Antibacterial Activity of Methanol Extract of Boal Fish (*Wallago attu*)

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ABSTRACT

In the present study, methanol extract of boal fish (Wallago attu) was tested against four human-pathogenic organisms such as Escherichia coli, Yersinia pestis, Salmonella spp. and Shigella sonnei. Among the four organisms, the highest antimicrobial activity was obtained against Yersinia pestis. Methanol extract of boal fish showed antimicrobial activities against almost all four microorganisms. The MIC values against Escheriachia coli, Yersinia pestis, Salmonella spp. and Shigella sonnei were found to be 16 μgml^{-1}, 8 μgml^{-1}, 64 μgml^{-1} and 16 μgml^{-1}, respectively.

Key words: Wallago attu, Methanol extract, Antibacterial activity.

INTRODUCTION

In spite of modern improvements in chemotherapeutic techniques, infectious diseases are still an increasingly important public health issue (WHO, 2002). It has been estimated that in 2000, at least two million people died from diarrhoeal disease worldwide (WHO, 2002). There is, therefore, still a need for new methods of reducing or eliminating pathogens, possibly in combination with existing methods (Leistner, 1978). Fish by-products are rich in potentially valuable proteins, minerals, enzymes, pigments or flavours (Durand and Lagoin, 1988; Faid et al., 1994, 1997; Cancre et al., 1999; Fouchereau-peron et al., 1999). Use of fish for research on biologically active compounds could be an interesting exercise.

Fish are in an intimate contact with their environment which can contain very high concentrations of bacteria and viruses. Many of these are saprophytic, some are pathogenic and both are capable of digesting and degrading the fish's tissues. However, under normal conditions, the fish can maintain a healthy state by a complex system of innate defense mechanisms. The innate defense mechanisms of fish against infectious bacteria include production of broad-spectrum anti-microbial substances and acute-phase proteins, non-classical complement activation, release of cytokine inflammation and phagocytosis. The nature and mechanisms of many of these defenses are published in many articles (Durand and Lagoin, 1988; Faid et al., 1994, 1997; Cancre et al., 1999; Fouchereau-peron et al., 1999).

The biological interface between fish and their aqueous environment consists of a mucus layer composing of biochemically-diverse secretions from epidermal and epithelial cells (Pickering, 1974; Ellis, 1999). This layer is thought to act as a lubricant (Rosen and Cornford,

1971), to have a mechanical protective function (Cameron and Endean, 1973), to be involved in osmoregulation and locomotion, to play a possible immunological role (Fletcher and Grant, 1969) and to have some function in intra-specific chemical communication (Saglio and Blanc, 1989). Over the past years, it has also been shown that mucus plays a role in the prevention of colonization by parasites, bacteria and fungi (Fletcher, 1978; Ingram, 1980; Austin and MacIntosh, 1988; Fouz et al., 1990; and Lemaitre et al., 1996; Ebran et al., 2000).

The antibacterial role of fish mucus has been known for many years (Fletcher, 1978; Ingram, 1980; Austin and MacIntosh, 1988; Fouz et al., 1990) but previous worked on anti-bacterial tests has been directed towards marine microbial strains. In this study, an aqueous methanol extract of fish species (*Wallago attu*) was screened for its in vitro activity against terrestrial pathogens.

MATERIALS AND METHODS

The extraction of test fish materials: The fish *Wallago attu* (boal) were collected in the month of February 2004 from Municipal Bazar, Kushtia, Bangladesh. Before collection of sample the fish were taxonomically identified by the Bangladesh Fisheries Research Institute (F.R.I), Cox's Bazer, Chittagong. After collection, fish were cut into small pieces, eighty gm of fish was weighed with the electric balance and transferred into 500ml conical flask. Then 400ml of methanol was added into conical flask. The conical flask was closed by foil paper and put in dark place at maximum 7 days. The crude methanol extracts were then filtered by passing the extracts through filter paper. After filtration, the extracts were placed in rotary vacum evaporator to concentrate.

Antibacterial screening: Four gram-negative organisms, (*E. coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei*), were used in the present study to determine the antibacterial activity of the crude extracts. Ten ml of distilled water was taken into the screw-cap tube and pure colony of freshly cultured bacteria was added into the tube and vortexed. The OD (optical density) was measured with the colorimeter and microbial population was confirmed to be within 10^7 ml^{-1} to 10^8 ml^{-1}. This suspension was used as inoculum. Then in vitro anti-bacterial activities of the test samples were carried out by disc diffusion method (Bauer *et al.,* 1966; Barry, 1980). In the disc diffusion method, nutrient agar (High media, India) was used as culture media and the discs were placed aseptically over the bacterial culture on nutrient agar plates and incubated at 37°C for 24 hours. After incubation for 24 hours, the zone of inhibition around the discs was measured by millimeter scale. Discs were impregnated with each treatment and control was assayed on duplicate agar medium plate for *E. coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei*. The antibacterial activities were determined by measuring the diameter of the zone in mm. The experiment was replicated two times to confirm the reproducible results. Sterile, blank paper discs impregnated with only sterile methanol served as negative control each time. Standard Nalidixic acid (30 μg/disc), Penicillin-G (10 IU) and Erythromycine (15μg/disc) were used as positive control for comparison of the antibacterial activity. Minimum Inhibitory Concentration (MIC) value of the extract of the boal fish was determined, following the serial dilution technique according to Reiner (1982).

RESULTS

Methanol extract of boal fish was found to be sensitive to *E. coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei*. Crude methanol extract produced zone of inhibition 6 mm against *E. coli, Salmonella spp.* and *Shigella sonnei*. (**Table-1**). However, it exhibited highest zone of inhibition (9 mm) against *Yersinia pestis* (**Figure-1a**). The MIC values were also determined against all the tested bacteria. The MIC values of methanolic extract were found to be 64μgml^{-1} against *Salmonella spp.* For *Shigella sonnei* and *E. coli,* the MIC values were found to be 16μgml^{-1} (**Table-2**). The MIC was found to be 8μgml^{-1} against *Yersinia pestis* (**Table-2**) Negative control (disc containing only methanol showed no zone against four different organisms (*E. coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei.*). All the positive control showed antibacterial activity against tested bacteria (**Table-1**).

Table-1. Activity of crude methanolic extract of boal fish on *E. coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei.*

Bacteria	Zone of inhibition of Crude (mm) Methanol Extract	Negative control	Positive control; mm		
			NA(30)	PG(10)	E(15)
E. coli	6	+	20	10	30
Yersinia pestis	9	+	10	16	32
Salmonella spp.	6	+	15	10	20
Shigella sonnei	6	+	10	11	25

Table-2. Comparison of minimum inhibitory concentration (MIC) values of methanol extract of Boal fish against *E. coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei.*

Bacteria	Methanol extract of Boal fish (μgml^{-1})									
	512	256	128	64	32	16	8	4	2	0
E. coli	-	-	-	-	-	-	+	+	+	+
Yersinia pestis	-	-	-	-	-	-	-	+	+	+
Salmonella spp.	-	-	-	-	+	+	+	+	+	+
Shigella sonnei	-	-	-	-	-	-	+	+	+	+

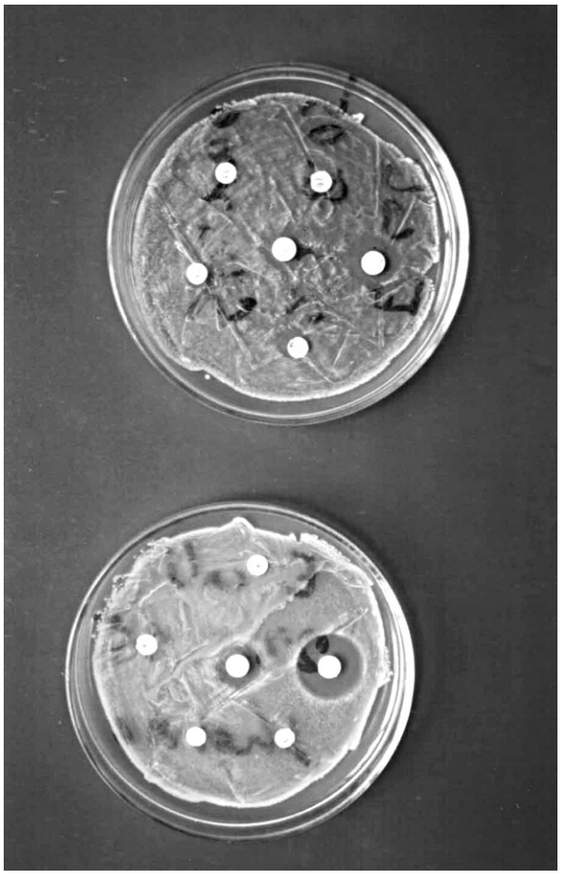

Figure-1(a). Zone of inhibition of methanol extract of boal fish (*Wallago attu*) on *Yersinia pestis* [9mm for crude and 16mm for PG(10)].

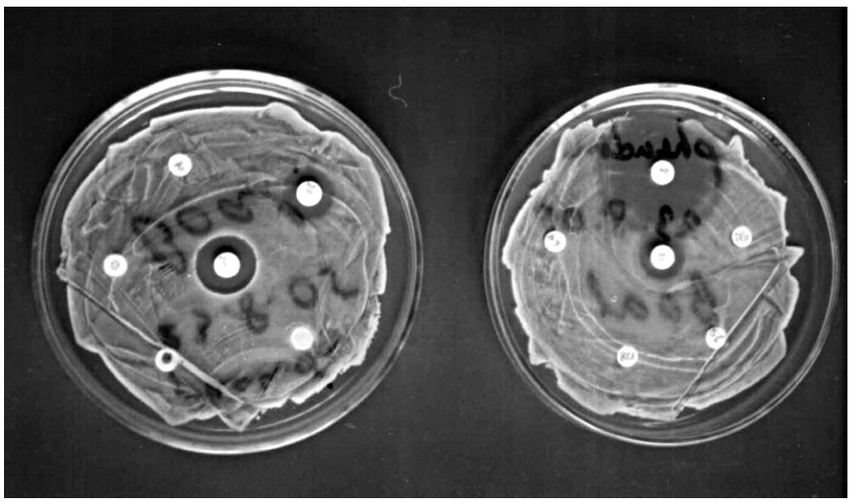

Figure-1(b). Zone of inhibition of methanol extract of boal fish (*Wallago attu*) on *E. coli* [6 mm for crude and 10 mm for PG (10)].

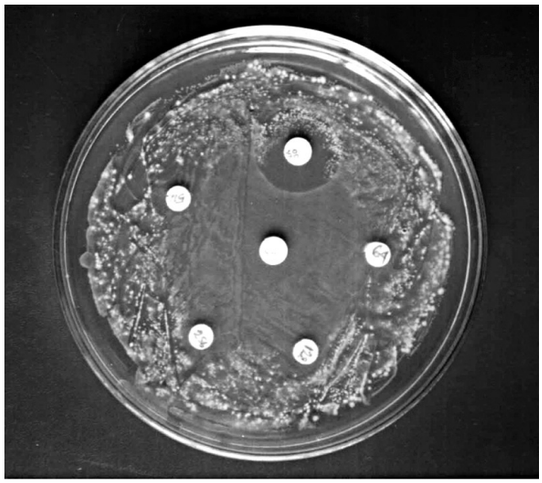

Figure-1(c). Zone of inhibition of methanol extract of boal fish (*Wallago attu*) on *Salmonella spp.* [6 mm for crude and 10 mm for PG (10)].

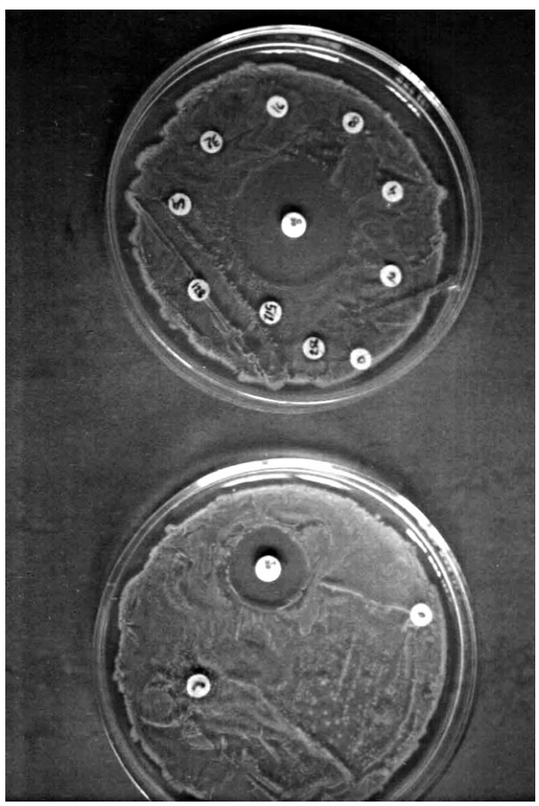

Figure-1(d). Zone of inhibition of methanol extract of boal fish (*Wallago attu*) on *Shigella sonnei* [6 mm for crude and 11 mm for PG (10)].

DISCUSSION

Microorganisms are the concealed enemies of mankind. They are small but cause a very profound damage in human body as well as other living organisms. The agents which have the capacity to kill the microbes or arrest the multiplication are called the antimicrobial agents or drugs. There are a lot of antimicrobial drugs of which some are discovered or established and some are hidden in the nature. Utilization of natural fish biomass offers a wide range of attractive methods for inducing and building protection against diseases. The main objective of this work is to increase the utilization of biomass from fish species in order to isolate new biologically-active compounds.

Fish play a vital role in economy and food habit of the people of Bangladesh. Fish and fishing have been linked to the development of man's earliest civilizations. Annual discards from the world fisheries were estimated to be approximately 20 million metric tones (25%) per year (Hellio et al., 2002). Fish tissues and body fluids contain naturally occurring proteins or glycoproteins of non-immunoglobulin (Ig) nature that react with a diverse array of environmental antigens and may confer an undefined degree of natural immunity to fish. They consist of microbial growth inhibitory compounds that include "acute phase" proteins such as transferrins, caeruloplasmin and metallothionein. Their actions are simply to chelate metal ions and deprive bacteria and other parasites of essential inorganic ion sources. Both serum and cellular interferons are found in fish, and this anti-virus protein has been demonstrated mainly in salmonids during viral disease studies. Enzyme-inhibitors (α2-macroglobulin and other α-globulins) detected so far in fish appeared to be antibacterial proteinases, and involved in the inhibition of extra cellular proteases secreted by fish pathogens (Alexander and Ingram, 1992). Fish also possess a variety of relatively specific lytic molecules that cause cell lysis, and some of these materials are hydrolase enzymes (lysozyme, chitinase, chitobiase) whose main actions are against bacteria and fungi. In addition, mucus contains trypsin-like proteinases which destroy gram-negative bacteria (Alexander and Ingram, 1992). Nonspecific lysins and agglutinins against erythrocytes and other cellular antigens are found in serum, eggs and skin mucus. The lysins, including toxins, some of which are bacteriolytic in activity, are, in their mode of action, natural or spontaneous, antibody-independent and noncomplement-mediated (Alexander and Ingram, 1992). In contrast, specific hemolytic antibodies (Ig) which complex with antigens, bind complement and cause complement-mediated immune lysis were reported to exist (Alexander and Ingram, 1992). Natural lysins and agglutinins behave in a similar way as antigen-induced antibodies or Igs, but exhibit a high degree of cross-reactions due to the occurrence of similar carbohydrate determinants on many types of microbial cell surface. As with mammals, both C-type (calcium-dependent) and S-type (thiol-dependent) lectins are present in fish. They are more resembling invertebrate lectins than those of higher animals. Fish lectins appear to play antibacterial or antifungal roles and in some instances seem to involve in egg-sperm fusion, polyspermy prevention and embryo development. Natural, non-Ig precipitins (e.g., a-precipitin and C-reactive proteins) are found largely, but not exclusively in fish serum and precipitate with simple monosaccharides or long chain polysaccharides of certain stereochemistry and glycosidic linkages. Their functions remain unknown but C-reactive protein is induced following stress-induction and exposure to inflammatory agents (Alexander and Ingram, 1992). Many of the above mentioned "defense" substances are present in skin mucus and possess the capacity to react with potentially- infective microorga-

nisms including parasites. Mucus thus acts as an immediate defense barrier to invasion and/or colonization of pathogens. Hellio et al., (2002) reported that lysozyme isolated from fish was an enzyme with bacteriolytic properties and was ubiquitous in its distribution among living organisms. This enzyme had antiviral, antibacterial and anti-inflammatory properties.

In this study, methanol extract of boal fish showed significant antibacterial activity against *Yersinia pestis* among the tested organisms (*E. coli, Salmonella spp.* and *Shigella sonnei.*)

In a recent study with another fish, two novel antibacterial muramidases were purified to homogeneity from skin exudates of rainbow trout fish (*Oncorhynchus mykiss*) (Fernandes et al., 2004). Unusually, one had an acidic isoelectric point and it was the first anionic muramidase to be reported for fish. Its molecular mass is 14 268 Da, as determined by mass spectrometry. The other muramidase is cationic with a mass of 14 252 Da. Partial N-terminal amino acid sequencing and peptide mapping strongly point to it being a C-type lysozyme, the first to be purified and characterised from skin of a salmonid. Its optimum pH ranged from 4.5 to 5.5 and its optimum temperature, at pH 5.0 was 33–49°C, although it still exhibits activity at 5°C. It is strongly bactericidal to the gram-positive bacterium *Planococcus citreus,* with a minimum bactericidal concentration of 100 U/ml, but is neither chitinolytic nor haemolytic. These two muramidases probably contribute to epithelial defense of the fish against microbes, either alone or in synergism with antibacterial peptides (Fernandes et al., 2004).

In another study, it was reported that the presence of male tilapia (*Tilapia hornorum*) at a biomass not lower than 300 g/m^3 in the green water culture system (or fin-fish–shrimp integrated culture system) efficiently inhibited the growth of luminous bacteria *Vibrio harvey* in shrimp (biomass=80 g/m3) (Eleonor et al., 2004).

Yersinia pestis is the causative agent of plague. Plague is mainly a disease of wild rodents and is spread from animal to animal by fleas.In human, *Yersinia pestis* causes bubonic and pneumanic plague. As the methanol extract of boal fish showed satisfactory antibacterial activity against *Yersinia pestis,* therefore, methanol extract of boal fish could be used in the treatment of plague. This is the first report on bioactivity of methanol extracts of biomass from Wallago *attu* in Bangladesh.

It has been reported that the prevention of colonization by aquatic parasites, bacteria and fungi is mediated both by immune system compounds (IgM, lysozyme, etc.) and by antibacterial peptides and polypeptides. In fish, a layer of mucus contributes to the defense system of fish. Ebran et al., (1999) showed that only the hydrophobic components of crude epidermal mucus of fresh-water and sea-water fish exhibit strong pore-forming properties which were well correlated with antibacterial activity. They have isolated novel glycosylated proteins from the hydrophobic supernatant of tench (*Tinca tinca*), eel (*Anguilla anguilla*) and rainbow trout (*Oncorhynchus mykiss*) mucus. The study of their secondary structure was performed by circular dichroism and revealed structures in random coil and α-helix in the same proportions. When reconstituted in planar lipid bilayer, they induced the formation of ion channels. This pore-forming activity was well correlated with a strong antibacterial activity (minimal inhibitory concentration<1 μM for the three proteins) against both gram-negative and gram-positive bacteria (Ebran et al., 1999). In another report, it was also found that fish secrete antibacterial glycoproteins able to kill bacteria by forming large pores (several hundreds to thousands of pores) in the target membrane (Ebran et al., 2000).

In our study, the MIC values of methanol extract of boal fish against *Escherichia coli, Yersinia pestis, Salmonella spp.* and *Shigella sonnei* were found to be 16 μgml^{-1}, 8 μgml^{-1}, 64 μgml^{-1} and 16μgml^{-1} respectively. More detailed work is needed to improve the extraction procedure of boal fish and to determine its antibacterial activity for further therapeutic development and to provide an additional weapon in the overall strategy of disease control.

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page 332 none