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Research article

Symphytum asperrimum as anti-septic treatment improves survival in lethal sepsis induced in mice

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ABSTRACT

The leaves and fruits of plant *Symphytum asperrimum SA*. (*Boraginaceae*) in Brazil/ India have been used by native people to treat infectious diseases, diabetes & stomachache. Since the bactericidal activity of *Symphytum asperrimum* has been confirmed *in vitro*, the aim of this work was to evaluate the effect of the prophylactic treatment with *Symphytum asperrimum* in *vivo* polymicrobial infection induced by cecal ligation and puncture (CLP) in mice. C57Bl/6 mice were treated by the subcutaneous route with a hydro alcoholic crude extract (HCE) from fresh leaves of *Symphytum asperrimum* . After 6 h, a bacterial infection was induced in the peritoneum using the lethal CLP model. The prophylactic HCE treatment increased the mice survival, the neutrophil migration to infectious site, the spreading ability and the hydrogen peroxide release, but decreased the serum tumor necrosis factor (TNF) and nitrite. The HCE treatment induced a significant decrease on the bone marrow cells number but did not alter the cell number of the spleen and lymph node. We conclude that the treatment with *Symphytum asperrimum* has a potent prophylactic anti-septic effect that is not associated to a direct microbicidal effect but it is associated to a recruitment of activated neutrophils to the infectious site.

Keywords: sepsis, tumor necrosis factor, cecal ligation and puncture.

INTRODUCTION

Many species have been used in the folk medicine to treat infectious diseases, and some of their anti-microbial activities have been proved. In fact, the anti-microbial activity has been recognized in different species of vegetal families, and this activity is usually due to the presence of secondary metabolites.

ymphytum asperrimum is commonly known in india sweet olive or java plum. This species, from the myrtle family (Boraginaceae), has been used to treat illnesses caused by bacterial, fungal and viral pathogens, ulcers in genitourinary tract caused by Candida albicans, as well as cold, cough, fever and skin problems such as rashes and the mouth, throat and intestines. In India, it has been used, in a mix with honey or milk, to treat diabetes and digestive diseases and the fresh fruits has been taken orally to treat stomachache. Symphytum asperrimum leaf and physical description

The anti-microbial activity of Symphytum asperrimum has been confirmed in-vitro by some authors using bacteria strains However, there are no results about the effect of this species on the in vivo bacterial infection. Based on this, the aim of this work was to evaluate the effect of the prophylactic treatment with a hydroalcoholic extract from fresh leaves of Symphytum asperrimumin the lethal sepsis induced by cecal ligation and puncture in mice [1].

MATERIAL AND METHOD

Male C57Bl/6 mice (10/group), seven to twelve-weeks-old, weighing 20–25 g have been maintained for many generations in the Animal Breeding Unit (sapience bio- analytical research laboratory, Bhopal, India) under standard conditions. The animals were kept in well cross ventilated room at 26 ± 2°C, relative humidity 44– 56%, light and dark cycles of 12 h. The animals had free access to sterilized food and acidified water. All procedures described were reviewed and

approved by the Committee for the purpose of control and supervision of experiments on animal CPCSEA (Ministry of environment & Forests, India).

Plant material

Leaves of *Symphytum asperrimum SA*. (*Boraginaceae*) were collected and identified at the forest of bhedaghat near Jabalpur district, india. The fresh leaves (200 g) were extracted with 1 L of ethanol (70%) and mixed every 8 h during 24 h. The same procedure was repeated four times. After this period the hydro alcoholic crude extract (HCE) was filtered using a cotton funnel and it was concentrated under low pressure. The yield obtained was 5.2% (w/w). Finally, the HCE was dried and the remainder was later lyophilized. For the experiments, the lyophilized dry residue was diluted in an isotonic phosphate buffered saline (PBS) at a concentration of 1 mg/ml. The animals were then weighed to adjust the dose of HCE to 5 and 50 mg/Kg (mg of dried plant material/Kg of body weight). These doses were chosen based in pilot experiments of neutrophil recruitment.

Experimental design

Polymicrobial sepsis was induced using cecal ligation and puncture (CLP) according to previously described methods. Briefly, under deep anesthesia, a laparotomy was performed and the cecum was mobilized and ligated below the cecal valve, punctured 8× with an 18-gauge needle to induce the lethal sepsis. The cecum was replaced into peritoneal cavity and the abdomen was closed in two layers. Saline (0.5 mL/10 g body weight) was given subcutaneously to CLP animals for fluid resuscitation.

The animals were initially divided into 4 groups that were treated by subcutaneous route 6 hours before the CLP induction. In group 1 (Control) the mice received a control vehicle (saline solution). In groups 2 and 3 the mice received the HCE treatment at the doses of 5 (HCE 5) or 50 mg/Kg (HCE 50) respectively. In group 4 (Sham), the cecum was not perforated and the mice were not treated.To evaluate the lifespan the mortality of the animals was recorded every 12 h until the 5th day. The mice which remained alive were followed by one month. In all the subsequent assays, the blood of anesthetized mice was collected 12 h after the CLP, and the animals immediately sacrificed [2].

Blood Glucoses Concentration

The quantification of glucoses was made in peripheral blood using a digital glucometer with specific material. The values obtained represent the concentration of glucoses (mg/dl).

TNF bioassay

The serum TNF was measured by a modification of the standard L929 *in vitro* cytotoxicity assay using actinomycin D-treated target cells as previously described method.

Determination of serum nitrite concentration

Serum nitric oxide levels were determined by the measurement of nitrite and nitrate after enzymatic reduction of nitrate with nitrate reductase, as previously described.

Peritoneal cell harvesting

The peritoneal cell harvesting and the assays to evaluate the spreading, the hydrogen release and the nitric oxide production by peritoneal cells were performed according to previously described methods.

Platelets, spleen, lymph node and bone marrow's cells counting - platelets count and the lymphoid cells quantification were performed according to previously described methods.

Colony forming units (CFU) determination

The mice were killed 12 hours after the CLP. The skin of the abdomen was cut open in the midline after thorough disinfection and without injury to the muscle and the peritoneal cavity was washed with 2 mL of sterile phosphate buffered solution (PBS). Aliquots of serial log dilutions of the peritoneal fluid obtained were plated on Mueller-Hinton agar dishes (Scan Laboratories, Bhopal, India); colony- forming units were counted after overnight incubation at 37°C, and the results were expressed as $\log_{10}$ of the number of colony-forming units per peritoneal cavity.

Histopathology

To evaluate the inflammatory infiltrate to the cecum walls from mice submitted to CLP, 3 animals from each group were killed 12 h after surgery; cecum fragments were removed, fixed in 10% phormol for 24 h, dehydrated in alcohol, and embedded in paraffin. Serial 5-mm sections were stained with hematoxyline-eosin for analysis of the inflammatory response.

Statistical Analysis

Results are expressed as the mean ± standard error of mean (SEM) deviation from 10 animals per group [3].

Statistical evaluation was done by ANOVA test followed by Neuman-Keuls. Mice lifespan was demonstrated using the Kaplan-Meier curve and the log-rank statistical test was applied to compare the curves. Differences were considered significant at P = 0.05 and are represented by an asterisk. All experiments were repeated for at least two times [4].

RESULTS

Effect of prophylactic HCE treatment on survival in CLP-induced sepsis

The CLP induced the death of 80% of the mice until the 24th hour in the control group. However, the prophylactic HCE treatment improved the mice survival when compared to the control group. The survival was followed by one month and that mice which had survive until the 5thday remains alive by all the period. Based on this result, in the rest of experiments, the mice were treated with HCE and killed 12 h after the CLP induction to investigate the mechanisms of protection.

Effect of Symphytum asperrimum HCE treatment on lethal

sepsis induced by CLP. The cecum was perforated 8× with an 18 G needle. The treatment with HCE at the doses of 5 (HCE 5) or 50 mg/Kg (HCE 50) was done 6 hours before the CLP.

The mice survival was observed until the 6th day. The results were expressed as mean ± S.E.M of 10 animal/group.

Effect of prophylactic HCE treatment on the cellular influx to peritoneal cavity induced by CLP The cell recruitment to the peritoneal cavity, constituted mainly by neutrophils, was enhanced in the HCE treated mice when compared to the control group. The treatment with HCE also induced an intense infiltration of inflammatory cells to the cecum walls which was more evident than that observed in the control.

Effect of prophylactic HCE treatment on lymphoid organs cellularity and on platelets counting in mice submitted to CLP

The HCE treatment induced a significant decrease on the bone marrow cells number and in the platelets count in peripheral blood when compared to the control group. However, it did not alter the cell number in both the spleen and the lymph node. that [5].

The CLP induced, *per se*, a decrease in the blood glucose levels after 12 hours, what was observed in the control and in the HCE- treated groups when compared to the sham group (Sham: 94.3 ± 1.5, Control: 46.1 ± 6.7 mg/dL). The HCE prophylactic treatment did not alter the glucose levels when compared to the control group (Control:

46.1 ± 6.7, HCE 5: 44.1 ± 2.9, HCE 50: 36.3

± 5.1).

DISCUSSION

Some in vitro evidences demonstrated that Symphytum asperrimum has a microbicidal activity (2-7). To evaluate the in vivo anti-microbial effect of this species we used the model of CLP which resembles the clinical situation of bowel perforation and mixed bacterial infection of intestinal origin which seems to be the most realistic model of sepsis. The present study demonstrated that the prophylactic treatment with HCE from the leaves of Symphytum asperrimum effectively reduces the mortality of CLP-induced lethal sepsis in mice. However, this effect was not related to a direct anti-microbial effect of the HCE since the CFU was not decreased. In fact, the HCE was used by subcutaneous route, so, it had no direct contact with the bacteria in the peritoneum, what can justify the absence of a microbicidal effect in the first hours post CLP. It is reasonable to suppose that 12 hours post CLP the bacteria were already not controlled in the initial focus. One of the consequences in the sepsis is the hypoglycemia. In fact, we found that CLP induced per se a decrease in the blood glucose levels which was not aggravated by Symphytum asperrimum treatment [7].

CONCLUSION

The results obtained here show that the HCE of the leaves from *Symphytum asperrimum*has a prophylactic effect in the sepsis induced by CLP. This effect is related to an increased migration of neutrophil to the primary infection focus and also to the activation of those cells. These effects could justify the popular use of this plant in the treatment of infectious diseases. Further studies are in progress in order to characterize the bioactive compounds involved in the biological action of *Symphytum asperrimum* has.

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