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An Assessment of the Effect of Aqueous Extract from *Thymus fontanesii*on Growth, Aggregation and Biofilm Formation of Pathogenic and Probiotic bacteria

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ABSTRACT

Polyphenols are a group of organic molecule ubiquitously distributed in plant kingdom. Several of these polyphenols appear to be effective antimicrobial agents. the purpose of this study was to optimized the method of polyphenolic extraction from *Thymus fontanessi* by using of four technic of extraction (overnight maceration at room temperature, two cycle of maceration at 70°C during 2h, extraction by Soxlet and extraction by sonification at 52 Khz during 45min) and to evaluated of the effect of this extract as prebiotic on the growth and aggregation of some isolated probiotic (*Lactobacillus bulgaricus*and *Streptococcus thermophilus*) and on growth and biofilm formation of pathogenic bacteria *(Staphylococcus aureus 25922, Bacillus cerius 11798, Bacillus subtilis 3633, Pseudomonas aerugenosa 27853 and Escherichia coli 10536*). The extraction by sonification was found to have the most significant efficiency of extraction (41.5%) and the most content on polyphenols (156mg EAG/g of extract). This extract was exhibited a prebiotic activity and antibacterial effect; adhesion tests were shown the stimulatory effect of natural extract on probiotic aggregation and inhibitory effect of biofilm formation of pathogenic. Therefore these molecules can be used as alternative or additive agents to improve the probiotic bacteria activity and to prevent the pathogenic bacteria colonization.
KEYWORDS: polyphenols- *Thymus fontanesii*- probiotic- prebiotic- antibacterial activity- biofilm-aggregation.

INTRODUCTION

Mucosal surfaces are home to a complex microbiota, which rapidly colonizes the tissues at birth and bestows important nutritional, metabolic and protective functions which benefit the host. The numerous and diverse microbial communities residing at the vast intestinal mucosal surface have a particularly important role to play in human health, any *dysbiosis*of this microbiota being an underlying factor in a variety of clinical diseases **[1]**.

Divers factors are able to alter intestinal microbiota balance by favoring the growth of pathogens such as *Salmonella, L. monocytogenes, Staphylococcus aureus, C. perfringens type A, Clostridium difficile*and *Candida albicans*, which can affect the host health; An alternative strategy to modulate or renovate this balance is the use of probiotic which are bacteria with beneficial proprieties, present in great quantity in the intestine, they permit to keep the microflora balance by reduction of its pathogenic members and increasing the potentially benefitial ones **[2];** There are also food probiotic bacteria that exhibit specific beneficial properties through microbiotamodulation. The protective role of probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms have received special attention. Pathogen inhibition by lactic acid bacteria might provide significant human health protection against pathogens **[3].** It is currently accepted the probiotic definition formulated in 2001 by FAO/WHO **[4]**"Live microorganisms which when administered in adequate amount confer a health benefit to the host". From them **Havenaar et al. [5],** defining probiotics as mono or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the proprieties of the indigenous microflora.

It is important to guarantee the viability of these strains during the passage through the gastrointestinal tract, the maintenance of the beneficial effects and, the preservation of their characteristics particularly adhesion and capability of colonization **[6].**

Auto-aggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells, and co-aggregation abilities may form a barrier that prevents colonization by pathogenic mico-organisms **[7]**.

The mechanism to increase the number of beneficial bacteria in the gut is the ingestion of prebiotics. Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively

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stimulating the growth and/or activity of one, or a limited number of bacteria in the colon that can improve the host health" **[8].**

Prebiotics are present in many edible plants such as chicory, artichoke, onions, leeks, garlic, asparagus, wheat, bananas, oats, soybeans and other legumes. Many commercial prebiotics are obtained from vegetable raw materials **[6].**

Recently, the scientific interest in the study of plant materials as sources of new compounds for their processing into therapeutic agents antimicrobial and prebiotics has increased considerably. Also **Alberto et *al.* [9]**indicate that polyphenolic compounds had a stimulatory effect on probiotic growth. From them **Khalil et *al.* [10]** confirmed that Flavonoids may also affect growth and metabolism of bacteria.

Thymus (Lamiaceae) is one of the eight most important genera with regard to the number of species include comprising about 215 species particularly prevalent in the Mediterranean region **[11].**Considerable work has been done on the antibacterial activity of different species of *Thymus* extracts.

The aim of current research was to evaluated the effect of aqueous extract (the most richer on polyphenols) obtained from Thymus *fontanesii*as prebiotic (on growth and aggregation of probiotic bacteria) and as antimicrobial agents (on development and biofilm formation of pathogenic bacteria).

2. MATERIALS AND METHODS

2.1 *Thymus fontanesii*collection

The selection of the plant is related firstly to these medicinal effects then at bibliographical considerations and experimental work which showed his chemo-diversity, his richness in polyphenols and his remarkable antimicrobial activity through experiments carried out on various samples.

*Thymus fontanesii*is an aromatic plant, spontaneous belonging to the family Labiatae and originating in Algeria and Tunisia **[12].**

*Thymus fontanesii*was collected from the region of Ammari of Tissemssilt (south- west of Algieria) in juin 2011 when plants were flowering. This plant was identified according to African Flowering Database. The plant material was confirmed by a local expert and a voucher specimen (T 038) was deposited at the herbarium center of the department of Biology. Mascara University (North West Of Algeria) for future reference. The leaves and flowers of the harvested plant were cut into small species and allowed to dry at room temperature for two weeks according to the standard procedures. They were then grounded to powder (0.250 mm of diameter) in a ball mill.

Fig.1. *Thymus fontanesii used*

2.2. Preparation of extracts

The extract was prepared according to four techniques:

Maceration at room temperature: 5 g of fine powder (0.2 mm) of *Thymus fontanesii*were subjected to maceration overnight at room temperature in a volume of 100 ml of distilled water **[13].**

Maceration at high temperature: 5g of fine powder (0.2 mm) were extracted with 100 ml of distilled water twice at 70°C for two hours **[14].**

Extraction by Soxhlet : 10 g of dry matter, finely ground (0.2 mm) of *Thymus fontanesii*were extracted with 200 ml of water distilled in a Soxhlet apparatus for 3 h. The mixture is then subjected to evaporation at low pressure to + 40 ° C **[13].**

Extraction by sonification: Extraction with ultrasound is performed by adopting the technique described by **Wen et al. [15]**with some modification, 5 g of fine powder (0.2 mm) were first macerated in 100 ml of distilled water for 2.5 h and then sonicated by ultrasonic probe at 52 KHZ frequency for 45 min the filtrate was collected and the retentate (plant material) was used for a second extraction on the same terms (volume of water distilled, frequency, temperature and duration of exposure).

After filtration and evaporation of the solvent at 70 ° C, the extracts obtained by four techniques were then reconstituted in same solvent (distilled water), sterilized by miltiport filter and stored at + 4C ° and protected from light.
The yield of each extract (**Y %**) was estimated from its weight and expressed in percentage: **Y (%)=(W/W0)100** (W0, W are the weight in grams of plant material treated and the extract obtained respectively) **[16].**

2.3. Analytical methods

2.3.1. Total phenolic content

The total phenol contents (TP) of the crude extracts were determined spectrophotometrically using the Folin-Ciocalteu's method following the procedure described by **Lister and Wilson, [17].** 100µL of sample was mixed with 500µL of the 1/10th FolinCiocalteu reagent and 1000 µL of distilled water, this mixture was incubated 1min then 1500µL of an aqueous sodium carbonate solution (20%) were added. The final mixture was incubated during 2h at home temperature, after which the absorbance was read at 760 nm against a blank (solution with no extract added) using UV-visible spectrophotometer (JENWAY 7305). All determinations were performed in triplicate.

Results were expressed in gallic acid equivalents per gram of extract (mgGAE/g of extract) using a standard curve which was prepared by gallic acid.

2.3.2. Total flavonoids content

The AlCl3 method, as described by **Brighente et *al.* [18],** was used for the determination of total flavonoid content (TFlav) of extract. To 2mL of sample, the equal volume of the 2% AlCl3 solution (2g in 100mL methanol) was added. The mixture was vigorously shaken, and absorbance was read at 430 nm after 1h of incubation at home temperature. Results are expressed in quercetin equivalents per gram of extract (mgQE/g of extract). All determinations were carried out in triplicate.

Aqueous extract used in this study was screened for the presence of high concentration of polyphenols and flavonoids.

2.4. Microbial tests

2.4.1. Microorganism and culture conditions

Probiotic strains used *Lactobacillus bulgaricus and Streptococcus thermophilus, were separated from the mélange collection and properly examined from Gram coloration, catalase activity, oxidase reaction, gas production, sugar metabolism; these tests are confirmed by API strp and API50 CHI (Biomérieux, Marcy l'Etoil, France)*. These strains were also characterized by the principal criteria of selection of probiotics as (Growth at different temperature, at acid pH, resistance to bile salts, anti-bacterial propriety and bacteriocin production, aggregation and adhesion capacity, resistance to antibiotic).

Pathogenic strains ATCC (American Type Culture Collection) used were: *Bacillus subtilis (3633), Bacillus cereus (11798), S. aureus (25922), Escherichia coli (10536)* and *P. aerugenosa (27853)* The microbial strains were maintained at **-20°C** in culture broth with 15% (v/v) of glycerol Subcultures were freshly prepared before use by inoculation of a loop of stored culture and incubation overnight at 37°C **[19].**

2.4.2. Inoculum preparation

The probiotic bacteria were grown to exponential phase for 18 h in appropriate media and the optical density at 625 nm was adjusted by dilution in fresh media to a turbidity of 0.5 Mac Farland standard (107CFU/ml), for pathogenic bacteria, the suspensions were diluted 10 times to give 106CFU/ml **[20].**

2.4.3. Influence of selected extract on aggregation of probiotic bacteria used

Theevaluation of the effect of aqueous extract on auto and coaggregation of both probiotic bacteria was performed according to the technique of **Kos et al. [21],** with some modification. Bacteria were grown in MRS / M17 liquid enriched by two different volume of aqueous extract (100 and 200 microliter), and incubated at 37 ° C for 18h, they were harvested by centrifugation at 5000 g for 15 minutes and washed three times with PBS. After vortexing for 10 s of 4 ml of bacteria suspensions containing 108 cell/ml were decanted at room temperature and the autoaggreagation was determined. For the coaggregation, equal volume 2ml of each cell suspension of tow probiotic bacteria (2ml) mixed by vortexing for 10s and then decanted at room temperature. After five hours of decantation, the absorbances A at 600nm were estimated for both tests. The results were expressed as a percentage by the following formulas:
Autoaggregation: %= $1-(A_t/A_0)*100$, where $A_t$was the absorbance after decantation, A_0 the absorbance at t0.
Coaggregation: %= **: % =** $[(A_X + A_Y)/2 - (A_{X+Y}) \times 1/ (A_X + A_Y)/2] \times$ **100.**; Where x and y represent the OD of each of the two strains in the control tubes, and (x+y) the OD of mixture.

2.4.4. In vitro antibacterial activity

Well-diffusion assay

The agar well diffusion method was realized according to **Damintoti et al. [22]**with some modifications**,** petri plates (9 cm) were prepared with 20 ml Mueller Hinton agar. Each Petri plate was inoculated with 100µl of each bacterial suspension (106 CFU/ml). After drying in a sterile hood, wells (8 mm in diameter) were made in the agar and 50, 100, 150, 200 microliter of aqueous extract were loaded in the wells.. The plates were incubated for 24 h at 37°C. The diameters of the inhibition zones were evaluated in millimeters and the inhibition in percentage (I%) was calculated according to: I% = (dz/d0)*100, where dz was the diameters of the inhibition zones; d0 was the diameters of petri plates used **.**

The extract inducing inhibition zone around disc were considered as antibacterial according to the ladder cited by **Mutai et al. [23].** The diameters of inhibition zones (D) of the microbial growth were classified in five classes:

-Very strong response, zone diameter ≥ 30mm
-Strongly response, zone diameter 21mm - 29mm
- Moderate response, zone diameter 16mm -20mm
- Week response, zone diameter 11mm- 16mm
-No inhibitory: zone diameter <8mm

Determination of minimum inhibitory and bactericidal concentrations (MIC, MBC)

Minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no visible growth was observed after 24 h incubation at 37°C and Minimum bactericidal concentration (MBC) was recorded as a lowest extract concentration killing 99.9% of the bacterial inocula after 24 h incubation **[24].** MIC and MBC were determined by measuring optical density of suspensions in presence of differents concentrations of aqueous extracts, 2.8 ml of medium was inoculated by 0.2ml of bacterial suspension *(S. aureus, E. coli, B. cerius and B. subtilis),* extracts were added to this mixture to obtain a final concentration of 1 to 10^{-9} mg/ml.Tubes controls were prepared without extracts. Incubation was performed at 37 ° C for 24 hours and absorbance was taken at 625nm with a UV-Vis spectrophotometer.

The percentage of inhibition (I%) of strains was also estimated according to: I%= (A-At/A)*100, where A is the absorbance for the negative control (containing no extract), and At is the absorbance for the sample treated with extract **[25].**

2.5. Effect on biofilm formation of pathogenic bacteria

The effect of aqueous extracts on biofilm formation of pathogenic bacteria (*S. aureus*, *E. coli*, *B. cerius*and *B. subtilis*) was evaluated as described by **O'Tool et *al.* [26],** with some modifications. 100microliter of cell suspension from overnight culture were dispensed into each well of microtiter plates in the presence of 100 microliter of aqueous extract. The microtiter plates were incubated at 37°C for 24 h.

After incubation the medium was then removed, the wells were rinsed three times with phosphate-buffer saline (PBS) to remove non-adherent cells and dried during 1h. 125 microliter of gentian violet have been added to each well and plates were incubated at room temperature during 15 minutes, then the wells were washed 3-4 times with distilled water.

After photo taking, the adhered microbial strains were lysed by incubation at room temperature for 15 min in presence of 125µl acetic acid (30%). The content of wells were then aspired and the optical density was measured with at 550 nm to quantify the microorganisms adhered.

The reduction percentage of biofilm formation of each bacteria in the presence of aqueous extract was calculated according to following formula: 100- [(OD492 nm with extract / OD 492nm without extract)*100] **[27].**

2.6. Statistical analysis

All of the experiments were carried out in duplicate for exception the experiments of extracts analysis which were performed thrice with three replicates. The results are reported as mean ± SEM standard deviation. For determining the significant inter group difference each parameter was analyzed separately and one-way analysis of varience (ANOVA) was carried out and p inferieurou equal 0.05 was considered statisticaly significant.

3. RESULTS AND DISCUSSION

The extraction of phenolic compounds was performed using four different techniques (classical and alternative method). The best performance is recorded by ultrasound namely 41.5% (52 KHZ, 45min, 50 ° C), whereas that obtained by Maceration at room temperature, at hig temperature and by soxhlet were respectively 21, 30 and 32%.

Previous study carried out by **Wen et al. [15]**using four methods of extraction from *F.eucommiae*was reported that the highest yield was obtained by ultrasonic method (41%). **Da portor et al. [28]**were also

confirmed the efficacity of ultrasound, compared to conventional extraction methods, for the extraction of plant compounds (oil/ polyphenols) with a lower solvent consumption and a shorter extraction time. They were reported that the action of ultrasonic vibrations producing the bubbles collapse enhancing the dispersion of the cells, and so improving the extraction efficiency. The results of the quantification of total polyphenols and flavonoids are illustrated in figure 2.

From figure 01, it obvious that the high levels of total polyphenols and flavonoids are registered in the extract obtained by ultrasound. These contents being of EAG 156mg / g of crude extract and EQ 14mg / g of crude extract, respectively.

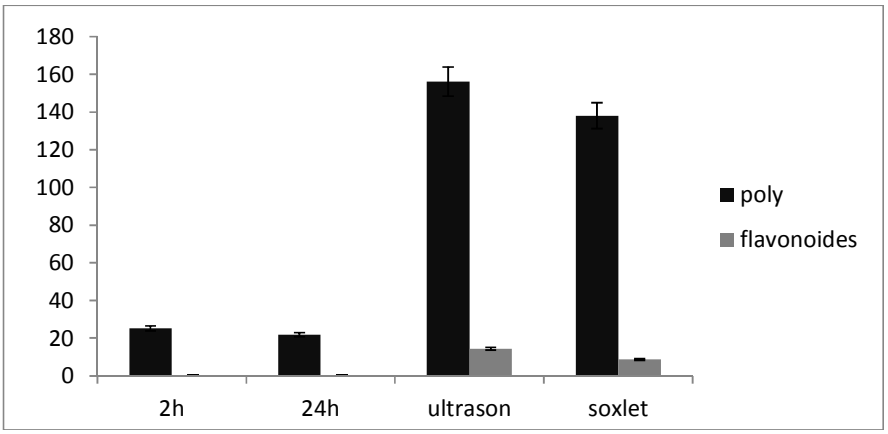

Fig.2. Percentages of polyphenols and flavonoids of aqueous extract of *Thymus fontanesii*

Khan et al. (29) concluded that the high total phenolic content (275.8 mg of gallic acid equivalent/100 g FW), flavanone concentrations (70.3 mg of naringin and 205.2 mg of hesperidin/100 g FW) and extraction yield (10.9 %) obtained from optimised UAE proved its efficiency when compared with the conventional methods.
Other studies have also shown that the concentrations of phenolic compounds are most important when the extraction method used is ultrasound, moreover, **Jordan et al. [30**], using this technique have been observed contents polyphenols from 108 to 122mg EAG / g of dry extract in the species Thymus zygis ssp. gracilis. **Chemat et al. [31]**, using two extraction techniques: maceration and ultrasound has resulted in the order of levels of 89.6 ± 2.3 and 121 ± 3.8mg GAE / g dry extract, respectively. **Hossain et al. [32]** emphasizes that the highest concentrations of flavonols were observed in the extract of *Origanummajorana*obtained by ultrasound.

3.1. Effect on auto and coaggregation of probiotic strains

In this test, we characterized the in vitro effect of aqueous extract of *Thymus fontanesii*on probiotic aggregation (auto and coaggregation). The viability test which was carried out showed that the incorporation of 100ml of the aqueous extract improved the development of probiotic strains on agar and had no effect at 200ml. In recent research **Ming et al. [33]**demonstrated that phenolic extract from *Litseacoreana*stimulated growth of *S. thermophilus*, *L. acidophilus* et*L. casei*and was no effect on *L. delbrueckii*subsp. bulgaricus and Bifidobacteriumsp growth. In his study **China el al. [34]** confirmed that *Sesbaniagrandiflora* flower polyphenol extracts showed growth promoting effect on the common probiotic bacterium *L. acidophilus*.
Alberto et al. [9]reported that *L. hilgardii*from wine showed a growth stimulatory effect in the presence of gallic acid and catechin at concentrations normally present in wine.
The results of the effect of aqueous extract on auto and coaggregation indicated in figure3 , this results shown the stimulatory effect of aqueous extract on probiotic aggregation where the autoaggregation were increased from 30.625% to 60.06% % and from 45.93% to 68.5% for *Lactobacillus bulgaricus*and *Streptococcus thermphillus*respectively, the probiotic coaggregation between *Lactobacillus bulgaricus*and *Streptococcus thermphillus*was equally increased in presence of aqueous extract, from 72.35% to 92,3%.

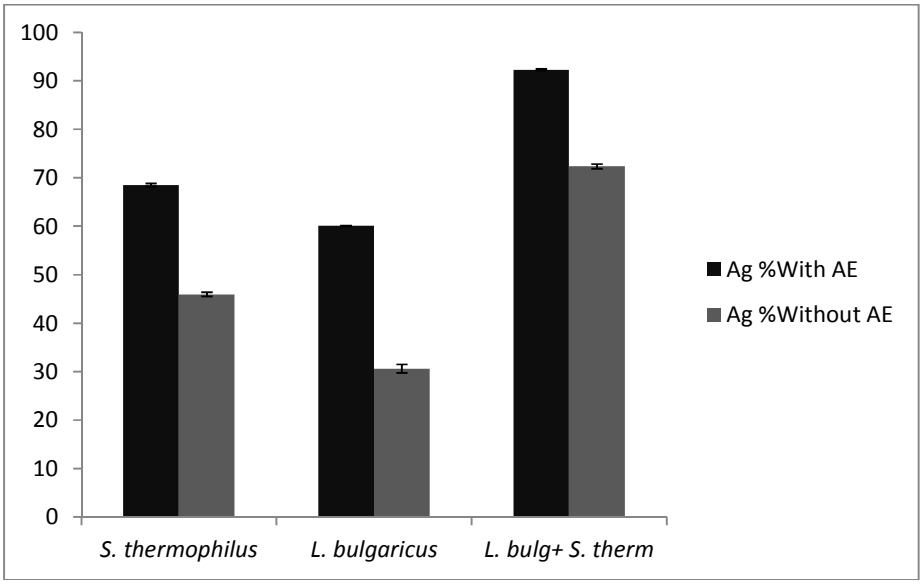

Fig.3. Effect of aqueous extract on percentage aggregation (Ag%) of probiotic bacteria.

Parkar et al. [35] demonstrated how several polyphenols, including caffeic acid, catechin, epicatehecin, coumaric acid, phloridzin, rutin, naringenin, daidzein, genistein, and quercetin, inhibited growth and adhesion of bacterial pathogens to human Caco-2 cells as well as enhanced the proliferation and adhesion of Lactobacillus rhamnosus as probiotic strain.

3.2. In vitro antimicrobial activities

The evaluation of the antimicrobial activity of the extract of *Thymus fontanesii*against five bacteria (*S. aureus, B. cerius, B. subtilis, E.coli and P. aerugenosa*) was made by the method of aromatogram. The antimicrobial property of the extract was obtained by measuring the percentage of inhibition using the diameters of the zones of inhibition (mm).

The percentages of inhibition of the microbial strains tested by the aqueous extract (50 and 100**µl**) were summarized in figure N04.

According to the ladder cited **Mutai et al. [23],** the aqueous extract of *Thymus fontanesii*had a very strong activity against bacterial strains tested (D> 30mm) except *Pseudomonas aeruginosa*(D <16mm).

The largest diameter of inhibition was obtained with *Bacillus cereus* (6_ + 0.2cm) and the lowest with *Pseudomonas aeruginosa*(1 to 1.5 cm). Thus the results showed that the aqueous extract of *Thymus fontanesii*had a broad spectrum of antibacterial activity against Gram + and Gram -.

The percentages of inhibition of the growth of *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*and *Pseudomonas aeruginosa*were respectively 69,4115%, 55,2935%, 47,05%, 40,588%, 17,882% in presence of **100 µl** of extract (figure 4).

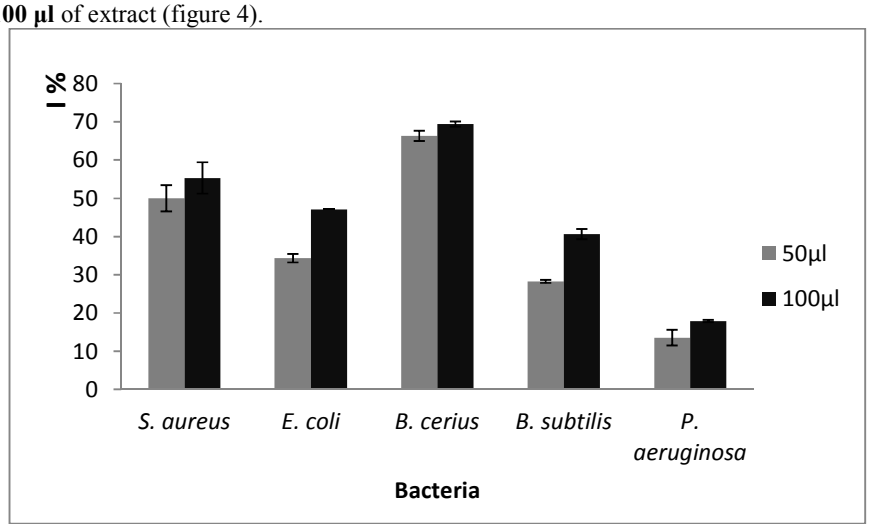

Fig.4. Percentage of inhibition (I %) of pathogenic bacteria growth on gelose by aqueous extract (50 and 100 µl)

3.3. Minimal and bactericid inhibitory concentrations

The results of the MIC determination of the aqueous extract of Thymus fontanesii against the bacteria tested (S. aureus, E. coli, B. cerius and B. subtilis) are shown in Table01

Table1: MIC determination in broth medium

Strains/ Concentration of Ae	1mg/ml	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	Control 0mg/ml
S. aureus	-	-	-	-	-	-	-	-	+	++	+++
B. subtilis	-	-	-	-	-	-	-	+	+	++	+++
B. cerius	-	-	-	-	-	-	-	+	+	++	+++
E. coli	-	-	-	-	-	-	+	+	++	+++	+++

According to the table1, the extract had an important inhibitory activity against bacteria tested. Thus, *S.aureus*proved to be the most sensitive, he was completely inhibited from a concentration of 10^{-7} mg / ml. The concentration of 10^{-6} mg / ml was sufficient button to inhibit completely the growth of *Bacillus subtilis*and *Bacillus cerius.* Whereas *Escherichia coli* was inhibited completely from a concentration of 10^{-5} mg / ml). It was confirmed that Gram positif bacteria such as enteropathogen*S. aureus*were more sensitive than Gram negative bacteria such as *E. coli* **[35], [36]. This difference can be explained by the wall structural diversity these different classes of bacteria. The low sensitivity of Gram-negative bacteria due to the presence of a second lipopolysaccharide membrane acting as a barrier. Thus, Ikigai et al. [37] proved that bactericidal catechins primarily act on bacterial membranes; they demonstrated that Gram-negative bacteria are protected by the presence of negatively charged lipopolysaccharide.**
The minimum inhibitory and bactericidal concentrations are represented in the table2.

Table2: minimum inhibitory and bactericidal concentrations determined on broth medium

	Strains Gram+			Strains Gram-
	S. aureus	***B. subtilis***	***B. cerius***	***E. coli***
MIC	10^{-8}	10^{-8}	10^{-8}	10^{-7}
MBC	10^{-7}	10^{-6}	10^{-6}	10^{-5}

In spite of changes in MIC and MBC from a microorganism to another, in some cases **(depending on the bacterial strain and the nature and effectiveness of the antibacterial agent)**they are equal and indicating a strong bactericidal effect. Indeed, when the ratio MBC / MIC is less than or equal to 1, the polyphenols are microbicide, when this ratio is greater than 1, polyphenols are microbiostatic**[22].**The table03 shows the different reports MBC/ MIC of the aqueous extract of *Thymus fontanesii*.

Table3: Different reports MBC/MIC

Srains	MBC/MIC	Interpretation
S. aureus	10	Bacteriostaticeffect
B. subtilis	10^2	Bacteriostaticeffect
B. cerius	10^2	Bacteriostaticeffect
E. coli	10^2	Bacteriostaticeffect

From the table3, we can say that the extract had a bacteriostatic effect on all strains used in this test.
Analysis of Table 2 shows that the MIC and MBC of the extract of *Thymus fontanesii*against Gram negative bacteria (*E. coli*) is slightly greater than that of Gram positive bacteria (*S .aureus*, *B. subtilis and Bacillus cerius*).
A number studies have documented the effectiveness of various polyphenols against pathogenic bacteria, particularly *S.aureus*and *E.coli*; the study of the effect of polyphenols on G + and G- bacteria confirms that **these phenolic compounds were inhibited several bacteria G + and G- including*E. coli*,*Salmonella enterica, Bacillus cerius and Staphylococcus aureus*with a differential susceptibility[38];** it has been shown that the propolis extract rich on polyphenols exhibited an important activity against several strains of *E.coli***[39]**; Another study reported that pure flavonoids and polyphenolic compounds of different wine were effective against several strains, among it *S.aureus***[40].**
On other hand, the influence of polyphenols on growth and adhesion of commensal (*E. coli*) and pathogenic bacteria (*S. aureus*and *S. typhimirium*) of humain gastrointestinal tract was confirmed **[35].**
Loziene et al. [36]confirmed that the potent antibacterial activity of large thyme extracts isolated from five chemotypes of *Thymus pulegioides L* depended on the plant chemotype, extract preparation, solvent used and finally the sensitivity of bacteria. Gram positive bacteria were the most sensitive. This difference of sensitivity can be explained by the structural diversity of the wall of these different classes of bacteria.The low sensitivity of Gram-negative bacteria is explained by the presence of a second lipopolysaccharide membrane acting as a barrier. In addition, the outer membrane of Gram-is richer in protein than Gram+

making them more hydrophilic which prevents hydrophobic compounds to penetrate into the phospholipid layer and exert their antimicrobial activity inside microbial cell.
The mechanisms of antimicrobial activities of polyphenols are very complex, it can be attributed also to the Inhibition of microbial enzymes so Inhibition of microbial metabolism (extracell enzymes) Sequestration of necessary substrate or chelation of metal such as iron. Another hypothesis can be suggested as the membrane disruption, ability to complex with bacterial cell well and adhesins, inhibition of nucleic acid synthesis (inhibition of DNA gyrase), inhibition of cytoplasmic membrane functions and bacterial motility **[27].**
In summary, several mechanisms could explain the inhibitory effect of bacterial growth by polyphenols , such as destabilization of the cytoplasmic membrane by the complex formation with the protein and some constituents of the cell wall leading to lysis, inhibition of enzymes extracellular microbial (enzymes leading to the formation of the extracellular polysaccharides)**;** the direct actions on microbial metabolism, or deprivation of substrates required for microbial growth, especially essential mineral micronutrients such as iron and zinc (chelation of metals), whose depletion can severely limit bacterial growth.

3.4. The results of the effect of the extract tested on biofilm formation pathogens

In this test, we evaluated the effect of aqueous extract on biofilm formation of pathogenic bacteria**.** The results of the effect of the extract tested on biofilm formation pathogens are shown in Figure 5.

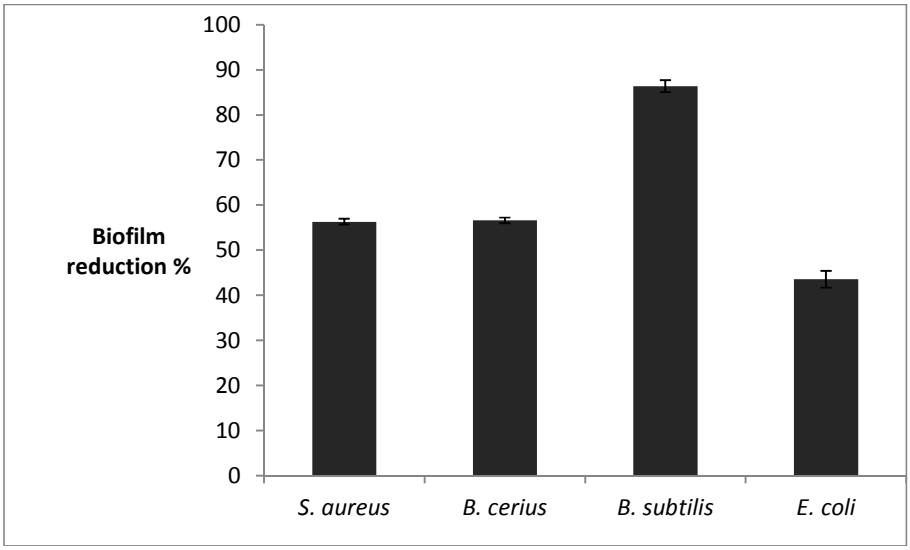

Fig.5. Percentages of biofilm reduction in presence of 100µl of aqueous extract

According to Figure5, biofilm formation of pathogenic strains tested was reduced in the presence of the extract of *Thymus fontanesii*. The extract reduced biofilm formation at a range of 43.54, 56.31, 56.59, and 86.36% respectively for *E. coli*, *S. aureus*, *B. cerius*and *B. subtilis.*
Marino et al. [27]in there study, were reported that the juniperus extract reduced biofilm formation of *S. aureus*ATCC 6538P at a range of 12-30% both at 7 and 24h.
On his part **Verhelst et al. [41]**reported that out of the three polyphenol extracts tested two exert inhibitory effects on the heat-labile toxin binding and all three extracts reduced the binding of ETEC to brush borders. Recently, **Verhlst et al. [42]**confirmed that three out of seven polyphenols inhibit effectively binding of LT to GM1, and inhibit induction of cAMP*in vero* cells.

Conclusion

our study of bacterial activity of natural polyphenols (of aqueous extract) of *Thymus fontanesii*reveal that the extract was shown an prebiotic activity by improving of probiotic bacteria (growth and aggregation) and antibacterial effect by inhibing of pathogenic bacteria (growth and biofilm formation) with significant difference; in this respect these molecules which has gained attention not only as a natural antimicrobial but is also thought to have prebiotic substances can be used as an alternative or additive agents to improve the probiotic bacteria activity (proliferation and colonization) and to prevent the pathogenic bacteria growth and colonization in hot.

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