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# *In Vivo* Antimicrobial Activity of Methanolic Extract of *Zygophyllum album* against *Bacillus cereus*

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## ABSTRACT

The principal objective of our study was to investigate the in vivo anti-*Bacillus* activity and the safety of methanolic extract of *Zygophyllum album (Z. album)*, a plant commonly used in Algeria by traditional healers. The methods use *Bacillus cereus*-infected rat model was used for the study. The physiological, and histopathological markers of possible side effects of this extract were studied using standard methods. The extract had a significant effect on the number of viable *Bacillus cereus* recovered from faeces, and could stop Bacillosis after 8 and 10 days of treatment for male rats, with non-toxic dose. However, the histopathological analyses revealed that at the same dose (800 mg/kg) the curatif technique with methanolic extract could induce better antibacterial effect than the preventif one, so all the overall results indicate that the methanolic extract of *Z. album* has the potential to provide an effective treatment for bacillosis. However, it is necessary to extrapolate these results in large animals, in further studies.
**KEYWORDS :** *Zygophyllum album ; Bacillus cereus* ; infection ; safety.

## INTRODUCTION

They are some works done which aimed at knowing the different antimicrobial and phytochemical constituents of medicinal plants and using them for the treatment of microbial infections (specialy food infection like bacillosis) as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistance [1]. 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.

*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 [2]. This plant used in traditional medicine as a remedy for rheumatism, gout, asthma and as a diureticand 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 [3]. The aim of this study deals with the antibacterial study of the plant as regards their effect *in vivo* and it is the first approch. 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 and intraperitoneal administration.

## 2. MATERIAL AND METHODS

### 2.1. 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.

### 2.2. 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 ºC and

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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.

**2.3. 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).

**2.4. 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 [4]; 180 g of powder divided over cartridge were extracted with 300 ml of dichloromethan 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.

**2.5. Acute toxicity test**

To assess the acute toxic effects of the methanolic extract, a measure of the lethal dose 50 (LD50) is required. Mature male albino rats have an average weight of 190-210± 5g were used which were obtained from the experimental farm of the faculty of sciences of nature and life, University of Mascara, Algeria. Rats are selected according to sex in cages each carries 5 animals. Different doses were used which were estimated to cause 0-100% deaths in the final test (100-400-800-1000-1200 mg / kg). The extract of the plant was suspended in physiological Water 9% of NaCl for 1 g of alcohol extract.

Rats were distributed in 06 groups of 10 animals (05 males and 05 females) who received 1 ml of the single doses of of the extract of *Z. album* and 9 ‰ NaCl (control) by gavage. After the oral administration of the extract, the rats were continuously monitored in the first, sixth and 24th hour after treatment, for any death or change in behavior. Deaths occurring during this period were recorded in 24, 48 and 72 h for each group. The number of dead animals is calculated and converted into a percentage [5].

**2.6. In vivo assay using rats:**

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 ºC), 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 [6].

Bacillosis was induced using the method proposed by Pan, *et al* [7], the rats were fasted overnight in the day before the experiment and given, by gavage, 1 mL of saline solution (0.9% NaCl) containing $1.5 \times 10^8$ 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 *B. 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 18h 24h, it means that treatment was beginning 24 hours after the administration of the germ.

**2.7. 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 [8]. *B. 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 hrs. Ten colonies were randomly selected from each plate and confirmed as *Bacillus cereuss* by biochemical test.

### 2.7. Mortality Rate and Pathological Manifestations

The mortality rate of the rats in the different groups was calculated as numbers of the rats that died during the course of the experiment in relation to all rats used in each group [9]. The animals were observed for consistency of faecal material. The frequency of defecation was noted from the transparent plastic dishes placed beneath the individual rat cages for up to 4 hours. Diarrhea was noted and scored based on consistency, color and the number of defecation. A daily score of water content that was >water content of neutrol group considered proof of diarrhea, while a score that was = or <water content was not. Cages and bedding were changed on a daily basis during collection of faecal samples to avoid cross-contamination. The animals were also observed for any abnormalities and pathological manifestation, and their weight was monitored daily during the period of the experiment [10].

### 2.8. Histopathological analysis:

After sacrificing the animals, small pieces of liver was fixed in 10% formalin, dehydrated in ascending grades of alcohol and cleared in xylene. The fixed tissue were embedded in paraffin wax and sectioned into five micrometres thick with the rotary microtome, then stained with hematoxylin and eosin. Then the sections were examined with light microscope and photographed using a microscopic camera.

### 2.9. 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 $p < 0.05$.

### 2.10. Ethics

This work was carried out with respect for the welfare of animals, as recommended by WHO [11].

## 3. RESULTS AND DISCUSSION

### 3.1. Extraction yield

Methanolic extract of *Z. 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 [12]. (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 [13].

### 3.2. Acute toxicity

After administration of phenolic extract of *Z. album* with gradual doses, observations over a period of 3 days (Table 01) showed no severe clinical symptoms of pain, despite some common signs seen as anorexia, hypoactivity, which are reversible and have appeared in rats for a short time and then they returned to their activity in the first three doses(100-400-800) The absence of mortality and clinical signs therefore indicates that the methanolic extract of *Z. album* devoid of acute toxicity in rats. but for doses of 1000 and 1200 mg/kg,

**Table 01:** Oral toxicity of the total alcoholic extract of *Zygophyllum album* L.f. in adult normal rats showing the number of animals that died during 72 h after oral administration of the extract. Each group consists of 10 rats.

| Mortality rate | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Acute | Delay | 24H | | 48H | | 72H | | | |
| Dose levels ($mg.kg^{-1}$) | No of tested rats | No | % | No | % | No | % | No | % |
| 100 | 10 | - | 0 | - | 0 | - | 0 | - | 0 |
| 400 | 10 | - | 0 | - | 0 | - | 0 | - | 0 |
| 800 | 10 | - | 0 | - | 0 | - | 0 | - | 0 |
| 1000 | 10 | - | 0 | - | 0 | 1 | 10.0 | 1 | 10.0 |
| 1200 | 10 | 1 | 10.0 | 3 | 33.33 | 1 | 16,66 | 5 | 50.0 |
| Total | 50 | 1 | 10.0 | 3 | 33,33 | 2 | 26,66 | 6 | 60.0 |

Moreover, the *in vivo* antibacterial activity revealed that the dose of extract (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. However, the extract may induce slight liver damage at high doses wich is in agreement with [14] and [15] who recorded that, *Zygophyllum microcarpum* was toxic and caused higher mortality in sheep. About 7.5-8.4 g $kg^{-1}$ b.w. of crude powdered plant was found to be toxic to the sheep and caused high

mortality [5]. Subchronic and chronic toxicity studies are necessary to further support the safe use of this plant. It is also necessary to extrapolate these results in large animals.

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

All the rats were found negative for *Bacillus cereus* in faeces before inoculation and treatment with plant extracts and an antibiotic drug (Vancomycin) for the positive control group. The percentage of albino rats that shed *B. cereus* in their faeces after inoculation and treatment with antimicrobial agents was presented in Figure 1.

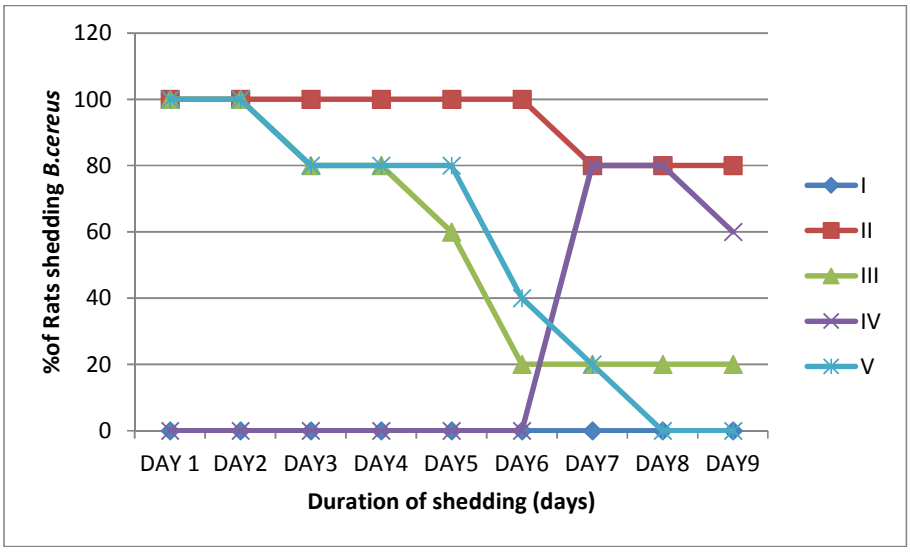


**Figure.1** The Percentage of Rats that Shed *Bacillus cereus* in their Faeces after Inoculation and Treatment with or without Plant Extracts

I = Rat group not infected and not treated, II = Rat group infected but not treated, III =Rat group infected and treated with antibiotic (positive control), IV= Preventif rat group infected and treated with methanolic extract of Z.album, V= Curatif rat group infected and treated with methanolic extract of Z.album.

The percentage faecal shedding of *B. cereus* following inoculation was highly variable among the individual rats, which is in agreement with previous studies [10, 16, 17].

The number of the albino rats shedding *B. subtilis* was higher in the infected non treated group (group I) during some of the sampling periods in this study. Consistent with our observations, other studies have shown that *Bacillus* spores are able to persist and occasionally germinate in the intestinal tract. Three *B. cereus* strains were shown to persist in the mouse GI tract up to 18 days postadministration [18]. Further one animal study showed that the number of spores of *B. cereus* excreted in faeces of mice in some case were higher than the original incoculum [19]. Together with the present study, these studies indicate that spores do occasionally germinate in the nutrient-rich small intestine, and subsequent spore formation may be a good strategy for survival in the more hostile and nutrient-scarce environment of the large gut [20]. Because a recent study shows that vegetative cells of Bacillus sp. are sensitive to simulated gastric fluid with a pH below 4.5 [21], whereas spores of the same strain are resistant to pH 1.5 for several hours.this resistance can be explaind that the spore surface is covered with appendages that may enable spores to adhere to the epithelial surface [22].Thus explaining the continuous shedding of bacillus in the infected non treated rats group throughout the experiment.

In the other hand, the preventif and curatif 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 *B. cereus*. There were differences in percentage of inhibition which the curatif extract were the most inhibitor and another difference in time because the preventif methanolic extract eliminated the pathogenic strain quickly than the curative one because after administration of the pathogenic strain in this group in the day 6 we observed a important diminution since two day after but not completely eliminated, in contrary with the curative group;*Bacillus cereus* was completely eliminated in all rats of group V but in a longer period of time . This difference in time of reaction could be due to the quantity bioactive ingredients present in the plant extract because just after inoculation of *B. cereus* these ingredients were more important in the organism of rats treated prevently than those treated curately. So, it is difficult to juge between the two mode of treatment with the methanolic extract,

Therefore, our study was the first approach testing the antibacterial effect *in vivo* of the plant used (*Z. album*) and the two ways of treatment extracts.

### 3.4. Mortality rate and pathological manifestations

**Table 2:** Mortality Rate/Pathological Manifestations observed in Rat Groups during the Course of the Experiment

| | Number of rats affected / total number of rat in each group | | | | |
|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | **V** |
| Mortality rate | 0/5 | 1/5 | 0/5 | 0/5 | 0/5 |
| Watery diarrhoea | 0/5 | 5/5 | 3/5 | 1/5 | 1/5 |
| Loss of weight | 0/5 | 5/5 | 3/5 | 1/5 | 1/5 |
| Body weakness /slow movement | 0/5 | 5/5 | 3/5 | 1/5 | 0/5 |

The results in Table 2 show the mortality rate and pathological manifestation observed in the different rat groups respectively. Mortality rate in group II and was 20%, while zero mortality rates was recorded among rats of the other groups of the experiment. None of the rat group suffered from bloody diarrhoea. However 100% of the infected non treated group (group II) and 60% and 20% of the infected antibiotic treated group (group III), preventif and curatif group respectively manifested the symptom of watery diarrhea a day after inoculation with *B. cereus* cells (Table 2). It was observed that there was more reduction in the number of rats defecating watery stool over time among the infected and treated groups of rats than the infected not treated and antibiotic treated groups. Thus, the defecation of watery diarrhea by the rats lasted between some hours to1 day in group IV and V and 4 to 5 days in group II and III. In this two last groups; the rats that suffered from diarrhea and abnormalities such as general weakness with slow movement, loss of appetite and loss of weight were observed in them. No pathological changes were observed in other rat groups all through the course of the experiment. Thus all the rats treated in methanolic extract of *Z. album* were protected against diarrhoea that is usually induced by *Bacillus cereus* infection. This suggests that the extracts at a dose of 800 mg per kg of rat body weight suppressed the accumulation of fluid in the intestinal wall of the rats. Previous reports have demonstrated the anti-diarrhoeal activity of tannin [23], flavonoid [24], alkaloids [25], Saponins, sterols, and terpenes [26] containing plant extracts.

Preliminary phytochemical analyses of the plant extract used in this experiment showed the presence of all these compounds. These constituents may be responsible for the anti-diarrheal activity of the plant extracts [10].

### 3.5. Histopathological analysis

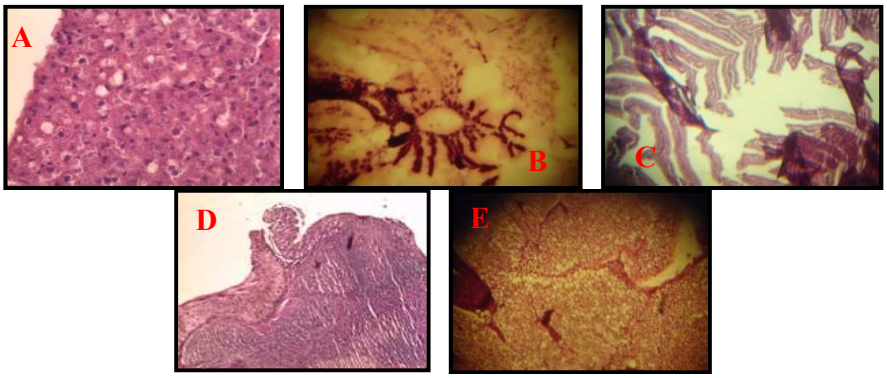


**Figure02:** Histopathological changes in liver of infected rats after the period of treatment (100X)

A: a section of methanolic extract -treated male rat's liver showing excess portal tract infiltration group 4
B: enlarged hepatocytes with numerous vacuoles and dissociated hepatic cords in group 2; C: Slightly inflammatory cells infiltration and vascular congestion in group 3;
D: Intact lobular architecture and normal hepatocytes of neutral control group 1; E: Hepatocytes with well-preserved nuclear to cytoplasmic ratio and very slight inflammation in group 5

The histological architecture of liver sections of the animals treated with doses of 800 mg/kg of methanolic extract of *Z. album* showed injury pattern like significant inflammation of the parenchyma and the portal space, and vascular congestion. This may be explained by the fact that at relatively high doses, the extract had the ability to induce liver damage. Portal tract infiltration by lymphocytes and a focus of dysplasia with atypic cytology were observed in preventif-treated liver of rats(A) while curatif group at the same dose solely caused

mild portal tract infiltration by lymphocytes( B). However, the apoptosis or necrosis of hepatocytes remains one of the major signs of liver damage due to toxic compounds [27], and this was not observed in this study

**Conclusion**

The overall results of the present work provide baseline information for the possible use of the methanolic extract of *Z. album* in the treatment of Bacillosis, especially infection caused by *B. cereus*. In addition to antibacterial activity, the data reported from acute toxicity showed that the extract may be non toxic. These observations can justify the traditional use of the plant in the treatment of typhoid fever so *Z. album* was found to be safe up to dose 800mg/kg ,The safety margin of the methanolic extract of the plants under investigation is highly encouraging the biological evaluation.

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