BIOACTIVE COMPOUNDS FROM CHICOREUS RAMOSUS
ANTIBACTERIAL ACTIVITY - IN VIVO

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INTRODUCTION

Marine invertebrates have been subjected to massive screening for a wide variety of bioactive substances since utilization of marine bio-toxic extracts have been realized to be more powerful than their pharmacologically terrestrial counterparts (Marion et al., 1973). This has further led to careful appraisal and as a result a broad spectrum of antibacterial agents has been isolated from various marine molluscs. This process of isolating the active components involves the initial screening of the tissue extracts for bacteriostatic activity in vitro, as it is considered to be the normal way of assessing the sensitivity of the microorganisms to antibacterial agents. This is followed by testing the efficacy of the extract further in experimental animals (in vivo) to uncover any possible evidence of toxicity, since toxicities of naturally occurring substances are reported to be useful indicators of biological activity (John, 1973). However, the general approach to in vivo testing has appeared pragmatic in the recent past. Despite this practice, potent antibacterial components from the molluscan extracts of Strombus gigas (Queen Conch), Tegula gallina (Top shell), Crassostrea virginica, Crassostrea rhizophora, Mercenaria mercenaria have been tested in vivo (Baslow, 1977). Crude abalone juice (Paolin) found to exhibit antibacterial activity in vitro, has been tested in vivo, in which intra peritoneal injections of 2 mg of Paolin/mouse was reported to lower the death rate of mice infected with S. pyogenes by 25% approximately (Li, 1960).

Extensive screening of the tissue parts of the muricid gastropod C. ramosus revealed that the gonad and hypobranchial gland extracts exhibited significant anti-bacterial activity in vitro (Emerson and Ayyakkannu, 1992). Therefore, an attempt was made to test the active gonad extract of C. ramosus in vivo. Toxicity tests of the hypobranchial gland extract revealed it to be toxic and lethal to mice. Therefore, only the gonad extract that elicited a reasonable degree of efficiency in vitro was examined to determine whether it protects experimentally infected mice, with Staphylococcus aureus, without causing appreciable damage to the host.

MATERIALS AND METHODS

Collection of samples

Samples of C. ramosus were collected either by skin diving or lobster nets at a depth of 15-20 m in Mandapam, Gulf of Mannar region, South-East Coast of India. The collected animals were kept in sea water for one or two days before use. After 2 days, the shells were broken with a hammer and the animal carefully removed without causing any damage to the gonad region. The gonad part was excised from the animals and preserved in alcohol.

Preparation of tissue extracts

For chemotherapy, the ethanol preserved tissues were homogenized in a Waring blender in an equal volume of ethanol and distilled water. The homogenates were filtered. The filtrate was re-extracted with ethanol and distilled water after overnight percolation, and filtered. The filtered solution was centrifuged. The supernatant solution was evaporated to dryness at reduced pressure (40 ± 3°C). The concentrated extract was weighed and used to prepare test solutions.

Preparation of test solutions

For intraperitoneal injections into mice, test solutions of the concentrated extracts (800 mg ml⁻¹) were prepared by emulsifying a known aliquot of the concentrated extract with a 1% saline solution of Tween 80.
Preparation of culture for inoculation

For in vivo evaluation, a potentially pathogenic bacterial culture of *Staphylococcus aureus* was used. Pure stock culture of *Staphylococcus aureus* was transferred to fresh agar slants. After 24 hrs incubation, suspension of microbial cells was made by submerging the agar slant culture in a small volume (4 ml) of sterile saline and gently scraping off the surface of the slant and mixing it with the saline. A microbial inoculum of 0.1 ml of this suspension containing about $10^7$ ml$^{-1}$ bacterial cells was injected into experimental mice to produce infection.

In vivo evaluation of antibacterial assay

Six groups of 5 male Swiss Wistar mice weighing of 20-26 g were used as the test animals for antibacterial chemotherapy tests. Since female mice were reported to be more resistant to bacterial infections than the male (Wheatier and Hurst, 1961) they were not preferred for testing. The challenge dose of the infecting culture ($10^7$ ml$^{-1}$) and the extract (0.1 ml) assumed to be the drug for protection from infection was kept constant. The first set of 5 mice were each injected intraperitoneally with 0.1 ml of extract in Tween 80 (control I); the second set with 0.1 ml of $10^7$ ml$^{-1}$ of *Staphylococcus aureus* culture (control II). Controls were maintained in order to check the side effects of the challenging doses (culture & extract).

In the third set, 0.1 ml of the gonad extract of *Chicoreus ramosus* was mixed with 0.1 ml of the inocula incubated for 1 hour and injected intraperitoneally in order to check the potency of the extract before reaching the physiological system of the mice. In the fourth set, 0.1 ml of the gonad extract was given in advance followed by the culture after 1 hour, in order to measure prevention rather than cure.

In the fifth set, 0.1 ml of the inocula was injected intraperitoneally followed by the gonad extract after 1 day.

In the sixth set, 0.1 ml of the inocula was given intraperitoneally and the therapy was given subcutaneously after one day. These tests were carried out to determine the best therapeutic effect of the extract via two different routes.

All the treated mice were marked with different colour codes for identification and kept in separate cages with feed and water and observed for 20 days.

RESULTS

The various sets of experimental mice injected with *Staphylococcus aureus* and treated with the gonad extract revealed the following results. It was noted that five mice of each set represented 20% of the set’s response. The first control of mice injected with extract in Tween 80 showed 100% survival whereas the second control of mice infected with *Staphylococcus aureus* ($10^7$ cells ml$^{-1}$) showed 60% mortality within 24 hrs. The surviving mice were noticed with heavy damage in the abdomen region near the right hind limb close to the area of infection. They appeared to be very weak and they tended to huddle in groups. The third set of mice injected with a mixture of the extract and the culture, exhibited 100% mortality within 18 hrs. Mice treated with this mixture appeared to be distressed for 4-5 hrs and the activity weakened gradually. The fourth set of mice treated with the extract followed by the infection later to measure prevention of infection did not afford significant result and this set was noted by 80% mortality. Mice treated with culture (infection) followed by the extract after 1 day to determine the therapeutic effect of the extract exhibited appreciable results and this set was noted by 80% survival, but was accompanied by slight damage of the skin in the abdomen region. However, the last set of mice in which infection was given intraperitoneally, followed by extract subcutaneously was identified with 60% survival and marked injury was caused by infection in the abdomen region. The animals inoculated with the bacterial culture were noticed by their ruffled fur and tendency to huddle in groups.
From this results, it was inferred that infection followed by the extract protected the mice to a considerable extent with less injury, than the set administered in two different routes consecutively.

**DISCUSSION**

In search for a chemotherapeutic agent a number of essential properties must be considered: biological activity, selective action, and reasonable dosage of the agent. Since the extracts of the gonad exhibited a high range of activity against gram +ve and gram -ve bacteria *in vitro*, antibiotics against a selected pathogen *Staphylococcus aureus in vivo* has been established. Mice were taken as the test animals for this evaluation as it is considered to be an inexpensive mammal of which a large background of information on infections and therapy is known (Miller, 1971). Bacterial population suspended in saline for inocula was preferred since broth medium was reported to increase the virulence of the microorganisms (Cameron 1957). The number of organisms considered for inoculation was $10^7$ ml$^{-1}$. The lethal dose of *Escherichia coli* was reported to be $10^8$ organisms (Bullen et al., 1968). Animals inoculated with the culture alone (control II) had delayed lethal effects some days after infection when the bacterial count increased to $10^8$ or $10^9$. This can be compared with the fact that animals infected by *Salmonella typhimurium* died 4-5 days after infection (Berry 1955). The lethal effects on mice treated with a mixture infection & extract, and the extract followed by the infection, with little lapse of time, might be due to virulence of these organisms. The concentration of the drug must have altered the host's resistance resulting in fatal infections.

Significant survival rate by the fifth set of mice tested for therapeutic effects indicates the protective nature of the extract. In this test it was inferred that 0.1 ml of the extract per mouse lowers the death rate of the mice considerably. Li (1960) reported that when crude preheated abalone juice was added to liquid cultures of *Staphylococcus aureus*, bactericidal action was observed. Moreover, *in vivo*, injection of two 1 mg doses of the abalone extract given 3 hours before and after the bacterial infection of *S. pyogenes*, B-haemolytic strain has been reported to protect the mice by 25% (Li et al., 1962). However, the therapeutic effect of the gonad extract of *Chicoreus ramosus* which lowered the death rate of the infected mice reveals bacteriostatic efficiency against *Staphylococcus aureus* in live animals. Diffusion of antibiotic agents in the living tissues has been reported to increase the efficiency of the retention mechanism concerned (Bergquist and Bedford, 1978) and may enhance defence against bacterial infections.

It is also noteworthy to mention that administration of the gonad extract after 24 hrs of infection appeared to exhibit this significant result which indirectly reveals the importance of time factor in these tests.

**REFERENCES**


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*Different kinds of fishing crafts, Thailand*