Medical Journal of the
Islamic Republic of Iran

Volume 17
Number 4
Winter 1382
February 2004

EFFECT OF *AMYGDALUS COMMUNIS* ON GROWTH AND TOXIN PRODUCTION OF *CLOSTRIDIUM DIFFICILE*

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ABSTRACT

It is known that the major etiologic agent of pseudomembranous colitis in man is *Clostridium difficile*. With respect to traditional use of almond paste in the treatment of infantile diarrhea, we studied the effects of the aqueous extract of *Amygdalus communis* (AEAC) on the growth and toxin production of *Clostridium difficile* in culture medium and the rabbit ligated ileal loop.

Three groups of male New Zealand white rabbits (1.5 -2 kg) were used in this study and ligated segments of the small intestine (4 -5cm) were prepared and injected with 1 mL of 24 hour extract culture filtrate, a mixture of vegetative cells of *Clostridium difficile* and different concentrations of AEAC, 1 mL mixture of purified toxins (A and B) and AEAC, and 1mL suspension of bacterium (10000 CFU/mL) alone.

The results of this study revealed that AEAC at a concentration above 80 mg/mL completely inhibited the growth of *Clostridium difficile*. Although concentrations below 80 mg/mL of AEAC did not inhibit bacterial growth, synthesis or excretion of toxins A and B were inhibited. Injection of the mixture of AEAC and toxins A and B into the ligated segments of the small intestine yielded a positive result with no fluid accumulation at a level acceptable for diarrhea in comparison with positive controls ($p<0.01$).

In conclusion, although inoculation of bacterial suspension plus AEAC into the ileum of the animal model prevented colonization, growth, and toxin production, the results varied according to the concentration of both AEAC and number of viable bacteria. Thus, the significance of these results relative to the use of almond paste in prevention of gastrointestinal disease due to *Clostridium difficile*, requires further study.
MJIRI, Vol. 17, No. 4, 337-341, 2004.

Keywords: *Clostridium difficile*, Toxin A and B, Pseudomembranous colitis, *Amygdalus communis*.

INTRODUCTION

Bacterial infection and malnutrition are the two major causes of infant mortality in many undeveloped countries.[1,2] In recent years, some investigators have shown that the prevalence of disease associated with *Clostridium difficile* has increased.[3,4,5] Fifty-two percent of infants are normally colonized by *Clostridium difficile* while 78 percent of this group suffer from disorders affecting all or part of the gastrointestinal tract, such as diarrhea, colitis and pseudomembranous colitis. Mortal-

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ity and morbidity rates of 0-10 percent have been reported.[6,7] Antibiotic resistance has increased the rate of disease.[8,9] *Clostridium difficile* causes 300,000 to 3,000,000 cases of diarrhea and colitis in the United States every year.[10] Biotherapy and nutrition are two important factors influential in this regard.[11,12,13] Use of almond paste in the traditional treatment of infantile diarrhea,[14,15] led us to assess the effects of the aqueous extract of *Amygdalus communis* (AEAD) on *Clostridium difficile* growth and toxigenicity.

MATERIAL AND METHODS

Bacterial strains of a toxigenic variant of *Clostridium difficile* were kindly provided by Dr. N. Moazami (Iranian Research Organization for Science and Biotechnology, Tehran). Partial purified *Clostridium difficile* toxin A was prepared as previously described.[16] Polyclonal antibody against *Clostridium difficile* toxins and sodium nembutal were obtained from Sigma, Brain Heart infusion broth from Difco, New Zealand white rabbits from Pasteur Institute of Iran, ether and ethanol from Merck, Diazepam 5 IU, Gentamicin 40µg, Dextrose 10%, saline solution and povidone iodine 10% from Razi Co, 0.45µm filter from Sartorious, and silk 2-0 and syringes from Supa Co., were obtained.

Preparation of aqueous extraction of *Amygdalus communis*

100 grams of *Amygdalus communis* produced in Isfahan was added to 500 mL of distilled water. After 24 hours, they were shelled. The shelled almonds were disinfected via 70 percent ethanol and washed three times with distilled water. Fifty grams of shelled almonds were pulverized under aseptic condition and 100 mL distilled water was added. The milky aqueous solution was filtered (Watman no.1) and was kept at 4°C. Different concentrations were made. The solutions were dispersed in bottles (each bottle contained 5 mL of solution) then lyophilized and the dry weight of product determined.

The effects of AEAC on growth and toxin production of *Clostridium difficile*

Brain heart infusion broth was prepared according to the manufacturer, dispersed into tubes and autoclaved (121°C / 15psi, 15 min) and different concentrations of AEAC were added to each tube (containing final concentrations of AEAC of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 percent). A series of 12 tubes were prepared; ten of them contained different concentrations of AEAC, and two tubes without AEAC were used as controls. Contamination was checked by preincubation. The tubes were inoculated (100µL of preculture containing 5×10^5 CFU/mL) and anaerobically incubated at 37°C. Afterwards, the rate of bacterial growth and toxin production were determined. The rate of bacterial growth was determined by colony count. In this method serial dilutions were prepared (10^{-1} to 10^{-7}). Three 100mL samples from each dilute was placed on solid medium containing 5 percent agar and anaerobically incubated for 48 hours. Then the colonies were counted.[15]

To determine the effects of AEAC on *Clostridium difficile* [18]
In this method, 12 hours before operation the animal was fed only water. The animals were anesthetized by an intraperitoneal injection of pentobarbital sodium, 35 grams in 1000 mL of distilled water per kilogram weight, and followed by ether. An abdominal incision was done, and 3-5 cm closed ileal loops were formed. One ml of culture filtrate of *Clostridium difficile* was injected in each loop. After 12-24 hours, the animal was sacrificed and loops were removed from the abdomen and fluid accumulation and histopathological changes were assessed.[19]

Clostridium difficile was adjusted so that approximately 10,000 vegetative cells per mL and samples of 10-20 (g/mL semipurified *Clostridium difficile* toxin A which contain 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 percent of AEAC were carried out.

The accumulated fluid in each loop was removed and washed three times with distilled water. Then loops were fixed via 10 percent formaldehyde. Then they were washed three times. The loops were treated by placement in 50, 60, 70, 80, 90, and 100 percent ethanol for one hour respectively to dehydrate the tissues. After that, they were placed in xylene for one hour. Then the blocks were made and thin sections were prepared. Giemsa was used for staining the sections[19] and histopathological changes were determined microscopically.

RESULTS

The *Amygdalus communis* used in this study is shown in Fig 1. The AEAC prepared in this study was a milky

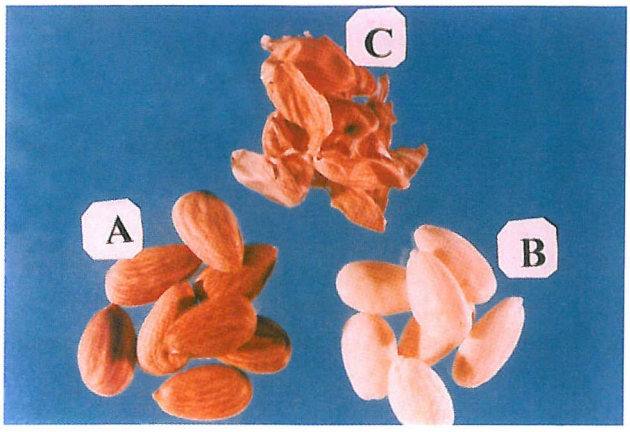

Fig. 1. The almonds. A: Before shelling out. B: After shelling out. C: The shells.

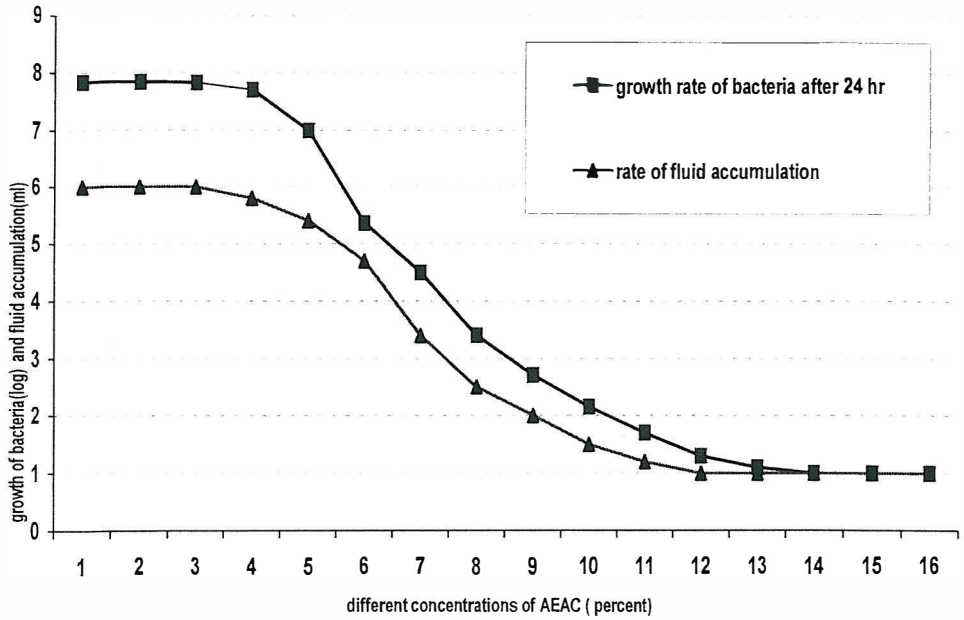

Fig. 2. The effects of different concentrations of AEAC on growth and toxin production from *Clostridium difficile*; growth was determined by colony count and toxin production was measured by the amount of fluid accumulation in rabbit ligated ileal loop and inhibited by specific antibodies.

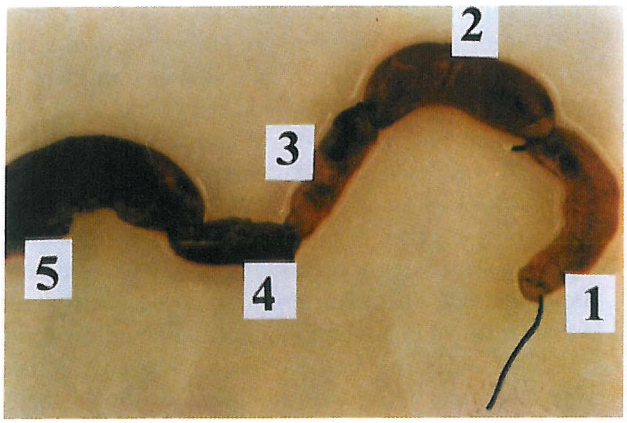

Fig. 3. The effects of 10000 cfu/mL of *Clostridium difficile* containing different concentrations of AEAC on rabbit ligated ileal loop. As shown; loop 1 is control (10 µg toxin A plus antitoxin). Loop 2 was treated by a mixture of 10000 cfu/mL of bacterium and 10 percent of AEAC. Loop 3 was treated by a mixture of 10000 cfu/mL of bacterium and 16 percent of AEAC. Loop 4 was treated by a mixture of 10000 cfu/mL of bacterium and 20 percent of AEAC and loop 5 was treated by 10000 cfu/mL of bacterium alone.

aqueous extract and it's dry weight was calculated as 487 mg/mL.

The effects of different concentrations of AEAC on bacterial growth and toxin production showed that less than 10 percent (48 mg/mL) of AEAC could not inhibit the growth of bacteria or fluid accumulation. Concentrations greater than 10 percent inhibited toxin production and also fluid accumulation (Fig. 2). Increased concentrations of AEAC up to 16 percent (80 mg/mL) inhibited the growth of bacteria and also completely blocked toxin production. As shown in Fig. 2, the higher the AEAC concentration, the lower the bacterial growth and fluid accumulation.

Injection of the suspension of 10000 cfu/mL of bacterium and different concentrations of AEAC in to the rabbit ileal loops showed that concentrations less than 10 percent of AEAC can not inhibit the growth of bacteria and toxin production, based on enumeration of bacteria and fluid accumulation in the ileal loops. Concentrations above 14 percent inhibited fluid accumulation (Fig. 3).

The results of histopathologic examination confirm that samples without AEAC accumulated fluid or bloody fluid. Infiltration of lymphocytes, disrupted mucous membranes and deformation of villi were also seen. AEAC decreased the effect of these symptoms. However an AEAC concentration of 16 percent or greater inhibited histopathological symptoms completely (Fig. 4).

DISCUSSION

In recent years, investigators have confirmed that

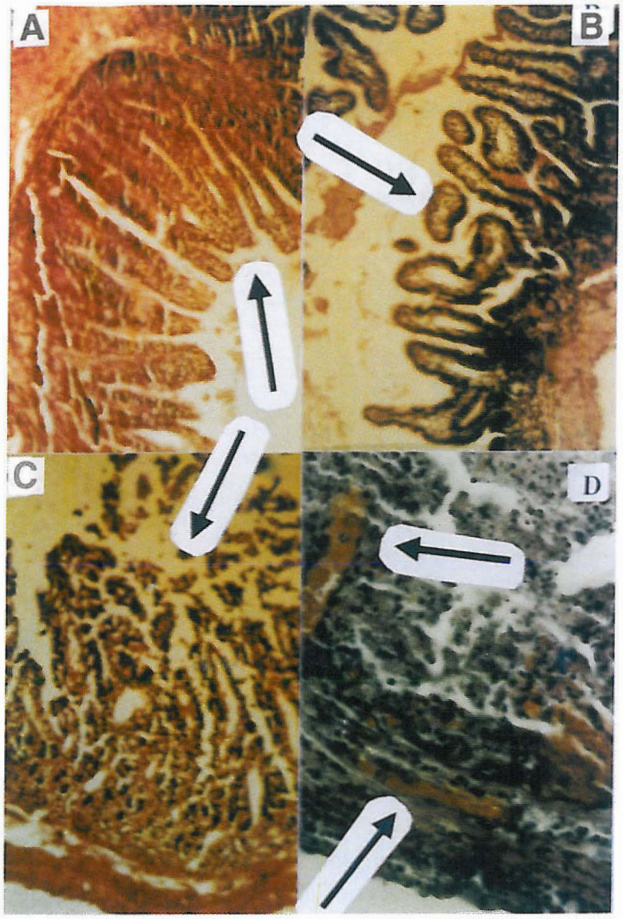

Fig. 4. Histopathological effect of *Clostridium difficile* toxin A on rabbit ligated ileal loop. A: Loop treated with a mixture of 10000 cfu/mL of bacterium and 20 percent AEAC. B: Loop treated with a mixture of 10000 cfu/mL of bacterium and 10 percent AEAC. C: Loop treated with a mixture of 10000 cfu/mL of bacterium and 6 percent AEAC. D: Loop treated by 10000 cfu/mL of bacterium alone.

Clostridium difficile is the leading cause of nosocomially acquired intestinal infection and it has been reported to colonize 21% of hospitalized patients[20] and be responsible for as many as 20% of patients with antibiotic- associated diarrhea.[21,22] In addition, treatment of *Clostridium difficile* has been shown to increase the average length of hospitalization by 2-3 weeks and increase health care costs. However the problem of resistance to antimicrobial agents has now reached global proportions with the pathogens responsible for gastrointestinal tract infections and also malnutrition in children.[23] Thereafter, the most important side effects of antibiotic resistance are diarrhea, colitis, and pseudomembranous colitis in man. Because *Clostridium difficile* pseudomembranous colitis occurs almost exclusively after the use of antibiotics, the most obvious explanation of this association is that antibiotics disrupt the colonic microflora, which normally suppresses *Clostridium difficile*.[24] It appears that use of other methods such as biotherapy[25] and traditional medicine for treatment warrant further research. However, with respect to traditional use of almond paste in the treatment of infantile diarrhea, this study began to investigate the effect of the aqueous extract of *Amygdalus communis* on growth and toxin production of *Clostridium difficile.*

In a separate series of experiments, 1 mL of 24 hours of *Clostridium difficile* culture filtrate that contained different concentrations of AEAC were injected in rabbit ligated ileal loops. The results have shown that samples containing 10 percent of AEAC or less have no effect on bacterial growth and toxin production. Thus, 5-7 mL fluid had accumulated in each ligated loop. Microscopic examination showed histopathological changes such as lymphocyte infiltration, mucous influx and villi disruption [Fig. 3]. Specific antibody against toxin A completely inhibited this episode. These results confirm that AEAC in concentrations less than 10 percent can not obviously affect growth and toxin production of *Clostridium difficile.* But AEAC in concentrations of 16 percent and above inhibited toxin production completely, and bacterial growth was decreased and histopathological examination did not show damage. These findings indicated that concentrations above 16 percent of AEAC can delay bacterial growth but we do not know whether toxin production is inhibited or toxin was produced and inactivated by AEAC.

Although the bacteriostatic or bactericidal activity of AEAC was not described in this experiment, it is obvious that the process of toxin production and it's biological activity are complex. According to this study AEAC not only affects toxin production but also decreases its biological activities. Bacterium exposed to 16 percent AEAC resulted in inhibition of toxin production. Mixing of AEAC and partial purified toxin A was able to inactivate biological activity of the toxin. However, the reason is not clear.

The mechanisms of action of enterotoxin are various.[26] However, the specific receptor was confirmed. The lack of pathological damage in the presence of AEAC may be due to modification of the specific toxin receptors. This is the first time that the protective efficacy of AEAC on the growth and toxin production of *Clostridium difficile* was studied. Perhaps, novel strategies for blocking of bacterial pathogenesis may be required to combat the rise in *Clostridium difficile.* Recent advances in the understanding of the mechanisms of gastrointestinal disease and treatment have focused on the importance of natural substances such as AEAC, which has anti-enterotoxin activities.

In this investigation, AEAC decreased the rate of bac-

terial growth and toxin production. Thus, it may be useful in baby and elderly diets and also may be protective against bacterial gastroenteritis.

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