, Singh, Shekhar Prasad & Kishore Dubey, 2008; Liu, 2004; Wang, Li, Cao & Jiang, 2010). Therefore, it is necessary to develop new alternative molecules from medicinal plants for their biological activity, such as the treatment of infectious diseases (Boominathan & Ramamurthy, 2009). At the present time, many new studies have confirmed the antimicrobial activity of polyphenols occurring in medicinal plants. They act as anti-tumour agents, antimicrobials and anti-inflammatory agents (Jayaprakasha, Singh & Sakariah, 2001; Muanda, Soulimani, Diop & Dicko, 2011). *Zizyphus lotus (L.)* was investigated in the present study. *Ziziphus lotus* (Jujube) is a medicinal plant found in the Mediterranean region, including Algeria (Pottier, 1981). The *Zizyphus lotus* is a fruit-bearing, thorny shrub belonging to the family Rhamnaceae (Rsaissi & Bouhache, 2002). In North Africa it is commonly called "Sedra" (Borgi, Ghedira & Chouchane, 2007). The different species of *Zizyphus* are widely used in the pharmaco-biological field in the treatment of certain diseases such as inflammatory diseases, digestive disorders, weakness, liver diseases, obesity, ur-inary disorders, diabetes, skin infections, fever, diarrhea and insomnia (Abdel-Zaher, Salim, Assaf & Abdel Hady, 2005; Abu Zarga, Sabri, Al-Aboudi, Saleh Ajaz & Sultana, 2004; Suksamrarn et al., 2005).

The present study aims first to investigate the intestinal bacterial community in populations with colorectal cancer and compare it to the bacterial community of healthy adults. Secondly, the objective is to compare the phytochemical screening and evaluation of *in vitro* antioxidant and antibacterial activities of phenolic extracts from the leaves and stems of *Zizyphus lotus (L.)*. Finally, the effect of this extract as a prebiotic on the aggregation of probiotic and inhibitory effects of pathogenic bacteria isolated from adult patients with colorectal cancer will be investigated.

2 Materials and Methods

2.1 Subjects

A total of 20 adults (12 male and 8 female) were included and divided into 2 groups: group 1 of patients with colorectal cancer (n=10) and group 2 of healthy controls (n=10). Their age ranged between 21 and 34 years, with a mean of 27.6±2.73 years for cancerous patients and 26.2±4.53 years for healthy controls. Body Mass Index (BMI) had a mean and standard deviation of 22.10±0.65 kg/m² for cancerous patients and 22.05±1.10 kg/m² for healthy controls.

Clinical Characteristics

Clinical characteristics of groups included in this study are shown in Table 1.

All adults included in this analysis (male or female) were between 19 and 40 years old, having only colorectal cancer disease (Group 1); not having acute or chronic infectious diseases or problems that may affect the gut microbiota (diarrhea, constipation); and not having been treated with antibiotics or antifungals (three months), and chemotherapy.

Table 1: Clinical characteristics of groups (means ± SD)

Characteristics (n=10)	Cancerous patients (n=10)	Healthy controls (n=10)
Male/Female	6/4	6/4
Age (years)	27.6±2.73	26.2±4.53
Weight (kg)	61.4±2.4	64.8±6.53
Height (m)	1.66±0.027	1.72±0.04
Body Mass Index (kg/m^2)	22.10±0.65	22.05±1.10

Ethics

This work was carried out with the patients' consent and the permission of the director of Yessad Khaled hospital of Mascara.

2.2 Research and isolation of probiotic and pathogenic Bacteria in patients with colorectal cancer

Sample preparation

A 10^{-1} dilution was prepared by adding 1g of fecal samples to 9 ml of sterile saline. The suspension was mixed for 2 minutes by a vortex. From this suspension serial decimal dilutions were performed up to 10^{-6}, and 0.1 ml of each dilution was spread on different selective agar media in duplicate.

Clinical isolation and enumeration of bacterial candidates

Microbiological analysis of the fecal samples was carried out, using various appropriate culture media, to characterize the gut microbiota of different populations. *Lactobacillus* on MRS (Man Rogosa and Sharpe agar), *Streptococcus* on M17agar, *Total Aerobic* and *Anaerobic Flora* was isolated on GN (Nutrient agar), *Enterobacteria* was isolated on EMB (Eosin Methylene Blue agar), *Staphylococcus* on CHAPMAN agar, *Enterococcus* on BEA (Bile Esculin agar), *Clostridium* on Liver Meat agar and *Bacteroides* Blood agar (Béraud, 2001). The plates were incubated at 37 ^oC for 24 to 72 hours under aerobic conditions for *Lactobacillus, Streptococcus, Total Aerobic Flora, Enterobacteria, Staphylococcus* and *Enterococcus*, and anaerobically for *Total Anaerobic Flora, Clostridium* and *bacteroide*. Identification of bacterial candidates found in each culture medium was confirmed by macroscopic examination, Gram staining and use of biochemical tests, API system. Probiotic strains were identified by API 50 CH and characterized for their probiotic potential by evaluation of growth at different temperatures and gastric pH, tolerance of bile salts, resistance to antibiotics, antibacterial activity, aggregation and adhesion capacity. The enumeration of viable bacteria in fecal specimens was performed on dishes with 30-300 colonies and expressed as log colony forming unit (CFU) per gram of fecal sample for statistical reasons and for better mathematical interpretation according to the following formula (Béraud, 2001):

$$\text{Log CFU/g} = \frac{Number\ of\ colony}{Dilution \times Volume\ seeded} \quad (1)$$

2.3 Phytochemical analysis of *Zizyphus lotus*

The plant material consisted of leaves and stems of *Zizyphus lotus (L.)* from Sidi Benyekhlef in the region of Mascara (Algeria). The plants were collected in July – August 2015. Taxonomic identification was performed by Pr. Najat ELKHIATI (Biological and Health Laboratory, Faculty of Science Aïn Chock-University Hassan II, Casablanca).

Preparation of plant extracts

The collected plant samples were air dried for a few days, and then the leaves and stems were crushed into a powder and stored for use.

a **Preparation of aqueous extract**
A 5 g sample of plant material was suspended in 100 ml of distilled water and macerated for 24 h (Xiao, Han & Shi, 2008). The macerated material was filtered, and the filtrate was lyophilised and stored at ambient temperature until further use.

b **Preparation of methanolic extract**
The powder of plant material (10 g) was extracted with 100 ml of methanol (maceration) during 24 h. After filtration, the filtrate was concentrated under reduced pressure at 40 ^{o}C by rotary evaporator to eliminate the methanol, and the extract was then lyophilized and stored at ambient temperature until further use (Diallo, Sanogo, Yasambou, Traoré & Maiza, 2004).

Determination of total Phenolic Content

The amount of total polyphenols was determined according to the Folin-Ciocalteu method. Briefly, 200 μl of extract was mixed with 1 ml of 1/10th Folin–Ciocalteu reagent. After 4 min, 0.8 ml of Na_2CO_3 (7.5%) was added. The mixture was incubated at room temperature for 2 hours and the absorbance was then read using a spectrophotometer at 765 nm (SHIMADZU-1240 UV/ visible). All determinations were carried out in triplicate. A standard curve was prepared using Gallic acid. Total polyphenolic values are expressed in Gallic Acid Equivalents (GAE) per gram of dry weight (mg GAE/ g DW) (Li et al., 2007).

Estimation of total Flavonoid content

The determination of total flavonoid content in the various extracts was carried out by a method using aluminium trichloride ($AlCl_3$) (Barros, Carvalho & Ferreira, 2011). Briefly, a 1ml aliquot of the different extracts was mixed with 0.3ml of $NaNO_2$ solution (5%). After 5 min, the resulting mixture was added to 0.3 ml of $AlCl_3$ solution (10 %), and then after 6 min, this was mixed with 2 ml of NaOH (1M) and the total volume made up to 10 ml with distilled water. The absorbance was measured at 510 nm using a spectrophotometer (SHIMADZU-1240 UV/ visible). A standard curve was prepared using Catechin. Total flavanoid was expressed in milligram Catechin Equivalent (CE) per gram of dry weight (mg EC / g DW).

Estimation of total condensed Tannin content

The tannin content of plant extracts was determined by the method described by Heimler, Vignolini, Giulia Dini, Vincieri and Romani (2006), using Quercetin as a reference compound. Briefly, 400 μl of extract was added to 3 ml of methanolic solution, with 4% of vanillin, and 1.5 ml of concentrated hydrochloric acid. After 15 min of incubation, the absorbance was read at 550 nm using a spectrophotometer (SHIMADZU-1240 UV/ visible). The condensed tannin was expressed in milligram Quercetin Equivalent (QE) per gram of dry weight (mg EQ / g DW) (Heimler et al., 2006).

HPLC Analysis

Standardization of Phenolic extracts of *Zizyphus lotus (L.)* by High Performance Liquid Chromatography HPLC-UV was carried out using the optimized conditions. In this analysis, *Agilent Technologies 1200 series* chromatography equipment was used. For this system, 10 μl of each extract was injected onto a C18 reverse phase column. The mobile phase consisted of two eluents, water / methanol mixture (95/5), and the flow rate of the mobile phase was1 ml / min. The temperature was set at 25 ^{o}C. The detector used was a UV detector at a wavelength of 280-320 nm, for 70 min. The results are expressed by a comparison of the chromatograms of the standard with that of the sample.

Antioxidant activity evaluation: Scavenging ability towards DPPH radical

This technique depends on the reduction of the free radical DPPH (purple color) to a yellow coloured diphenyl picrylhydrazine in the presence of the antioxidant. Briefly, 25 μl of different concentrations of extract were mixed with 2.5 ml solutions of DPPH (0.004%). Reference antioxidant solutions (ascorbic acid) were also prepared under the same conditions to serve as a positive control. An equal amount of methanol and DPPH served as negative control. After 30 min of incubation at room temperature in the dark, the absorbance was recorded at 517 nm. The experiment was performed in duplicate (Es-Safi, Kollmann, Khlifi & Ducrot, 2007). The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH Scavenging activity}(\%) = \left[\frac{(A1 – A2)}{A1}\right] \times 100 \tag{2}$$

where:
A1: Absorbance of negative control
A2: Absorbance of sample.

Determination of IC$_{50}$

The concentration of sample required to scavenge 50% of DPPH (IC$_{50}$) was determined graphically from the percent reduction versus concentration curve (Samartha et al., 2008). A decrease in the DPPH solution absorbance indicates an increase in the DPPH radical scavenging activity.

2.4 Antimicrobial activity evaluation of extract from *Zizyphus lotus*

Inoculum preparation

From a culture of 18 h, a bacterial suspension was prepared to obtain a density equivalent to the standard of 0.5 Mac Farland. This density corresponds to a concentration of 10^6 - 10^8 CFU / ml (Wade et al., 2001).

Antimicrobial Resistance Testing (Agar Diffusion Method)

The resistance of the strains to different antimicrobial agents was determined by the disc diffusion method and the antibiotic was chosen according to CASFM (2016). After 24 h of incubation at 37 ^{o}C, the diameter of inhibition zones was measured.

Agar Disc Diffusion Assay

The *in vitro* evaluation of antimicrobial activity was carried out using a disc diffusion method. The plant extracts were dissolved in DMSO at 200 mg / ml with binary dilution. The sterile paper discs (6 mm diameter), impregnated with 10 μl of extract of plant per disc, were sterile deposited on the agar surface. The discs were kept at 4 ^{o}C for 1 h and then incubated for 24 h at 37 ^{o}C (Warda et al., 2009). The disc impregnated with DMSO was used as a negative control. The diameter of the inhibition zone around each disc was measured for three replicates (Kumar et al., 2008).

Determination of MIC by microdilution

The minimal inhibitory concentration (MIC) of the plant extracts against the different strains was assessed using the microdilution method. Each well of microtiter plates was inoculated with 50 μl of Broth Muller Hinton (BMH), 50 μl of the extract (dissolved in DMSO) and 50 μl of bacterial suspension containing 10^8 CFU / ml adjusted to 0.5 McFarland so the final volume in each well was 150 μl. The positive control was prepared by 50 μl BMH and 50 μl of inoculum. The negative control was prepared with 50 μl of the extract in solution and 50 μl of BMH without inoculum. The microplates were then covered and incubated at 37 ^{o}C for 24 hours (Abdelrahman, Skaug & Francis, 2002). The turbidity was measured every 2 hours using a microplate reader at 620 nm. Each plant extract was run in duplicate.

Determination of minimal bactericidal concentration (MBC)

The minimum bactericidal concentration corresponds to the lowest concentration of extracts yielding negative cultures after incubation at 37 ^{o}C for 24 h. It was determined by re-plating 10 ul from culture negative wells on Muller Hinton Agar. After 24 h incubation at 37 ^{o}C, the number of colonies on the streaks was compared with those of the bacterial inoculum. All tests were done in triplicate. The MBC / MIC ratio was calculated for each extract. If the result was less than 4, the extract was considered bactericidal (Guinoiseau, 2010).

2.5 Effect of extracts from *Zizyphus lotuson* auto-aggregation and co-aggregation

Autoaggregation and coaggregation abilities of each strain were evaluated. The autoaggregation assay was performed according to the method of Collado, Meriluoto and Salminen (2007) and Tuo et al. (2013) with little modification. The *Lactobacillus* strains were grown for 18 h at 37 ^{o}C in MRS broth. Bacterial cells were recovered by centrifugation at 5000 g/15 min, washed twice and resuspended in phosphate buffered saline (PBS, pH 7.2) to obtain an optical density of 0.5 at 600 nm. Cell suspensions (4 ml) were enriched separately by three different volumes of aqueous extract (50, 100 and 200 μl). The absorbance at 600 nm of cell suspensions incubated at 37 ^{o}C was monitored with a spectrophotometer (SHIMADZU-1240 UV/ visible) for different times (2 h, 4 h and 24 h).

The results are expressed as a percentage by the following formula:

$$Autoaggregation(\%) = \left[1 - (\frac{A_t}{A_o}) \right] \times 100 \quad (3)$$

where:

A_t represents the absorbance at time t = 2 h, 4 h, 24 h

A_o the absorbance at time t = 0 h.

The assay was carried out in triplicate (Collado et al., 2007; Tuo et al., 2013).

Coaggregation assays were also prepared as described for autoaggregation analysis. Briefly, 2 ml of each cell suspension of the different probiotic and pathogen strains were mixed and incubated at 37 ^{o}C. Samples were taken in the same way as in the autoaggregation assay. Absorbance was determined for the mixture and for the bacterial suspensions alone. The absorbance (600 nm) was monitored at different times (2 h, 4 h and 24 h). Percentages of coaggregation were determined as:

$$Coaggregation(\%) = \left\{ \frac{\left[\frac{(Ax+Ay)}{2-A(x+y)} \right]}{Ax} + \frac{Ay}{2} \right\} \times 100 \quad (4)$$

where x and y each represents one of the two strains in the control tubes, and (x + y) represents the absorbance of the mixed bacterial suspension.

2.6 Adhesive interaction assay

The adhesion ability of each strain was evaluated according to the method of Collado et al. (2007). The hydrophobicity of *Lactobacillus* strains was determined by Xylene and Toluene extraction. Overnight cultures were harvested by centrifugation, washed twice with PBS (pH 7.2), resuspended in the same buffer, and $A600$ of the cell suspension was measured to standardize the number of bacteria (approximately 10^8 CFU/ml). An equal volume of Xylene and Toluene was added, and the suspension was vortexed for 4 min. The phases were separated, and the absorbance at 600 nm of the aqueous phase was measured using a spectrophotometer (SHIMADZU-1240 UV/ visible). The affinity of the bacterial strains to hydrocarbons was reported as adhesion percentage according to the formula:

$$Hydrophobicity\% = \left[\frac{(A_0-A)}{A_0} \right] \times 100 \quad (5)$$

where A_0 and A are absorbance values measured before and after Xylene / Toluene extraction.

3 Statistical analysis

All experiments were done in triplicate. All data are presented as means ±SD or as frequency in percentage (%). The means were compared using multivariate analysis of variance (ANOVA). P values <0.05 were considered significant.

4 Results and discussion

4.1 Microbiological analysis

The quantitative and qualitative distribution of the different microbial groups included in this study are shown in Figure 1.
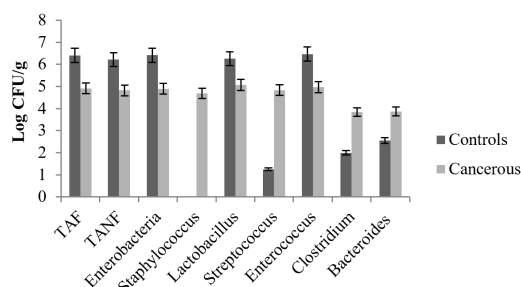
Figure 1: Intestinal microbiota composition in selected groups (cancerous and controls)

The results show that the gut microbiota of patients with colorectal cancer is significantly different than that of the healthy controls. The most difference is the presence of *Stapylococcus* for the group of cancer patients (4.69 log CFU/g) and its absence in the group of healthy controls. The also was a marked difference for *Streptococcus*, *Clostridium* and *Bacteroides* which were more abundant in patients with colorectal cancer (medians of 4.83 log CFU/g, 3.84 log CFU/g and 3.87 log CFU/g respectively) than in controls (medians of 1.24 log CFU/g, 1.99 log CFU/g and 2.54 log CFU/g respectively). However, the microbial groups belonging to the genus *Enterobacteria*, *Lactobacillus* and *Enterococcus* are signific-

antly more abundant in healthy controls (medians of 6.41 log CFU/g, 6.25 log CFU/g and 6.46 log CFU/g respectively) than in patients with colorectal cancer (medians of 4.89 log CFU/g, 5.06 log CFU/g and 4.96 log CFU/g respectively).

4.2 Firmicutes/Bacteroidetes ratio

In this study for the Firmicutes/Bacteroidetes ratio, there was a significant difference between healthy controls and patients with colorectal cancer (0.6 and 0.7 respectively). The ratio is significantly higher in cancer patients that in healthy controls. Similar findings were reported in the study of Chen et al. (2012) which showed more abundant Firmicutes and less abundant Bacteroidetes in groups of patients with CRC. Gao, Guo, Gao, Zhu and Qin (2015) showed that cancerous tissue had a significantly higher abundance of Firmicutes and Fusobacteria than tissue found in healthy individuals. Another study reported that a reduction in *Bacteroidetes* diversity and an increase in Firmicutes and Fusobacteria diversity were observed in CRC samples, suggesting that specific bacteria could play a major role in CRC (Allali, 2017).

4.3 Microbial strains selection for aggregation and adhesion capacity

After identification, the following pathogenic microorganisms *Citrobacter braakii*, *Enterobacter cloacae*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Clostridium leptum*, and the probiotic strains, *Lactobacillus salivarius* were selected for aggregation and adhesion tests.

4.4 Phytochemical analysis of *Ziziphus lotus* l

Extraction Yield

The results obtained show that the yield of extracts from *Ziziphus lotus* varies according to the solvent used. The greatest yield was observed

with the aqueous extract (32.92%), followed by the methanolic extract (30.84%). The yield of extracts depends on several factors, such as the origin of the plant (Ebrahimzadeh, Pourmorad & Hafezi, 2008), the variety, the harvest season, the climatic and environmental conditions, the geographical location, the different diseases that can affect the plant, the maturity of the plant (Park & Cha, 2003) and the method of extraction.

Phytochemical analysis

Aqueous and methanol extracts were prepared to examine the content of total phenolics, flavonoids and tannins. The results for extractive values are tabulated in Table 2.

The total phenolics content varied among the extracts as shown in Table 2. *Zizyphus lotus* extracts showed the higher polyphenol content (225.40 mg AGE / g DW) in the aqueous extract than in the methanolic extract (63.04 mg GAE/gDW). This could be due to different degrees of polarity of the solvents used for the extraction of the polyphenolic compounds. AE and ME extracts of leaves of *Zizyphus lotus* appear richer in polyphenols. In a study by Hossain, Uddin and Islam (2015), the extract of *Zizyphus lotus* contained a total phenolic content of 97.18 ± 12.81 mg of gallic acid equivalents /g DW. *Zizyphus lotus* extracts showed the higher flavonoids content (14.88 mg CE/gDW) in the methanolic extract than in the aqueous extract (11.30 mg CE/gDW). The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (Gao & Liu, 2005). The presence of flavonoids in leaves of *Zizyphus lotus* extracts have been reported to be responsible for antioxidant activity (Braca et al., 2003).

For condensed tannin contents, higher values were observed in the aqueous extract (10.66 mg QE/gDW) than in the methanolic extract (8.66 mg QE/gDW). The presence of tannins in some plant extracts indicates that the plant may have the ability to be an antioxidant, antifungal and anti-inflammatory agent, and possess healing properties (Araujo, Alencar, Lucia Cavalcanti de Amorim & Albuquerque, 2008).

HPLC analysis

Preliminary analysis for the extracts of the aerial parts of this plant showed chromatographic profiles almost similar from a qualitative point of view but with a quantitative difference in the abundance of their majority compounds. The chromatographic profiles of the extracts EA and EM are shown in Table 3 and 4 respectively.

The chromatographic profile for quantitative and qualitative analysis (HPLC) of extracts is shown in Tables 3 and 4. During these analyses, 13 components were identified. The phytochemical analysis by HPLC showed that all extracts are rich in phenolic compounds. The aqueous extract is mainly composed of Quercetin glucosid (36.35%), Resveratrol (34.31%), Kaempferol glucosid (8.18%), Miricitine (3.19%), Kaempferol (2.9%), Gallic acid (1.06%) and Quercetin (1.03%). Other components such as Caffeic acid (0.70%), Epigalocatechin (0.34%), Epigalocatechin gallate (0.28%), Catechin (0.25%) and Procyanidin B2 (0.23%) are also present but with lower concentrations.

A comparison with the methanolic extract showed it to be composed of Quercetin glucosid (33.27%), Resveratrol (16.89%), Kaempferol glucosid (6.26%), Quercetin (3.33%), Kaempferol (3.13%), Miricitine (3.09%), Caffeic acid (2.87%), Catechin (2.83%), Procyanidin B2 (2.24%), Epigalocatechin gallate (1.1%) and Gallic acid (0.45%).

Other components such as Epigalocatechin (0.10%) and Catechin hydrate (0.08%) are also present but with lower concentrations. Some trace compounds can significantly increase the biological activity of medicinal plants (Vamanu & Nita, 2013). Plants with high levels of phenolic compounds have been shown to exhibit high antioxidant capacity (Razali, Razab, Junit & Abdul Aziz, 2008).

Antioxidant activity

The antioxidant activity of our extracts is expressed in IC_{50} (Table 5). This parameter has been used by several research groups to present their results. It defines the effective concentration of the substrate that causes the loss of 50% of the activity of DPPH. Lower IC_{50} values in-

Table 2: Total phenolic, flavonoid and tannin contents in the plant extracts

Sample	Polyphenol content (mgAGE/gDW)^a*±SD	Flavonoid content (mgCE/gDW)^b*±SD	Tannin content (mgQE/gDW)^c*±SD
Aqueous extract (AE)	225.40 ± 0.28	11.30 ± 0.20	10.66 ± 0.33
Methanolic extract (ME)	63.04 ± 0.58	14.88 ± 0.37	8.66 ± 0.42

*Mean±SD of three determinations
^{a} mg acid galic equivalent/g dry weight. ^{b} mg catechin equivalent/g dry weight.
^{c} mg Quercetin equivalent / g dry weight

Table 3: Chromatographic profile of aqueous extracts

Components	Retention time (min)	Percentage (%)
Gallic Acid	7.30	1.0609
Catechin	17.59	0.3466
Epigalocatechin	17.92	0.3466
Procyanidin B2	19.94	0.2393
Caffeic acid	21	0.7062
Epigalocatechin gallate	22.54	0.2861
Resveratrol	36	34.3188
Quercetin glucoside	36.7	36.3544
Miricitine	38.5	3.1961
Kaempferol glucoside	39.7	8.1833
Quercetin	42	1.0331
Kaempferol	46	2.5935

Table 4: Chromatographic profile of methanolic extracts

Components	Retention time (min)	Percentage (%)
Gallic Acid	7.30	0.4573
Catechin hydrate	17	0.0807
Catechin	17.59	2.8301
Epigalocatechin	17.92	0.1047
Procyanidin B2	19.94	2.2410
Caffeic acid	21	2.8711
Epicatechin gallate	27.88	1.3186
Resveratrol	36	16.8941
Quercetin glucoside	36.7	33.2753
Miricitine	38.5	3.0955
Kaempferol glucoside	39.7	6.2677
Quercetin	42	3.3308
Kaempferol	46	3.1372

dicate higher antioxidant activity (Pokorny & Schmidt, 2001).

The IC_{50} value was determined from a graph of scavenging activity against the different concentrations of *Zizyphus lotus* extracts and ascorbic acid. The 50% inhibition concentrations (IC_{50}) are shown in Table 5. They are relatively low, reflecting the presence of compounds that can reduce the DPPH radical.

According to the results shown in Figure 2, our mostly methanolic extracts have more interesting DPPH free radical neutralization capabilities (195±0.02 μg/ml) than aqueous extracts (227±0.01 μg/ml). This finding is supported by the study of Sun, Powers and Tang (2007) which showed that methanol remains the best solvent to extract antioxidants from a plant, although still significantly lower than ascorbic acid (36±0.03 μg/ml). Much of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds. They act as reducing agents, hydrogen donors and singlet oxygen scavengers (Hakkim, Gowri Shankar & Girija, 2007).
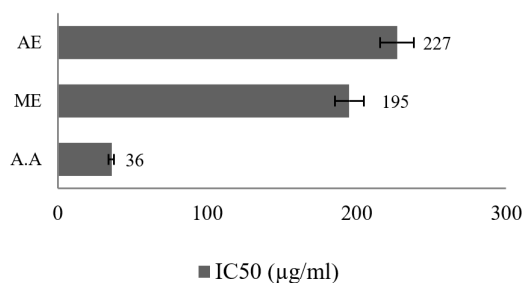

Figure 2: Inhibition concentrations (IC_{50}) of the plant extracts

4.5 Antimicrobial activity

Antimicrobial activity of selected antibiotics

The antibiotic resistance of strains was determined from the diameter of the zone of inhibition (mm) according to the Committee of the Antibiogram of the French Society of Microbiology (CASFM, 2016). The results of the antimicrobial activity of selected antibiotics are reported in Table 6.

Disc diffusion test

The results of the antimicrobial activity of extracts against the strains of bacteria tested by the method of aromatogram are shown in Table 7.

From the disc diffusion test results, it appears that all bacterial strains tested are inhibited by phenolic extracts, with various degrees of antimicrobial activity. This confirms the broad spectrum of antimicrobial activity of these extracts against Gram+ and Gram- bacteria. Large zones of inhibition appear with the methanolic extract against *Escherichia coli* and *Citrobacter braakii* (35-29 mm), respectively. The aqueous extracts are also more active against *Escherichia coli* (32mm). This plant extract exhibited a significant antibacterial activity against the different strains of antibiotics. However, these activities of extract are due to its content of active compounds.

The antimicrobial activity of these extracts against bacterial strains could be attributed to the presence of biologically active components such as flavonoids, phenolic acids and terpenoids as described in the chromatogram (Doudach et al., 2012).

Minimum inhibitory concentration (MIC)

Results of the antimicrobial activity of extracts against the strains of bacteria tested by microdilution are shown in Table 8.

According to Table 8, the plant extract had an important inhibitory activity against bacteria tested. The highest activity against strains tested with MIC was 6.25-25 mg/ml for aqueous extract and 12.5-25 mg/ml for methanolic extract. However, AE was more active against *E.coli, Enterobacter cloacae* and *Citrobacter braakii* (6.25 mg/ml) and ME were more active against *Enterococcus faecalis* and *Clostridium leptum* (12.5 mg/ml).

The antibacterial activity can be explained by

Table 5: The antioxidant activity of the plant extracts

Sample	DPPH (IC$_{50}$, μg/ml)
Aqueous extract (AE)	227±0.01
Methanolic extract (ME)	195±0.02
Ascorbic acid (AA)	36±0.03

Table 6: Antibiotic resistance profile of tested bacterial strains

	AMC	CN	OX	V	C	TE	SXT	L	ATM
Escherichia coli	R	I	/	/	S	R	R	/	R
Enterobacter cloacae	R	I	/	/	I	R	R	/	R
Citrobacter braakii	R	I	/	/	S	R	R	/	I
Staphylococcus aureus	/	I	R	R	S	I	R	R	/
Enterococcus faecalis	/	I	/	R	S	S	I	R	/
Clostridium leptum	R	I	/	R	I	/	/	R	R

R: Resistant, I: Intermediate, S: Susceptible, AMC: Amoxicillin+ Clavulanic acid, CN: Gentamycin, OX:Oxacillin, V: Vancomycin, C: Chloramphenicol, TE: Tetracyclin, SXT: Trimethoprin/Sulfamethoxazol, L: Lincomycin, ATM: Aztreonam

Table 7: The diameter of the zone of inhibition (mm) from plant extracts for pathogenic bacteria

	Inhibition diameter (ø mm)			
	Aqueous extract (AE)	Methanolic extract (ME)	OX 5 μg	CN 10 μg
Escherichia coli	32±0.8	35±0.12		14±0.1
Enterobacter cloacae	20±0.06	21±0.3		11±0.8
Citrobacter braakii	18±0.2	29±0.01		13±0.2
Staphylococcus aureus	16±0.17	22±0.15	–	
Enterococcus faecalis	23±0.35	20±0.08	–	11±0.09
Clostridium leptum	16±0.57	20±0.03		10±0

- :No inhibition zone

the mechanism of toxicity towards the half-organisms, which is done either by non-specific interactions such as the establishment of hydrogen bridges with the proteins of the cell walls or the enzymes, either by the chelation of metal ions (such as iron) and the imprisonment of the substances necessary for the growth of bacteria (Karou et al., 2005).

Minimum bactericidal concentrations (MBC)

Minimum bactericidal concentrations of extracts are reported in Table 9.

The highest activity against strains tested with MBC was 25-50 mg/ml for aqueous extracts and 25-100 mg/ml for methanolic extracts (Table 9). For the MBC / MIC ratio, the extract is validated as bactericidal when it is less than or equal to 4. Values of this ratio are variable for the various extracts according to the bacterial strains tested (Table 9), which makes it possible to establish a classification of these extracts according to their spectrum of action. Indeed, some work has shown that Flavonoids such as Quercetin and Apigenin are involved in the inhibition of D-alanine: D-alanine ligase, thus disrupting the synthesis of the bacterial wall (Wu et al., 2008). Other authors have speculated that certain Flavonoids belonging to the class of Flavonols (Cushnie & Lamb, 2005), the flavan-3-ols (Sirk, Brown, Sum & Friedman, 2008) cause an alteration of the cytoplasmic membrane leading to its lysis, or the inhibition of topoisomerase by Isoflavonoids, thus blocking the synthesis of deoxyribonucleic acid (DNA) (Gradisar, Pristovsek, Andreja & Jerala, 2007; Wang et al., 2010).

Effect of extract on autoaggregation and coaggregation

The *in vitro* effect of aqueous extract of *Zizyphus lotus* in auto and coaggregation of bacteria strains was evaluated. The viability of *Lactobacillus salivarius* was carried out on agar. The incorporation of 200 μl of aqueous extract had no effect on the development of probiotic strains tested.

As shown in Figure 3, autoaggregation of *Lactobacillus salivarius*, without aqueous extract, increased in time from 45.9% to 55.6% for 2 h and 4 h respectively to 79.6% and 91.2% for 2 h and 4 h respectively, in the presence of aqueous extract. After 24 h, the highest autoaggregation capacities were recorded of 81.20% and 93.4% respectively, without and with aqueous extract. The difference is statistically significant (P<0.05) compared to control (without AE).

The high autoaggregation capacity suggests that the probiotic strain is good at forming biofilms and/or gastrointestinal tract colonization, which are the ways of forming barriers against colonization by pathogenic microorganisms (Schachtsiek, P. Hammes & Hertel, 2005).

Coaggregation of *Lactobacillus salivarius* with potential gut pathogens could contribute to the positive properties of the probiotic. As shown in Figure 4, the strain *Lactobacillus salivarius* showed the highest coaggregating ability with *Clostridium leptum*, which without aqueous extract was 72.61% and in the presence of aqueous extract was 73.20%. Coaggregation is a process by which bacteria are attached to each other by means of specific molecules (Rickard, Gilbert, J. High, E. Kolenbrander & S. Handley, 2003). The percentage of coaggregation, without aqueous extract, of *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter brakii*, *Staphylococcus aureus* and *Enterococcus fecalis* respectively was 8.66%, 57.75%, 72.40%, 56.65% and 48.98%. However, the respective percentage of coaggregation increased to 69.75%, 64.80%, 77.32%, 70.40% and 52.64% in the presence of aqueous extracts from *Zizyphus lotus (L)*. The difference is statistically significant (P<0.05) compared to control (without AE).

The coaggregation of probiotic-pathogen improved in the presence of the extract. A study by Alberto, Rinsdahl Canavosio and Manca de Nadra (2006) showed that flavonoids can prevent the expression of adhesion molecules, in particular human endothelial cells, and the inhibitory effect of the tumor factor NF-$\alpha\beta$ inducing the expression of adhesion molecules. This effect depends on the molecular structure, concentration and metabolic transformation of flavonoids. In another study, El Astal, Ashour and Kerrit (2005) suggested that plants have a variety

Table 8: The minimum inhibitory concentrations (MIC) of plant extracts and antibiotic

	Extracts of Zizyphus lotus (L.)(mg/ml)		Antibiotics (μg/ml)	
	Aqueous extract (AE)	Methanolic extract (ME)	OX	CN
Escherichia coli	6.25	25		62.5
Enterobacter cloacae	6.25	25		125
Citrobacter braakii	6.25	25		250
Staphylococcus aureus	25	25	62.5	
Enterococcus faecalis	12.5	12.5		125
Clostridium leptum	12.5	12.5		125

Table 9: The minimum bactericidal concentrations (MBC) and MBC/MIC ratio of plant extracts

	MBC (mg/ml)		MBC/MIC ratio	
	AE	ME	AE	ME
Escherichia coli	25	25	4	1
Enterobacter cloacae	50	25	8	1
Citrobacter braakii	25	25	4	1
Staphylococcus aureus	25	100	1	4
Enterococcus faecalis	25	25	2	2
Clostridium leptum	25	25	2	2

of potentially significant therapeutic compounds against human pathogenic bacteria.
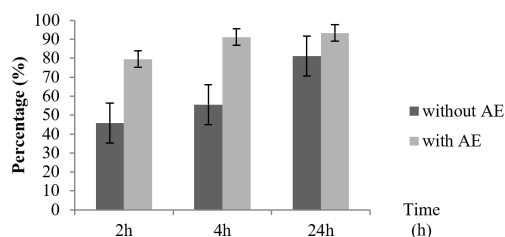
Figure 3: Effect of aqueous extract on autoaggregation of *Lactobacillus salivarius*

Figure 4: Effect of aqueous extract on coaggregation of bacteria strains tested

Capacity of Bacteria adhesion

As shown in Figure 5, *Lactobacillus salivarius* presents an important adhesion capacity of 67.52% and 65.31% for xylene and Toluene respectively. The hydrophobic nature of the outermost surface of microorganisms has been in-

volved in the attachment of bacteria to host tissue. This property could confer an important advantage for bacterial maintenance in the human gastrointestinal tract (Schillinger, Guigas & Holzapfel, 2005). One of the main criteria in the selection of probiotic bacteria is the ability to adhere to the intestinal mucus because the adhesion extends their permanence in the intestine, which

allows them to exert a salutary effect (Apostolou et al., 2001) and to improve the antagonistic activity against enteropathogens (Leahy, Higgins, Fitzgerald & Van Sinderen, 2005).

The adhesion of probiotics to the intestinal mucosa and enterocytes is considered an important factor in the colonization of the intestinal tract and the modulation of the immune system of the host (Ouwehand, Isolauri & Salminen, 2002).
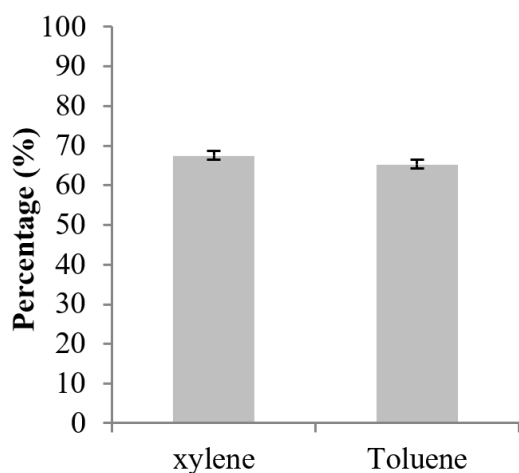

Figure 5: Percentage of adhesion of *Lactobacillus salivarius* to solvents

5 Conclusions

The presence of certain bacterial species has an impact on the development of CRC. Therefore, it is imperative to study the intestinal microbiota during CRC and the bacterial resistance to drugs, and to research a natural strategy that relieves the restoration and modulation of the gut microbiota.

This work confirms the prebiotic, antioxidant and antibacterial activity of extracts from *Zizyphus lotus*. It also confirms the stimulatory effect of the aqueous extract on probiotic aggregation and the inhibitory effect of *Lactobacillus salivarius* against the pathogenic bacteria tested (*Citrobacter braakii, Enterobacter cloacae, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Clostridium leptum*) which were isol-

ated from feces of patients with colorectal cancer. Autoaggregative and Coaggregative capacities can be used for preliminary selection of probiotic bacteria for potential applications in humans to modulate and keep the microflora balance by development of probiotics and the reduction of pathogenic bacteria responsible for many diseases.

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