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Anti-bacterial translocation effect of methanolic extract of *Zygophyllum album* from Algeria on infected model rats with *Bacillus cereus*

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Abstract

Many ethno-botanical studies suggest that plants provide natural source of antimicrobial drugs that will be employed in controlling some infections globally and that can be a consequence to decrease the phenomenon of bacterial translocation. The work presented in this thesis contribute to the recovery of a medicinal plant *Zygophyllum album* (*Zygophylaceae*) from Algerian Sahara by the identification of some phenolic compounds using chromatography and the evaluation of antimicrobial and anti-translocation activity *in vivo* against the intestinal infection caused by *Bacillus cereus*. Chromatographic identification conducted on the species allowed to characterize their polyphenolic extracts. The detected major active compounds have various biological activities that could play a recognized role in maintaining good health. The various plant extract was subjected to screening for their potential antimicrobial activity. The methanol extract of *Z. album* revealed a very strong antibacterial activity against *B. cereus*. This extract concentration of 800 mg / ml referred curative demonstrated a remarkable ability to treat infection of *B. cereus*, to prevent its translocation to the internal organs and blood and inhibiting intestinal permeability of residents germs while maintaining its low acute toxicity. The detected major active compounds have various biological activities that could play a recognized role in maintaining good health.

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Introduction

Translocation of bacteria (BT) and their products is an undeniable phenomenon that occurs naturally in healthy humans and its occurrence is increased in a certain number of clinical pathological conditions. In this way, BT is certainly involved in the physiopathological mechanisms of many diseases. However, it is probably not the most important factor in most cases. Progress in understanding the mechanism involved in the gut barrier function, BT and host response to this phenomenon will allow future clinical studies to provide answers about the actual impact of BT in various human diseases such as toxi-infection causes by pathogenic bacteria (Infectious Disease Epidemiology Section Office of Public Health, 2013). Bacillus cereus causes a toxin-mediated food poisoning. Bacillus cereus is an aerobic and facultatively anaerobic, spore-forming, gram-positive bacillus. The emetic syndrome is caused by a preformed heatstable toxin. The diarrhea syndrome is caused by *in vivo* production of a heat-labile enterotoxin (Balzan *et al.,* 2007).

Literature reports and ethno-botanical record suggest that plants are the sleeping giant of pharmaceutical industry. They may provide natural source of antimicrobial drugs that will provide novel or lead compounds that may be employed in controlling some infections globally and that can be a consequence to decrease the phenomenon of bacterial translocation. *Zygophyllum album* L. is one of the large world of beneficts plants belongs to *Zygophyllaceae* family, genus *Zygophyllum*. Four species of *Zygophyllum* are recorded in Algeria (Attyia and Ashour, 2002). This plant used in traditional medicine as a remedy for rheumatism, gout, asthma and as a diuretic and antidiabetic drug. Some Bedouins used it as hay or added it to the dry ration. However, it was found to be toxic to the sheep and caused high mortality (Tackholm, 1974).

The aim of this study deals the study of the chemical composition of the methanolic extract of the plant to have well a relation between these components and the highlighted biological activity which is the antibacterial and anti-bacterial translocation of the plant as regards their effect *in vivo* and it is the first approach. The acute toxicity of the methanolic extract of the plant was studied to determine the safety margin, qualitative features and quantitative assessment of toxic over dosage. This study was carried out using oral administration.

Materials and methods

Plant material

Fresh upper parts from *Zygophyllum album* (leaves, flowers and stems) were collected in April during the flowering stage 2014 from Sidi Khouiled region, Sahara of Ouargla, Algeria.

The sampling was done by a randomized collection of 15–20 sub-shrubs in an area of about 200 m^2 each areal parts of *Z. album* were isolated manually in our laboratory to obtain a weight of 500–700 g of each part. Botanical identification of this species was carried out according to Africain flowering plants database and by local experts.

Test bacterium and culture medium

The bacterial strain used in this study was *Bacillus cereus*, which was obtained from Microbiology Lab/ Department of Biology- University of Mascara, Algeria. Bacterial strain was maintained on agar slant at 4 °C and sub-cultured on a fresh appropriate agar plate 24 h prior to antimicrobial test. Mossel agar was used for the activation of *B. cereus*, and during *in vivo* assays in rats for bacterial counts and identification.

Antimicrobial Resistance Testing**:** The resistance of the *Bacillus cereus* strain to different antimicrobial agents was determined using the disk-agar method standardized in our laboratory of biology. The quality control strain used was *Enterococcus faecalis* ATCC 29212 (Belmimoun *et al.,* 2016).

Experimental animals

The Wistar rats used in these experiments were provided by the laboratory of the University of Mascara. Animals were housed at the cage, with water and food *ad libitum*, and the animal room temperature was kept at constant temperature of 20 ± 1 °C on a 12-hour light/12-hour dark cycle.

Adequate measures were taken to minimize pain or discomfort of the animals, and all experimental procedures were performed in accordance with the ethical guidelines of the Organization for Economic Cooperation and Development (OECD).

Preparation of plant extract

The upper parts of the plant (leaves, flowers and stems) were air dried and ground all together as a fine powder, The methanolic extract was prepared using the extraction with organic solvents of increasing polarity method; 180 g of powder divided over cartridge were extracted with 300 ml of dichloromethane until exhaustion under reflux condenser, the same operation was repeated with methanol except that it was applied to the marc. The extract collected in a flask was concentrated using the rotary evaporator, the extract was stored in a glass bottle, clean, sterile and sealed (Ferrari, 2002).

LC/ESI-MS analyses

The LC analyses were conducted using a Prominence Liquid Chromatograph (Shimadzu, Kyoto, Japan) equipped with an SLC- 10A controller, LC-20AD pump, SIL-10AF auto sampler, and SPDM10A PDA detector. A Phenomenex Luna C-18(2) column (250 x 4.6 mm, 5 mm) was used. The mobile phase consisted of methanol (A) and acetonitrile (B) at a flow rate of 0.8 mL min-1 using the following gradients: 0.1–23 min, 10–40% of solvent B in A; 23.01–40 min, 10% solvent B and 90% solvent A.

The detection was done on a DAD detector set at 340 nm. The mobile phase was prepared daily, filtered through a 0.45 mm membrane filter (Millipore), and sonicated before use (Da Silveira and Farias, 2014). The system was optimized in the positive mode for anthocyanins and in negative for the other phenolic compounds.

The flow-rate used was 0.4 ml/min. LC/MS accurate mass spectra were recorded across the range 100–3000 m/z. The DAD detector was set to a scanning range of 200–400 nm. The phenolic compounds were identified mainly by their UV-spectra and ESIMS spectra and by comparing with published data (Ksouri, 2013).

Acute toxicity test

To assess the acute toxic effects of the methanolic extract, a measure of the lethal dose 50 (LD50) is required (Belmimoun *et al.,* 2016).

Study design

Male Wistar Albino rats were used for this study. They were divided into 5 groups of 5 animals each. The rats were acclimatized [room temperature (23 ± 2 °C), and a 12 h photoperiod] in cages (1 rat/cage) for one week before the commencement of the experiment. Throughout the experiment, rats were provided with water that contained streptomycin (5 $mg.mL^{-1}$) in order to reduce the level of facultative anaerobic bacteria that normally colonize the mouse intestine (Myhal *et al.,* 1982).

Bacillosis was induced using the method proposed by (Pan *and al*, 2014), the rats were fasted overnight in the day before the experiment and given, by gavage, 1 mL of saline solution (0.9% NaCl) containing 1.5×10^8 CFU of *Bacillus cereus*, except animals of group 1 (which were neither infected nor treated, and used as neutral control; they received distilled water). Animals of group 2 (which were infected, but not treated) received distilled water during the treatment period, hence were used as negative control groups; and those of group 3 received a vancomycin, and thus were used as positive control groups.

The two remaining groups, one of them (group 4) is the preventive group which receives treatment 7days before induction of *Bacillus cereus* and 2days after appearance of the infection, the last group 5 is the healing one which animals were infected and treated with methanolic extract in 7days, time of incubation bacillus was a 18 h 24h, it means that treatment was beginning 24 hours after the administration of the germ.

Body temperature of Wistar rats

Body temperature was recorded with a rectal temperature probe and it was measured daily was throughout the duration of experiment (Luam *et al.,* 2014).

Body weight measurement

Body weight of all groups of mice was taken before the commencement of the first oral administration using digital electronic balance. These were considered to be the initial body weight. The body weights of all groups were also taken on the last day of oral administration (before dissection) and these were considered to be the final body weight.

Detection of Bacillus cereus

The faeces of the test animals were collected from transparent plastic dishes placed beneath the individual rat cages daily until 1 week after inoculation to determine the number of rats shedding the pathogen and the faecal counts shed (Aranda and Gianella, 1999). *Bacillus cereus* in each faecal sample was quantified as follows: 1.0 g of faeces was added to 9 ml of physiological water, vortexed and incubated at 37C° for 2 hours, after which the suspension was serially diluted (10^{-1} to 10^{-5}) in physiological water. Each tube is heated in a water bath at 80C°/10mn (heat resistance test).

Aliquots (0.lml) from each dilution were plated in triplicate by the spread-plate method onto Mossel agar. After incubation at 37C° for 8-24 hours. Ten colonies were randomly selected from each plate and confirmed as *Bacillus cereus* by biochemical test.

Animal dissection, organs weight measurement and tissue sampling

Animals of each group were sacrificed at the end of 10 days after body weight of rats were taken one by one on a digital electronic balance while under chloroform anesthesia. Animals laid on a clean paper towel and had all four extremities pinned to thin corkboard. A vertical midline incision with scissors cut from the neck to pubis opened the peritoneum. Then, 3–4mm wide strips of tissue samples were randomly taken from right lobe of liver and coronal section of right kidneys were cut lengthwise with a scalpel through the renal pelvis after each of these organs was weighed with 0.001 precision automatic balance. These tissue samples were taken from each organ and transferred by a blunt forceps to a test tube containing 10% buffered formalin that completely immerses the tissues for the purpose of fixation (Parkinson *and al.,* 2011).

Translocation of Indigenous Bacteria

Control animals, animals infected not treated and animals receiving methanolic extract of *Zygophyllum album* were anesthetized with chloroform and the blood obtained by heart puncture, the blood was transferred in Mossel Agar for detection of *Bacillus cereus*, and incubated for 48h at 37C°.In addition ,the translocation organs were homogenized for 15seconds in normal saline using a Tekmar tissue-mizer [14],one ml portions of the homogenate of each organ of animals groups were plated onto Mossel ,TGEA, BEA, MacKonkey, MRS, VF, agars for detection of *Bacilus cereus*, Total Aerobic Flora, *Enterococcus, Enterobacteria*, *Lactobacilles* and *Candida sp.* Respectively ((Stabb *and al.*,1994).

Statistical analysis

All extractions and determinations were conducted in triplicates and results were expressed on the basis of dry matter weight. Data are expressed as mean ± SD. The means were compared by using the one-way and multivariate analysis of variance (ANOVA). The differences between individual means were deemed to be significant at $p < 0.05$.

Ethics

This work was carried out with respect for the welfare of animals, as recommended by WHO (World Health Organization).

Results and discussions

Extraction yield

Methanolic extract of *Zygophyllum album* has a dark color and a strong odor, with a viscous aspect, it registered a higher yield (25,03±0,1 %), this result is higher than that quoted by Hussein *et al.*,2011. (14,30%); This may be due to the climatic conditions of the plant. The yield depends on the geographical origin of the plant, the season of harvest, method and conditions of the extraction. It is only relative (Benhamou, 2011).

Identification of Phenolic Compounds of Zygophyllum album

The content of phenolic compounds obtained by LC/MS is shown in Figure 1. nine compounds were

identified under the analytical conditions used, of which three constitute the major proportion: Isorhamnetin-3-O-rutinoside (11), Malvidin 3-rhamnoside (9), Quercitin-3-sulphate (6) and kaempferol 3-o-rutinoside (10) (table 1).

Table 1. Phenolic compounds of *Zygophyllum album* extract with LC/MS analysis.

Pic	m/z	Ions	Phenolic compound	Formula	Reference
1	135,2	$[M+H]^+$	N.I	/	
2	157,1	$[M+H]^+$	Gentisic acid 5-O-a-rhamnopyranoside	$C_7H_9O_4$	(Shehab *and al.*, 2015)
3	229,1	$[M-H]^-$	Tomentosin	$C_{10}H_{14}O$	(Quacem, 2015)
4	284,0	$[M-H]^-$	N.I		
5	337,2	$[M+H]^+$	N.I	/	
6	380,8	$[M-H]^-$	Quercetin 3-sulfate	$C_{15}H_{10}O_{10}S$	(Saleh and El-Hadidi, 1977)
7	441,3	$[M+H]^+$	N.I	/	
8	462,4	$[M-H]^-$	Quinovic acid 3-o-rhamnoside	$C_{18}H_{32}O_{16}$	(Duke, 2009 and Hassaean *and al.*, 1993)
9	482,4	$[M+H]^+$	Malvidin 3-rhamnoside	$C_{23}H_{25}O_{11}$	(Ksouri *and al.*, 2013)
10	594,2	$[M-H]^-$	kaempferol 3-o-rutinoside	$C_{27}H_{30}O_{15}$	(Hassanean and Desoky, 1992)
11	623,3	$[M-H]^-$	Isorhamnetin-3-O-rutinoside	$C_{28}H_{32}O_{16}$	(Hussein *and al.*, 2011)
12	809,4	$[M-H]^-$	3-O-[Glucuronic acid pyranosyl]-29-hydroxyoleanolic acid-28-o-[β-D-glucopyranosyl] ester (Zygophyloside K)	$C_{42}H_{66}O_{15}$	(Ksouri *and al.*, 2013)
13	831,6	$[M+H]^+$	N.I	/	
14	859,7	$[M+H]^+$	N.I	/	
15	873,4	$[M-H]^-$	3-O-[β-D-2-O-Sulphonylquinosyl]-quinovic acid-27-O-[β-D-glycopyranosyl] ester (Zygophiliside F)	$C_{42}H_{66}O_{17}S$	Hassaean *and al.*, 1993 and Ksouri *and al.*, 2013)
16	947,4	$[M-H]^-$	N.I	/	/

Table 2. Detection of Bacillus cereus in different organs and blood (-): Absence of *B. cereus*, (+): Presence of *B. cereus*.

organs	Batch	Blood	Liver	Spleen	Lungs	Kidneys	Heart	colon	intestine
Methanolic extract of *Z.album*	curative	-	-	-	-	-	-	-	-
	préventive	-	-	-	-	-	-	-	-
Control		-	-	-	-	-	-	-	-
Untreated infected rats		++	++	++	++	++	++	++	++
ATB		-	-	-	-	-	-	-	-

The negative ion mass spectrum exhibited $[M-H]^-$ at m/z 623.3, which makes it possible to propose the chemical formula $C_{28}H_{32}O_{16}$. Isorhamnetin-3-O-rutinoside is one of the main phenolic compounds in this species; it corresponds to that previously isolated from the Egyptian species of *Z. album*.

This compound appears to be a chemo-taxonomic marker in the genus Zygophyllum (Ksouri *et al.*, 2013).

The flavonoic compounds (9) (m/z 482.4 $C_{23}H_{25}O_{11}$) are temporarily determined in this species for the second time after the study of (Hassanean *et al.*, 1992). The remaining two (6) and (10)), They were already identified and described several times by [20-21]; Furthermore, two compounded triterpenoid saponins (12 and 15) with ions $[MH]^-$ at m/z 809.4 ($C_{42}H_{66}O_{15}$), 873.4 ($C_{42}H_{66}O_{17}S$) were respectively identified in this species, Zygophyloside K (12) Previously isolated and described from *Z. album* and *decumbens* (El Hadidi,1977;Ksouri *and al*,2013 and Hassanean *et al.*,1992) Zygophyloside F (15) was previously described by (Algamal *et al.*, 1995, Hassanean *et al.*,1993 and Hassanean *et al.*, 1992).

Acute toxicity

The dose of the methanolic extract of *Zygophyllum album* (800mg/kg wb) obtained from (or used by the) traditional healer may be considered as relatively safe, as shown by the results of subacute toxicity evaluation (Belmimoun *et al.*, 2016).

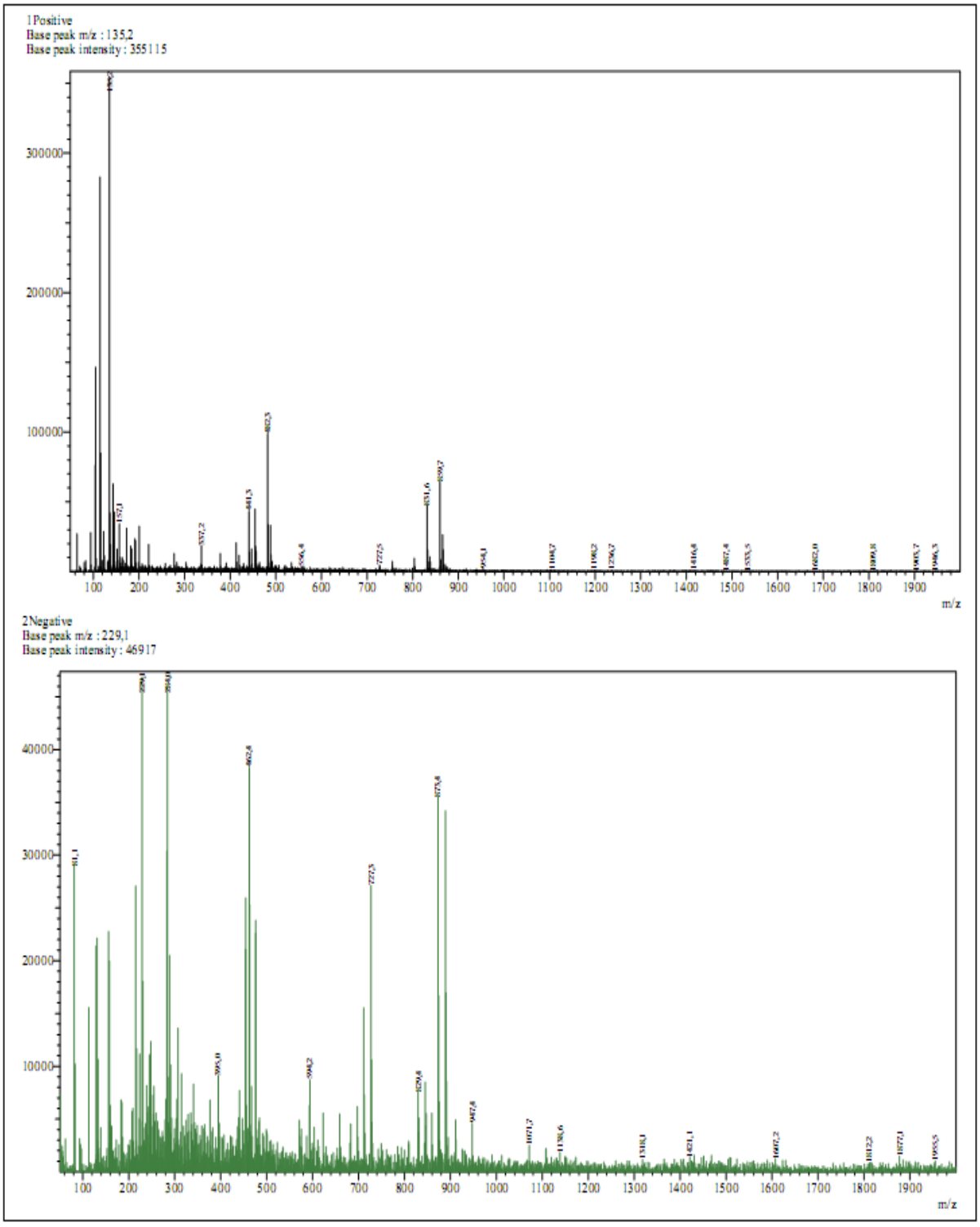

Fig. 1. Chromatogram of the relative content of phenolic compounds in *Zygophyllum album* plant (A : ESI+ ;B :ESI-

Body temperature of Wistar rats

According to the results presented in figure 02, the variability of the marked rectal temperature by an increase after the induction of the *Bacillus cereus* infection compared to the controls, that is to say the 2nd day for the treated and untreated infected rats, and the 8th day for pretreated rats corresponding to 24 hours after induction. This is quite normal as a sign indicating the acute phase of response to this infection (Mac carthy *et al.,* 1995).

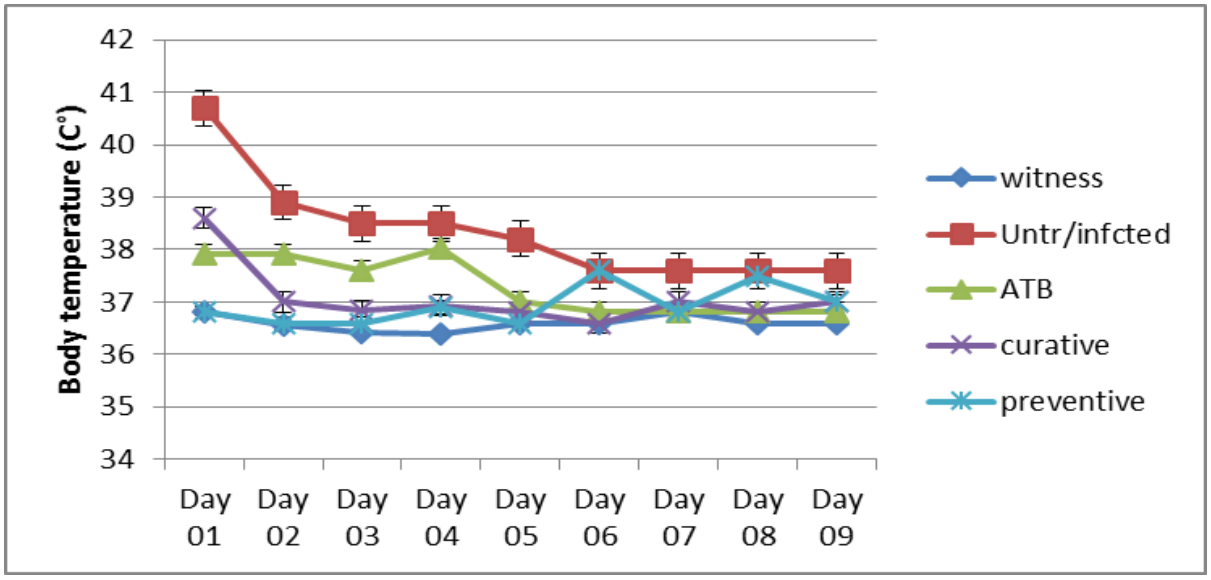

Fig. 2. Recorded daily rectal temperature (P≤0.05).

However, this increase varied from batch to batch by degree and days of persistence, in fact, for untreated infected rats (batch 2) the temperature rose to 39 ± 0, In a study by (Berzou *et al.,* 2013), rats infected with a strain Bacillus sp. Underwent a significant reduction in the production of their gastric acid with ligation of the pylorus.

And that these changes have been associated with fever. For treated batches the increase was minimal (± 1C °), which decreased significantly (p≤0.05) to normal temperature on the 9th day (38.9 ± 0.03C °). The pretreatment was not sufficient to lower the temperature to normal before the end of the experiment (±3°C) difference.

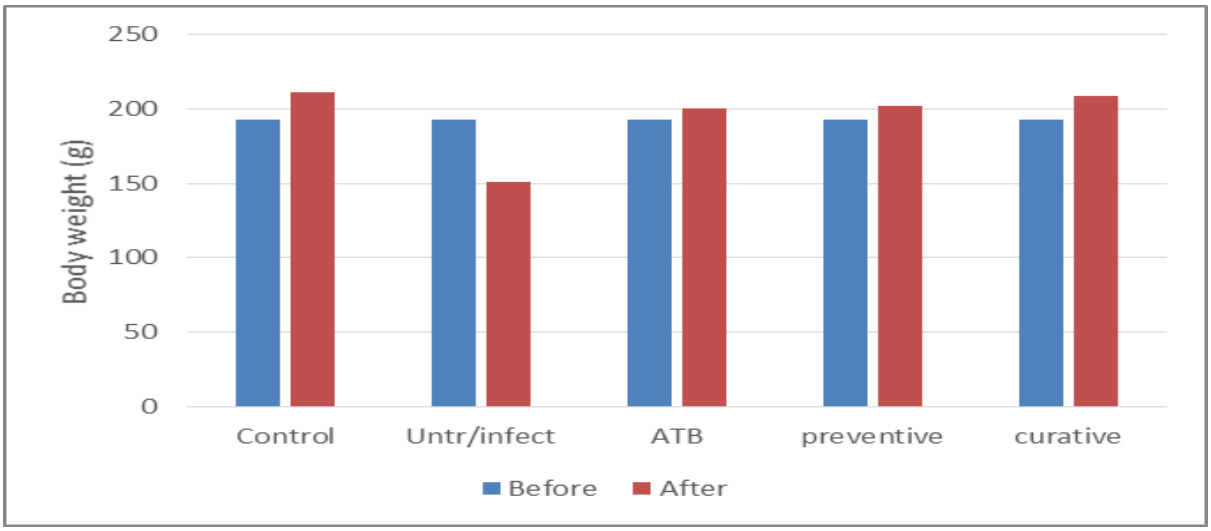

Fig. 3. Body weight of albinos rats before and after treatment with methanolic extract of *Zygophyllum album*.

For the control batches, a slight increase in temperature noted in the control groups (± 0.5C +) probably reflects food-induced thermogenesis (Asche and Butterfield, 1974). The thermoregulation property of essential oils has already been proved by Schnebelen and Goetz, 2007. The results of the Berzou study (2013) also proved the antipyretic effect of aqueous and ethanolic extracts of *Zygophyllum gaetulum*. In general, this power would be linked to the presence of the bioactive compounds in the two extracts (Deeni and Hussain, 1991, Kabore *et al.,* 1988). Nevertheless, the mechanisms of the antipyretic effect are not clearly elucidated (Mac carthy *et al.,* 1995).

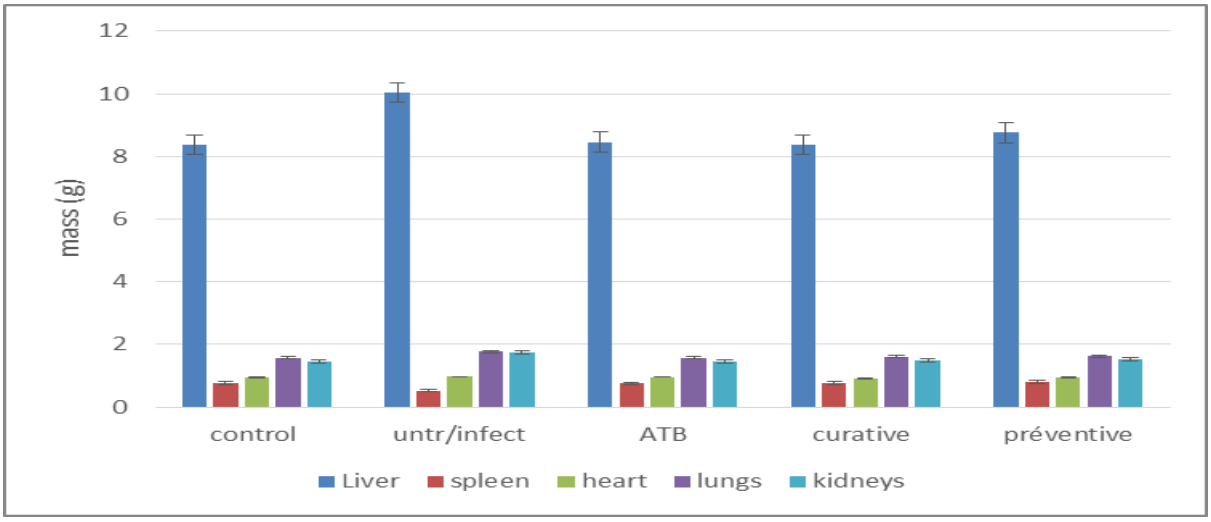

Fig. 4. Relative organs weight (p≤0,05).

Body weight measurement

The body weights of the control rats and those pretreated before infection induction and after 9 days varied (Figure 3). In rats receiving physiological water (batch 1), weight and nutrient intake with a gain of 18.46 g. In contrast, massive and significant weight loss was determined in untreated infected rats (batch 2) with (-41.75g). The administration of ZAM causes the highest weight gain of all treated rats. However, no significant difference was observed in the body evolution of treated rats compared to healthy animals.

The normal evolution of the body mass of the infected animals having received the methanolic extract of *Zygophyllum album* tested testifies the ability of these substances to keep the weight of the rats constant. This ability may be correlated with the attenuation of the infection causing imbalance in the physiological state of the diseased rats (Mac carthy *and al.,* 1995).

In vivo antibacterial activity of methanolic extract of Z. album in rat

In the other hand, the preventive and curative extracts of *Zygophyllum album* employed in this study exhibited significant antimicrobial activity against *Bacillus cereus* inoculated into the albino rats by reducing the concentration of the organism in their faeces to undetectable levels at different days after inoculation but not butter than the positive control which shows an important antibacterial activity against *Bacillus cereus*. Our study was the first approach testing the antibacterial effect *in vivo* of the plant used (*Zygophyllum album*) and the two ways of treatment extracts (Belmimoun *et al.,* 2016)..

Relative weight of internal organs

At the end of treatment, all rats were sacrificed and organs (liver, spleen, kidneys, lungs and heart) were collected, observed and weighed (Figure 4).

In the second batch (untreated infected rats), there was a highly significant increase in the weights of the livers, spleens and lungs compared with the controls, called the hypertrophy phenomenon. These results are in perfect agreement with those found by (Jothy and Lee, 2011) following intestinal infection with *Schistosoma mansoni* inmice.

This may be due to the effect of endotoxins produced by the pathogenic bacterium *Bacillus cereus,* in fact (Rasekh *et al.,* 2008) found that alterations in relative organ mass reflect toxicity after exposure to Toxic substance, heart, liver, kidneys, spleen and lungs are the first organs affected by the metabolic reaction caused by the toxic. The minimal elevations of kidney mass in pretreated batches may be related to congestion by reserving blood in the tissue, (Betti *et al.,* 2012) or by the presence of an inflammatory infiltrate (Merret *et al.,* 1994).

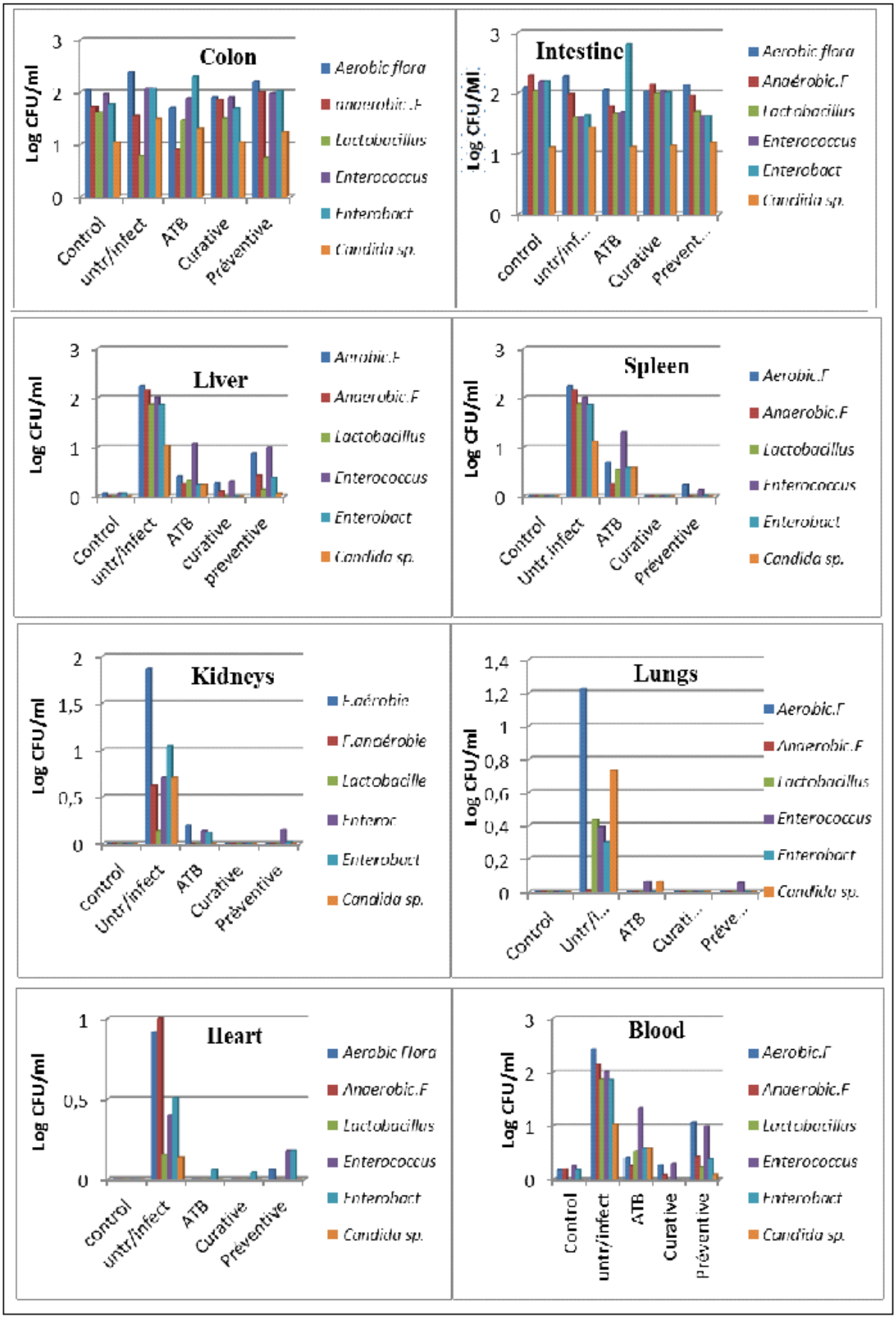

Fig. 5. Bacterial translocation.

Translocation of Indigenous Bacteria

What was first noticed in table 2 was the spread of *Bacillus cereus* in the various organs of all the untreated infected rats, intestinal involvement is sometimes associated with translocations and bacteremia (Plantefève and Bleichner,2001), thus the bacterial translocation of this germ was due to the alteration of the intestinal mucosa (Tir Touil Meddah *and al.*,2001).

Overall, the results show a total absence of bacterial translocation in the different batches treated preventively and curatively.

In batch 2 (untreated infected rats). Bacterial translocation was abundant in various internal organs and blood, which can be explained by *Bacillus cereus* toxins, which can alter the tight junctions of the intestinal epithelium by altering the protein structures and thus increase the para-cellular permeability. His would indicate that the disturbance of the intestinal barrier is a more important factor than the excessive growth of the intestinal microflora in the occurrence of bacterial translocation (Sori and *al.,* 1988).

For rats infected and treated with the antibiotic, a significant increase in enterobacteria was observed especially in the intestine (ileum) and in the colon, this is in perfect agreement with the work of (Joubert, 2015) who confirmed That ATB intake modifies the dominant normal flora by emergence and development of endogenous and exogenous bacteria in the digestive tract naturally resistant to ATB taken as the case of Vancomycin for *E. coli* (INSERM, 2013).

Moreover, the significant decrease ($p \leq 0.05$) in the majority of the rats pretreated with methanolic extract of *Zygophyllum album* of the germs of the resident flora, in particular the lactic acid bacteria and The strict anaerobes in the gastrointestinal tract can be explained by the inhibitory effect of the pathogenic organism on the growth of this flora, indeed (Lima *et al.,* 2015) have shown that if the spores of *Bacillus cereus* are able to germinate In the gastrointestinal tract, which could explain the effect on native intestinal flora, because vegetative cells are known to have antibacterial and antifungal properties. Another interesting study to prove that the spores of *Bacillus cereus* have the power to produce bacteriocins in the small intestine (Duc *et al.,* 2004).

The increase in the rate of enterobacteria and enterococci following infection with *B. cereus* is already demonstrated by another study showing that coliforms were significantly significant in the intestines of piglets fed with probiotic *Bacillus cereus* var Toyoi (Jamadus *et al.,* 2002).

Contrary to this, the methanolic extract of *Zygophyllum album* could not demonstrate any effect on the contamination of the internal organs and even the blood by the pathogenic germ and/or the resident flora by translocation phenomenon.

Conclusion

From the results, it can be concluded that the qualitative and quantitative phytochemical study demonstrated a richness of *Zygophyllum album* in bioactive compounds either in their polyphenolic extracts, which remains promising for the valorization of this plant in the field of phytotherapy.

The overall results of the present *in vivo* study provide basic information for the possible use of the methanolic extract of Zygophyllum *album* in the treatment of intestinal infections, particularly infection caused by *Bacillus cereus*.

In addition, the data reported acute toxicity showed that the extract might be non-toxic.

These observations may justify the traditional use of the plant in the treatment of typhoid fever, whereas *Zygophyllum album* was found to be on up to the dose of 800mg/kg, the margin of the methanol extract of the plants referred to Safety survey and very encouraging for biological evaluation.

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