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Research Article

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL EFFECT OF *ZIZYPHUS LOTUS* METHANOLIC EXTRACT AGAINST ORAL PATHOGENIC BACTERIA

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ABSTRACT

The aim of this *in vitro* study was to assess the antibacterial effect of the methanolic extract obtained from the roots of *Zizyphus lotus* (communally Sedra) collected in Mascara on bacteria responsible of oral pathologies, isolated from students aged from 20 to 28 years. Chemical qualitative analysis of this extract revealed the presence of polyphenols, flavonoids and tannins confirmed by a quantitative analysis which revealing 433,65µg EGA / mg dm of polyphenols, 247,87µg EQ / mg dm of flavonoids and 167.05 µg ECT/ mg dm of tannins. The microbiological analysis of samples collected from the oral cavity (10 students) revealed four bacterial groups (streptococci, staphylococci, lactobacilli and enterobacteria). The methanolic extract of *Zizyphus lotus* tested on agar medium and microdilution method showed an important inhibition diameter of 40 mm against *Serratia liquenfaciens* and MIC of 6.25mg/ml respectively towards *Lactobacillus sp.* and *Escherichia coli*.

Keywords: antibacterial effect; methanolic extract; flavonoids; oral bacteria; *Zizyphus lotus*.

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1. INTRODUCTION

Since the beginning of his history, Man was affected by caries, but with advent of sugar, he became the victim of the "third world plague ". The decline of caries begun in the 70s thanks to the use of fluorides and to the various campaigns on the oral hygiene led in dentist's surgeries and pharmacies [1]. Until recently, the dental health was almost only associated with the prevention of caries. We wrongly forgot that the periodontal diseases are one of two main causes of loss of teeth within adults; while the caries provoke toothaches, the periodontal diseases increase silently and show themselves only when the evil is already made [2]. The results of the epidemiological studies show clearly very strong prevalence of oral pathologies in the world population generally and in the Algerian population, in particular [3]. The presence of an infection generally and oral pathology in particular does not mean the systematic appeal to an antibiotic treatment. To avoid the development of the bacterial resistances, Nature has been providing a serie of remedies showing their proof as potential alternatives to antibiotic use.Plants represent an inexhaustible source of substances and bioactive natural compound [4]. Indeed, the secondary metabolites remain the object of numerous *in vivo* and *in vitro* researchs, in particular the search for new natural constituents such as phenolic compounds [5]. The African continent was endowed with a tremendous plant biodiversity, where large numbers are used as natural food or for therapeutic perposes. Numerous natural substances have been identified and many of them are used in traditional medicine for disease prevention and management [6]. In spite of the immense biodiversity of the African continent and Algeria in particular, only few efforts have been dedicated to the screening of plant bioactive ingredients. [7]. Our purpose was to evaluate the antimicrobial activity of the methanolic extract isolated from *Zizyphus lotus* (Sedra) roots, a plant collected in the city of Mascara, largely used In the local traditional medicine for the treatment of throat and bronchopulmonary irritations and as an emollient in the treatment of furuncle.

This *in vitro* study consists of:

- *In vitro* Chemical characterization of *Zizyphus lotus* root's methanolic extract (phytochemical screening, total phenolic, flavonoïds and tannins content)
- *In vitro* Evaluating the antibacterial effect of the extract toward bacteria isolated from the

oral cavity of students (20-28 years) presenting oral affections.

2. EQUIPEMENTS AND METHODS

2.1 Plant material

The roots of *Zizyphus lotus* collected in the city of Mascara (western Algeria), in March 2015 were identified by a botanist in the department of Biology of Mascara University. A referenced specimen (RH00001) was introduced in the WAMAP-base of the Laboratory of Bioconversion, Microbiological Engineering and Sanitary Safety (LBMESS) of our university. The collected roots were dried during about fifteen days and crushed in a traditional mortar, pulverized and then puted in a shaded glass box, hermetically closed for ulterior analyses.

Fig.1. *Zizyphus lotus* collected in Mascara (North West of Algeria)

2.2 Preparation of the methanolic extract of *Zizyphus lotus*

50 g of root powder were macerated in in 500 ml of methanol; and placed under agitation during 24h [8]. After filtration on wattman paper, the filtrate was then evaporated at 50°C under low pressure.The methanolic extract was lyophilized and conserved in shaded box for further analysis.

2.3 Phytochemical screening of *Zizyphus lotus*

2.3.1. Qualitative screening

The root powder of *Zizyphus lotus* was subjected to phytochemical screening, either in recation tubes or by CCM. . The chemichal characterization has been done as decribed in following works: cyanogenetic Heterosids [9], terpenoids, saponosids and coumarins, free quinines [10] and combined anthraquinones, alcaloides [11], tannins [12], flavonoids and reducing compounds [13].

2.3.2. Quantitative screening

The amount of polyphenols was carried out by the use of Folin-Ciocalteu reagent according to Rsaissi (2013) [12]. The concentrations of polyphenols were determined using a Galic acid calibration curve (0-200µg/ml) and were expressed in micrograms of Gallic acid equivalent per mg of dry matter (GAE µg /mg dm). The flavonoids content was calculated using a quercetin calibration curve (0-100µg/ml) with various concentrations under the same conditions as the samples. The results are expressed as micrograms of quercetin equivalent per milligram of dry matter (µg QE/mg dm) [13]. *Condensed tannins* were determined by the vanillin method. The results are expressed in micrograms of catechin equivalent per milligram of dry matter (CE µg/mg dm).

2.4 Oral sample collection

Oral samples (n=10) were collected with sterile swab from students aged between 20-28 years presenting oral pathologies (parodontite, dental caries), immersed in 1 ml of sterile nutritive broth for microbiological analyses. An experienced dentist has consulted these patients in order to detect and confirm oral pathology; we have been interested in the parodontits, the abscesses, the gum diseases and the dental caries. The inclusion criteria were as follows: students aged between 20 to 28 years, good medical health, presence of oral diseases, absence of any medical treatment in the three months preceding the study (as antibiotics). All students gave their informed consent.

2.5 Microbiological analyses of oral samples

The microbiological analysis relates to the research of the aero-anaerobic strains responsible for oral pathologies (enterobacteria, staphylococci; streptococci, enterococci; lactobacilles) on different medium (Blood agar, EMB, BEA, Chapman, Hektoen, Nutritive agar and MRS agar), incubated at 37 °C for 24 h, 48 h and 72 h according to the investigated strains and with the addition of 5% CO_2 (in dessicator) in the atmosphere for lactobacilli and streptococci. The isolated bacteria were identified with commercials kits (API Staph, API 20 E and API 50CH, Biomerieux, Marcy l'étoile, France) [14].

2.6 Antibiogram

All bacterial suspensions (culture of 18h on nutritive agar) were adjusted by the spectrophotometer at 650 nm to a final density of 10^5CFU/ml [15, 16].The inoculum was seeded on the surface of Muller Hinton agar; the dishes were left to dry 15mn at 37 °C. After this, the selected antibiotic discs were deposited with a pair of sterile forceps. The antibiotics used were: Gentamicin (CN), Colistin (CT), Spiramycin (SP), Penicillin (P), Aztreonam (ATM), Oxacillin (OX) and Cefazolin (CZ). Isolated and identified bacteria were classed as sensitive or resistant according to the French Society of Microbiology [17].

2.7 *In vitro* antibacterial activity

The antibacterial activity of the methanolic extract of *Zizyphus lotus*, were determined by the Diffusion agar method and the Microdilution method (determination of MIC)

2.7.1. Agar Diffusion method

100 µL of bacterial suspension (10^6 CFU/ mL) were spread on Muller Hinton agar surface (MH). The sterile discs (6 mm in diameter) impregnated with the methanolic extract were placed on the agar dishes inoculated with the tested bacteria [18]. Four concentrations were tested (binary dilutions in DMSO at 10%) as (200mg/mL, 100mg/mL, 50mg/mL, 25mg/mL and 12,5mg/mL).Discs with DMSO were used as a negative control. Amoxicillin (25 µg/disc) (Oxoid) was used as positive control. After 2 h at 4°C, plates were incubated at 37°C for 24 h [19]. Antibacterial activity was evaluated by measuring the diameter of the growth inhibition zones in millimeters and compared to control. Rsaissi *and al*. [12] have classified the microbial species in three categories (resistant, sensitive, very sensitive and extremely sensitive) according to the diameter of inhibition (Ø) measured.

- $Ø < 8mm$: Resistant $(-)$
- $9mm < Ø < 14mm$: Sensitive $(+)$
- $15mm < Ø < 19mm$: Very sensitive $(++)$
- $Ø > 20mm$: Extremely sensitive $(+++)$

2.7.2. Microdilution method

As recommended by National Committee for Clinical Laboratory Standards [19], in nutrient broth medium distributed in 96–well plates, binary serial dilutions of the tested extract

solution were performed. The obtained concentrations ranged from 4000µg/mL to 62,5 µg/mL in a 200 µL culture medium final volume. Each well was seeded with a 50 µL of calibrated bacterial suspension (0.5 MacFarland density, equivalent to 10^8 CFU/mL) [20]. In each test, a culture control (culture medium+ calibrated bacteria suspension) was performed. The microplates were incubated for 24 hours at 37°C. The optical density (representing bacterial growth) was measured at 620 nm using a Microplate Absorbance Reader Sunrise (Tecan Austria GmbH RC/ TS/TS) comparatively to control.

-Determination of minimum inhibitory concentration (MIC): represents the concentration that completely inhibit the growth of microorganisms

-Determination of minimum Bactericidal concentration (MBC): is the lowest concentration of the extract which can lyses bacteria (less than 0,01% of survivors).

100µl was taken from wells not presenting any visible culture and seeded on Mueller Hinton agar. The plates were incubated 24 h at 37°C. MBC/MIC was calculated.When this ratio is greater than 4, the extract has bacteriostatic and bactericidal if this ratio is less than or equal to 4. [21]

2.8 Statistical analysis

All experiments were done in triplicate. All data are presented as means ± SD. For *in vitro* antibacterial activity.A difference in bacterial growth equal or higher than 1 Log CFU is considered as significant [22].

3. RESULTS AND DISCUSSION

3.1. Characterization and yield of extract

The methanolic extract, presents a crystal aspect and a brown color, the yield of extraction was about 15.25%. This result was more important than that of *Zizyphus lotus* collected in Tlemcen (North west of Algeria) (13%) found by Ghalem, [23]. Another study in Batna (North Est of Algeria) revealed that the yield of fruit methonolic extracts of *Zizyphus lotus* was more less (6, 4%) [7].

3.2.Phytochemical screening

3.2.1. Qualitative analysis

The results of the phytochemical tests carried out mentioned in the table 01 showed the presence of polyphenols, flavonoïds, tannins, comarin,e saponosids, terpénoids, free quinons, combined anthraquinons, reducing compounds (glucosids), alkaloids and the absence of cyanogenetic heterosids in the root. These results were in agreement with study of Borgi *and al.* [24].

Table1. Results of qualitative screening of methanolic extract of *Zizyphus lotus* roots.

Chemical compound	Roots of *Zizyphus lotus*
Cyanogenetic Heterosids	-
Quinons	+
combined Anthraquinons	+
Tèrponoids	+
Saponosids	+
Coumarins	+
Tanins	+
Flavonoïds	+
Polyphenols	+
Alcaloïds	+
Reducing compounds (glucosids)	+

+: presence - : absence

3.2.2. Quantitative analysis

The methanolic extract obtained from *Zizyphus lotus* was riched in phenolic acids (33.65 µg EAG/mg dm). Ghalem (2014) reported 200 µg EAG/mg dm of the hydromethanolic extract of the roots of *Zizyphus lotus* in Tlemcen [23]. This extract was also riched in flavonoïds with 247,87µg QE/mg dm. These results corroborate those obtained by Ghalem [23] who etimates it to 200 µg QE/mg dm and a content of tannins about 167.05 µg CE/mg dm. The content of polyphenols, flavonoïds and tannins can be influenced not only by part used of the plant (root or fruits) but also by the method and the conditions of extraction (the polarity of the extract) and the environmental and geographical factors and the degree of maturation of the plant.

3.3 Identification of isolated oral pathogenic strains of students

An important bacterial diversity was found in students presenting oral pathologies. After enzymatic, biochemical and physiological identification; we have identified different groups (fig 2)

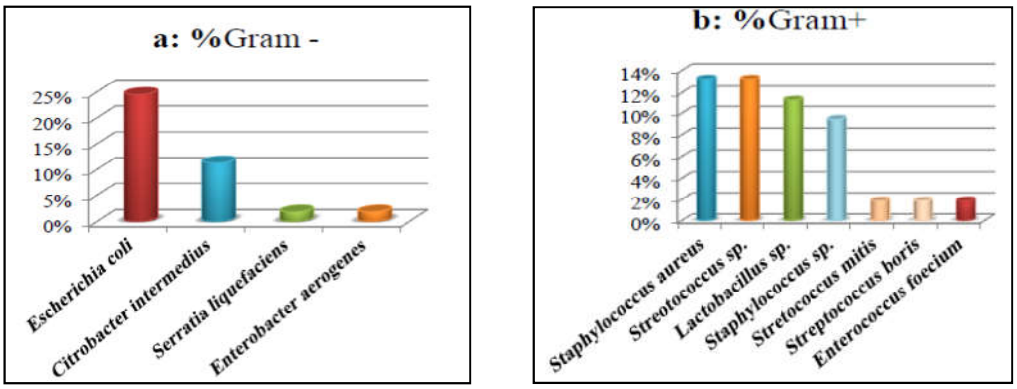


Fig.2. Bacterial groups identified from students presented oral pathologies.

For Gram negative bacteria, we observed a predominance of enterobacteria of enterobacteria in which *Escherichia coli* 24%, *Citrobacter intermedius*11%, *Serratia liquenfacians* 4% and *Enterobacter aerogenes*1%. The Gram positives are mainly represented by Staphylococci (*Staphylococcus aureus* 13%, and *Staphylococcus sp*. 9%), the Streptococci (*Streptococcus sp*13%, *Streptococcus mitis*1% and *Streptococcus boris*1%) and *Lactobacillus sp*. 11%. The Oral pathology develops when the balance of the bacterial community is broken in favour of the pathogenic bacteria, which replace the commensal microbiota [25]. *Escherichia coli*, *Citrobacter intermedius*, *Serratia liquenfacians*, and *Enterobacter aerogenes* belong to an inhabitual flora which resist to the acid pH are thus species acido-tolerants. The presence of these pathogenic bacteria not usually detected in oral pathologies is probably due to many factors which can influence the microbial ecology of the oral cavity of the students (dietary habits, oral hygiene) and other factors which are not yet known [26].

3.4 Antibiogram test

All identified strains were resistant to major antibiotics (table 02). *Staphylococcus aureus, Streptococcus sp* and *Escherichia coli* are among the multiresistant bacteria responsible of oral diseases.

Table 2. Antibiogram test of identified oral strains from selected students

Strains	**CN 10UI**	**CT 50µg**	**SP 100µg**	**P 10UI**	**ATM 30µg**	**OX 5µg**	**CZ 30µg**
Escherichia coli	S	R	R	R	S	R	R
Serratia liquenfaciens	S	R	R	R	S	R	R
Enterobacter aerogenes	S	S	S	S	S	R	R
Citrobacter intermedius	R	R	R	S	S	R	S
Streptococcus mitis	S	S	R	R	R	R	R
Staphylococcus aureus	S	R	R	R	S	R	R
Streptococcus boris	R	R	S	R	S	R	R
Streptococcus sp.	S	R	R	R	S	R	R
Lactobacillus sp.	R	S	S	S	R	S	S

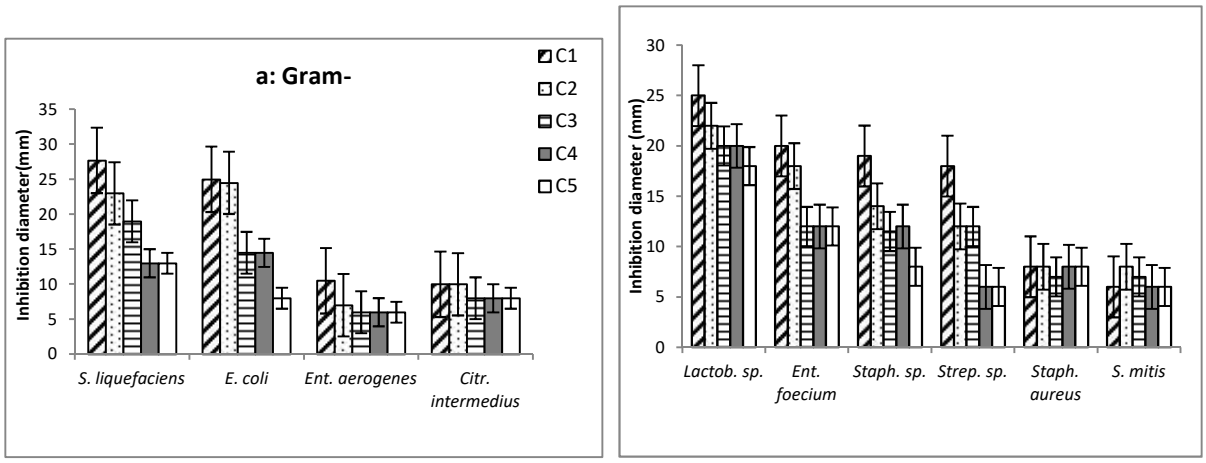
CN : Gentamicin CT :Colistin SP :Spiramycin P :Penicillin ATM :Aztreonam
OX : Oxacillin CZ :Cefazolin R :resistant S : sensitive

3. 5 I*n vitro* antibacterial activities

3.5.1 Determination of inhibition diameter

The antimicrobial activity of *Zizyphus lotus* root's methanolic extract estimated by agar diffusion method , testing the sensitivity of the isolated strains with methanolic extract of the plant [12]. The results showed that *Serratia liquenfaciens, Escherichia coli*, (Gram -) and *Lactobacillus sp., Enterocccus foecium* (Gram +) appear more sensitive at 200 mg/ml, and 100 mg/ml of root methanolic extract of *Zizyphus lotus* with inhibition diameter between (20 to 30 mm) (fig.3a, 3b). However, this extract strongly inhibited *Citrobacter intermedius (Gram -), Staphylococcus aureus* and *Streptococcus sp.* (Gram +) with a diameter of inhibition between (10 to 20 mm) at the same concentration and lower effect on *Enterbacter aerogens* (Gram-), *Staphylococcus aureus,* and *Streptococcus mitis* (0 – 10 mm) at 200 and

100 mg/ml .



S :Serratia E:Escherichia Ent:Enterobacter Lactob:Lactobacillus Citro: Citrobacter Staph: Staphylococcus S:Streptococcus

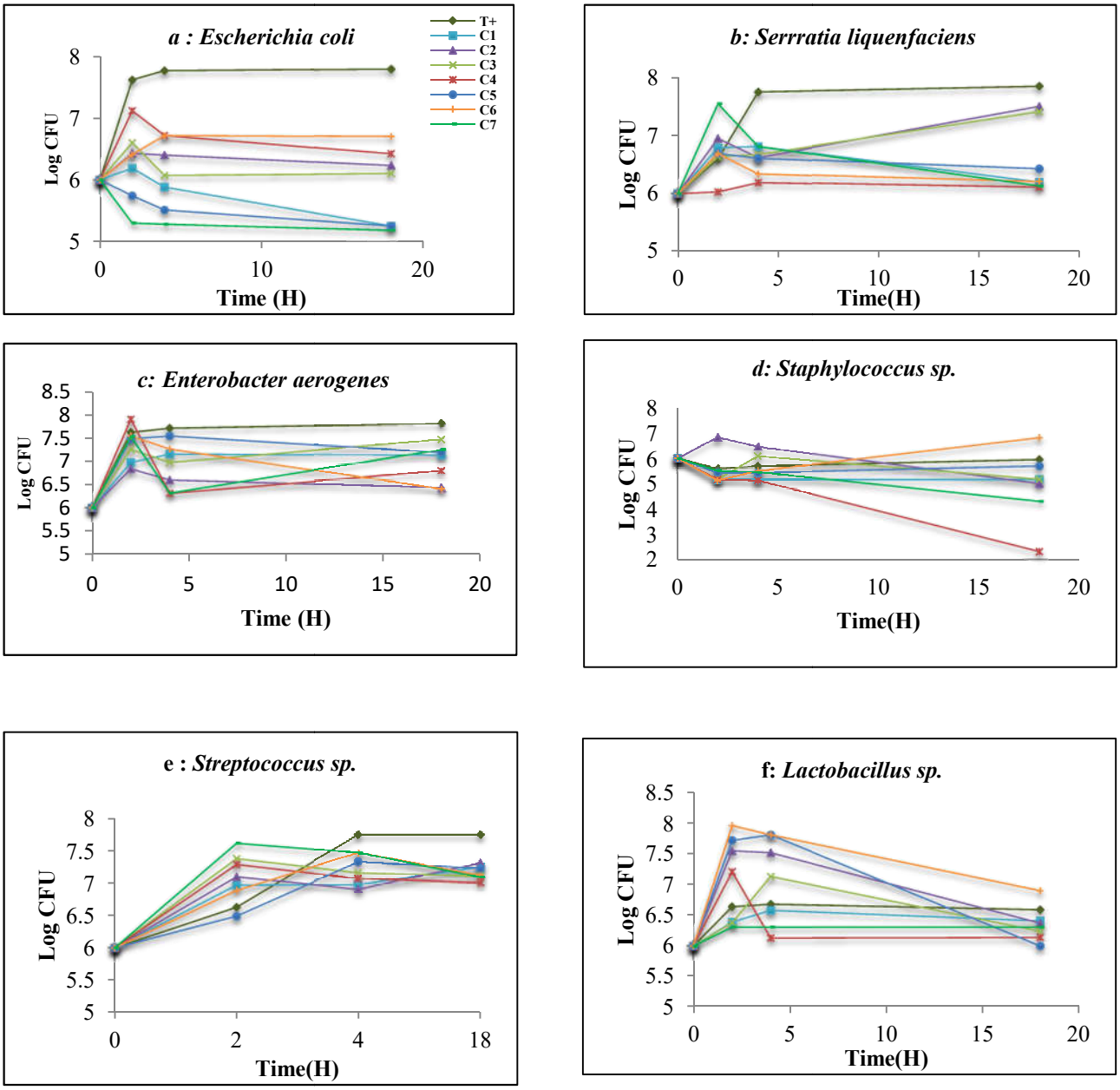
C1 :200mg/ml C2 :100 mg/ml C3 :50 mg/ml

C4 :25 mg/ml C5 :12,5 mg/ml

Fig.3. Average of inhibition diameter of methanolic extract of *Zizyphus lotus* against oral pothogenic bacteria isolated from students

3.5.2 Determination of bacterial growth (Microdilution method)

The bacterial growth of isolated strains was determined as: Log CFU= f (time) (fig. 4). After 2 hours of incubation, we observed a growth inhibition of most tested bacteria, at different degrees which was confirmed by the difference of the MIC. This sensitivity is related to the presence of the phenolic compounds (polyphenols, flavonoids, and tannins). Rsaissi *and al.,* confirm that the antibacterial activity of flavonoids can be attributed to: destruction the cellular walls or the enzymes, the chelating of the metallic ions, or the inhibition of the bacterial metabolism and the detention of substances necessary for the bacterial growth [12]. According to Scalbert, some tannin are able to inhibit the growth of bacteria causing diarrheas as *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus mutans* [27].



T +: Control, C1 : 4000 µg/ml, C2 : 2000 µg/ml, C3 : 1000 µg/ml, C4 : 500 µg/ml, C5: 250 µg/ml, C6: 125 µg/ml, C7: 62, 5 µg/ml.

Fig.4. Antibacterial effect of methanolic extract of *Zizyphus lotus* on oral pathogenic bacteria isolated from students.

3.5.3 Determination of MIC and MBC

According to table 03,: *Escherichia coli, Enterobacter aerogens* and *Serratia liquenfaciens* (Gram- bacteria) were sensitive with a MIC 62,5 µg/ ml, 2000µg / ml and 500 µg /ml

respectively. However, *Staphylococcus sp., Streptococcus sp., Enterococcus foecium* and *Lactobacillus sp*. (Gram +) was also sensitive to the methanolic extract of *Zizyphus lotus* with an MIC of 250 µg / ml for *Staphylococcus sp*., and 500 µg/ml for the other strains.This extract has a bactericidal effect (MBC/MIC≤ 4). These results were in agreement with the study of Djemai Zoughlache [7] who showed that the polar extracts of the *Zizyphus lotus* presented an antimicrobial activity with all bacteria except *Klebsiella pneumonia.*

Table 03. Antibacterial profile of selected multiresistant oral pathogenic bacteria

Strains	**MIC (µg /ml)**	**MBC (µg /ml)**	**MBC/MIC**	**Antibacterial effect**
Escherichia coli	62,5	62,5	1	Bactericidal
Enterobacter aerogenes	2000	1000	0,5	Bactericidal
Serratia liquenfaciens	500	500	1	Bactericidal
Staphylococcus sp.	500	250	0,5	Bactericidal
Streptococcus sp.	500	500	1	Bactericidal
Lactobacillus sp.	62,5	62,5	1	Bactericidal

4. CONCLUSION

The knowledge and the use of the medicinal herbs constitute a considerable heritage for the human being. Their importance in the field of the public health is much accentuated these years thanks to their therapeutic potential. The exploration of the antibacterial activity of *Zizyphus lotus* root's methanolic collected in the city of Mascara enabled us to get interesting results. This methanolic extract presented a yield extraction about 15.25% and phytochemical screening (qualitative study) highlights the presence of secondary metabolites in particular polyphenols, flavonoïds, tannins, quinons, saponosids, terpenoids, alkaloids, coumarins, and reducing compounds; with absence of cyanogenetic heterosides. The quantitative analysis of this extract is represented by the spectral proportion of the three bioactive substances: polyphenols, flavonoïds and tannins which are presented in considerable content about 433,65µg EGA/mg dm, 247,87µg EQ/mg dm and 167.05 µg ECT/mg dm respectively. The *in*

vitro antibacterial activity showed that *Zizyphus lotus* roots methanolic extract was active. A bactericidal activity was observed with all tested strains.

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