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ISOLATION AND CHARECTERIZATION OF AN ANTIMICROBIAL PROTEIN FROM THE LIVER OF GOAT (*CAPRA HIRCUS*)

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ABSTRACT : Antimicrobial proteins (AMPs) have become recognized as important components of the non specific host defense or innate system in a variety of organisms including bacteria, fungi, plants, insects, birds, amphibians, mammals and crustaceans. In the present study an AMP was isolated from the liver of *CAPRA HIRCUS* (Goat). Protein was isolated from the freshly collected liver tissue. Further acidic, basic and neutral fractions of protein were obtained and their antimicrobial activity was checked against *B. Subtilis, E. Coli, P. Vulgaris* and *Enterobacter*. The protein fractions having antimicrobial activity was subjected to SDS PAGE. The separated fractions were further processed for mass spectroscopy (LCQ-Fleet Mass Spectrophotometer) and their mass was deduced by the spectral data analysis. The amino acid sequence (FGGGMA) was deduced from the peaks obtained in mass spectroscopy which was having mass of 0.8 KD.

Keywords: Antimicrobial proteins (AMPs), liver tissue, *Capra hircus*, SDS PAGE, Mass spectroscopy.

INTRODUCTION

The continuous use of antibiotics has resulted in multi-resistant bacterial strains all over the world. The same is happening with animal-associated pathogens in commercially driven activities, such as aquaculture and confined poultry breeding, where the indiscriminate use of antibiotics is perceived as essential for industries survival. So there is an urgent need to search for alternatives to synthetic antibiotics (Marshall and Arenas, 2003). Anti microbial peptides (AMPs), are the new generation of native peptide molecules, also known as isolated from a full range of organisms and species from bacteria to man, seem to fit this description. They have been termed "natural antibiotics", because they are active against a large spectrum of microorganisms, including bacteria, filamentous fungi, protozoan and metazoan parasites (Li *et al*., 2003; Alberola *et al*., 2004).

Antimicrobial peptides (AMPs) are potent, broad spectrum antibiotics which are potential as novel therapeutic agents. AMPs are demonstrated to kill gram negative and gram positive bacteria, including the strains which are resistant to conventional antibiotics, mycobacteria, enveloped viruses, fungi and even transformed / cancerous cells. It may also have the ability to enhance immunity by functioning as immunomodulators (Moore *et al*., 1994). AMPs are unique and diverse group of molecules, which are divided into subgroups on the basis of their amino acid composition and structures (Sitaram *et al*., 2002). AMPs are generally between seven to fifty amino acids. Major types of AMPs falls into two categories: anionic and cationic peptides. The modes of action by which antimicrobial peptides kill bacteria is varied and includes disrupting membranes, interfering with metabolism, targeting cytoplasmic components, inhibition of cell wall synthesis, alteration of cytoplasmic membrane, activation of autolysin, inhibition of DNA, RNA, and protein synthesis and inhibition of certain enzyme (Brogden, 2005). In general the antimicrobial activity of these peptides is determined by measuring the minimal inhibitory concentration (MIC), which is the lowest concentration of drug that inhibits bacterial growth.

MATERIAL AND METHODS

Chemicals: All chemicals used in the present study were of analytical grade procured from Hi Media Laboratories Pvt. Ltd, Mumbai, S.D. Fine chemicals, Mumbai, India.

Source tissue: Liver tissue of Goat (*Capra hircus*) was obtained from registered local slaughter house, Anand, Gujarat, India. Tissue was brought to laboratory in sterile container at 4° C. Tissue was blotted free of blood and was further processed for biochemical and analytical experimentation.

Isolation and estimation of protein from source tissue: Known amount of liver tissue was treated with 10% TCA (trichloroacetic acid) and homogenized.

Separation of acidic, basic and neutral protein: The different fraction of protein was obtained by treating liver tissue with acid and base (Verma *et al*., 2008).Concentration of protein was estimated at 280nm by using nanodrop (Thermo Scientific). The content of protein was expressed in mg/100mg of fresh tissue weight.

Column chromatography: Purification of total protein sample was done by using Amberlite XAD. The total protein was treated and then eluted with known amount of acetate buffer (pH 7.0).

Column preparation: To remove manufacturing impurities, the XAD-2 resin was solvent extracted sequentially with a number of solvents, before use. The resin was placed in a large soxhlet apparatus and sequentially extracted for 24 hours with each solvent, first with methanol, then with acetone, hexane, and finally with dichloromethane. Then the resin was sequentially extracted for four hours with each solvent, first with hexane, acetone, and finally with methanol. The methanol was then displaced from the resin by numerous rinses with reagent water and then stored under reagent water at cool temperatures. After that the resin was ready to be packed into columns **(**Harwood and Moody, 1989**)**. The flow rate was maintained at 2 ml / min.

Antimicrobial activity of protein fraction: Antimicrobial activity of each fraction was determined by well diffusion method using N-agar. Zone of inhibition (ZOI) were obtained by using agar well diffusion method standardize by National Committee for clinical Laboratory Standards (NCCLS, 2002). Activity of three isolated fractions and total fraction along with antibiotic was checked against all of the four microorganisms. Control antibiotic used was Benzyl Penicillin (6mg/ml). Microorganisms taken for the present experimental were *Bacillus Subtilis* (MTCC 2423), *E. Coli* (MTCC 448), *Enterobacter* (MTCC 7087) and *Proteus Vulgaris* (MTCC 1771). The fraction which showed the antimicrobial activity was further processed for SDS PAGE.

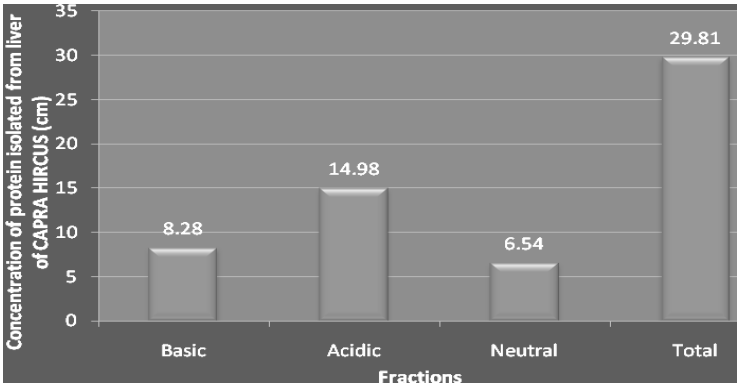
SDS- PAGE for isolated pure fractions: Denaturing (SDS) discontinuous gel electrophoresis was performed (Biorad, Hercules, CA, USA) with the total fraction of protein as well as the isolated fraction of protein (Gallaghar and Wiley, 2008). The protein sample to be analyzed was diluted 1:1 (v/v) with 2X SDS buffer. This was heated 3 to 5 min at 100 ºC in a sealed screw cap micro-centrifuge tube. Sample was loaded in to the well of SDS gel along with the standard marker. Sample was allowed to run at 120mV for 3 hrs. Gel was separated from plates, which was treated by fixatives, staining solution (Commasive Blue) then by destaining solution. Finally Bands are observed under Gel Doc.

Mass Spectroscopy (for molecular weight determination), LCQ FLEET for mass spectroscopy: Mass spectrometry is an analytical tool used for measuring the molecular mass of a sample. The fractions isolated by the column which showed the antimicrobial activity was further processed for amino acid sequencing (Bio-work tools).

RESULTS

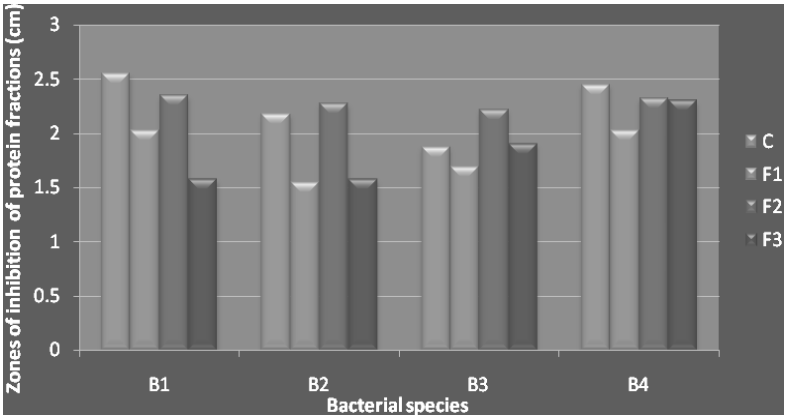
Graph 1, explains the concentration of protein (mg/ml) isolated from liver tissue of *Capra hircus*. Among all, acidic fraction was found to be maximum than that of basic and neutral fractions. Total protein concentration shown is the summation of all the three fractions.

GRAPH 1: EXPLAINS THE CONCENTRATION OF PROTEIN (mg/ml) ISOLATED FROM LIVER TISSUE OF *CAPRA HIRCUS.*

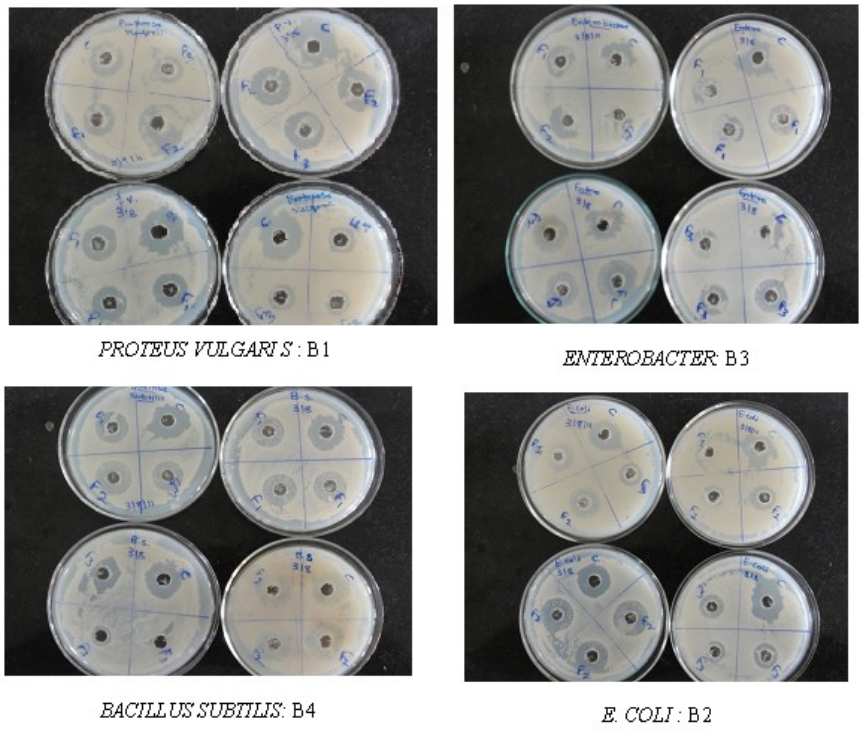


Graph 2, shows the zones of inhibition obtained by all the three protein fractions i.e. basic, acidic and neutral; against *Proteus vulgaris, E. Coli, Enterobacter and Bacillus Subtilis*. Out of all the microorganisms, maximum zone was obtained in acidic fraction. In case of *E. Coli* and *Enterobacter,* zones are significantly higher than standard control antibiotic Benzyl Penicillin (6mg/ml). Zone of inhibition of control was measured in cm. (Plates 1-4).

GRAPH 2: SHOWS THE ZONES OF INHIBITION OBTAINED BY ALL THE THREE PROTEIN FRACTIONS (BASIC: F1**,** ACIDIC: F2 AND NEUTRAL: F3) AND CONTROL (C) AGAINST *PROTEUS VULGARI S* : B1*, E. COLI :* B2*, ENTEROBACTER:* B3 *AND BACILLUS SUBTILIS:* B4.



PLATES 1-4: ZONE OF INHIBITION OF ISOLATED PROTEIN FRACTION (ACIDIC, BASIC AND NEUTRAL) AGAINST FOUR DIFFERENT BACTERIA



PROTEUS VULGARIS : B1

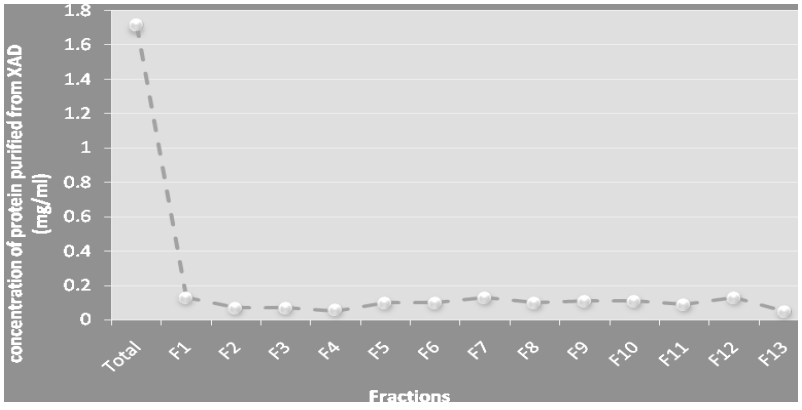
ENTEROBACTER: B3

BACILLUS SUBTILIS: B4

E. COLI : B2

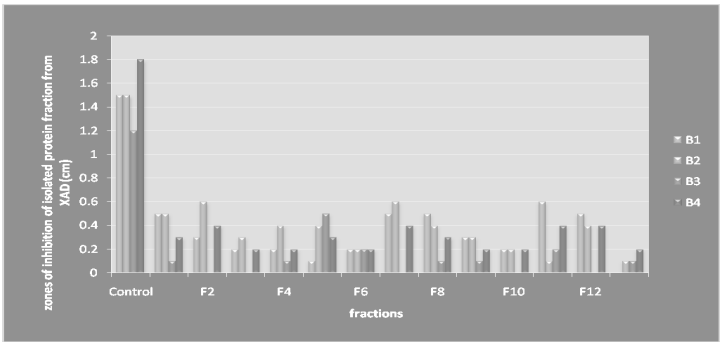
Graph 3, shows concentration of purified protein fractions isolated from XAD column chromatography. Concentration of each protein fraction was measured in mg/ml.

GRAPH 3: SHOWS CONCENTRATION OF PURIFIED PROTEIN FRACTIONS ISOLATED FROM XAD COLUMN CHROMATOGRAPHY.

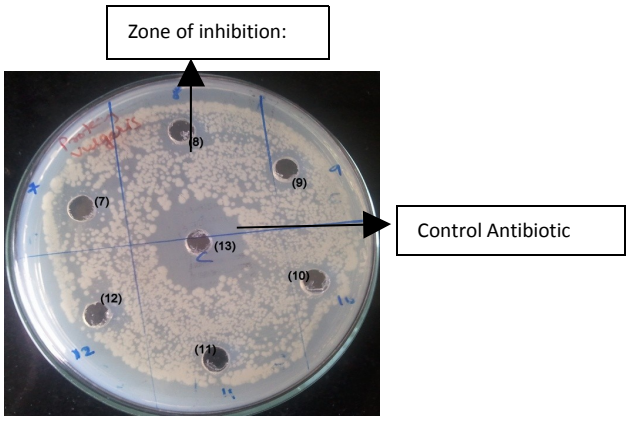


Graph 4, shows zone of inhibition obtained by protein fractions (F1 to F12), separated by XAD column. All the fractions were checked against different microorganisms (*Proteus vulgaris, E.Coli, Enterobacter, Bacillus Subtilis,* fraction (F8) showed the maximal antimicrobial activity. Antimicrobial activity was obtained maximum against *Proteus vulgaris* (Plate 5).

GRAPH 4: SHOWS ZONE OF INHIBITION OBTAINED BY PROTEIN FRACTIONS (F1 TO F12), SEPARATED BY XAD COLUMN. *PROTEUS VULGARI S* : B1*, E. COLI :* B2*, ENTEROBACTER:* B3 *AND BACILLUS SUBTILIS:* B4



PLATES 5: ANTIMICROBIAL ACTIVITY OF FRACTION 8 AGAINST *PROTEUS VULGARIS*.



The isolated purified protein fraction (F8), from column, was further separated by SDS-PAGE to check their purity and molecular weight (Fig 1). The molecular weight was found to be 0.84 KD. Then fraction 8 was processed further for amino acid sequencing by mass spectroscopy (Fig 2).

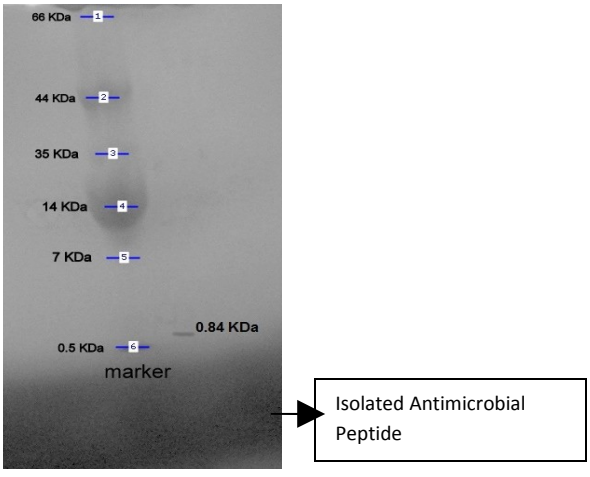


FIG 1: SDS-PAGE OF FRACTION 8

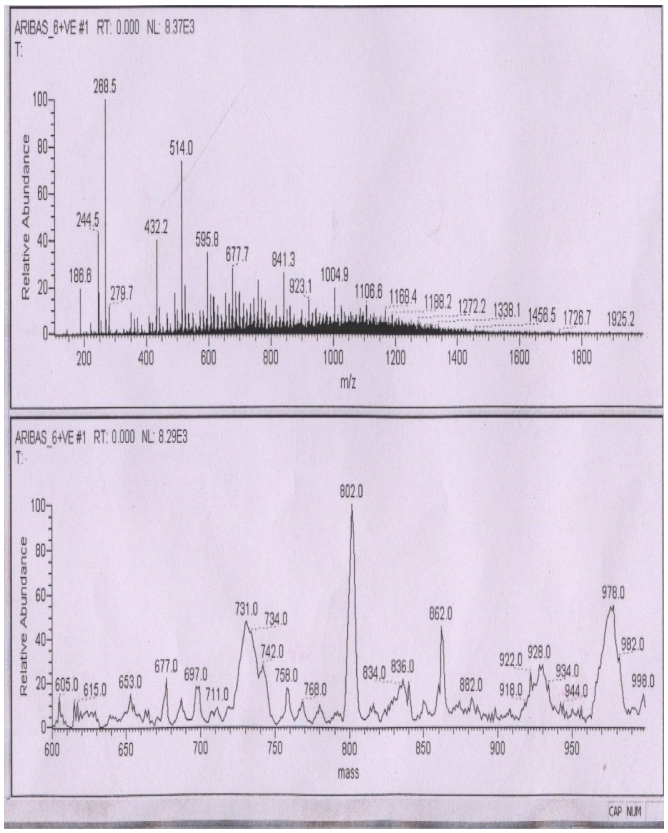


FIG 2: AMINO ACID SEQUENCING BY MASS SPECTROSCOPY- BIOWORK TOOL

Amino acid sequence deduction by mass spectroscopy:

From the peak obtained by mass spectroscopy (Fig 2), using bioworks**,** the amino acid sequence for this fraction was deduced. The deduced molecular weight of the isolated AMP was found to be 0. 80 KD containing a sequence of 6 amino acids. On using FASTA programming, it was found that the amino acid sequence showed 50.0% homolgy with antimicrobial peptide derived from *Lipepia sidoides* and 40.0% similarity with antifungal protein derived from *Cullen corylifolia*.

DISCUSSION

Earlier *Lippia sidoides* (*Ls*) have gained popularity and reputation as effective antimicrobial and anti-inflammatory agents. *Cullien corylifolia* have reported for inhibition of soybean trypsin and having antifungal activity against *R cerealis, A brassicae* and *A niger*, and weak antifungal activity against *F oxysporum*. Dental caries and periodontal disease are associated with oral pathogens. Derivatives from *Lippia sidoides* have been evaluated with respect to their antimicrobial effects against such pathogenic microorganisms. The leaves of *L. sidoides* are also extensively used in popular medicine for the treatment of skin wounds and cuts (Moreira *et al*., 2011).

Whereas, the extract of *Cullen corylifolia* was reported for having antifungal activity. It was also reported for inhibition of soybean trypsin. This extract was found to have antifungal activity against *R. cerealis, A. brassicae* and *A. niger,* and weak antifungal activity against *F. oxysporum* (Jamil *et al*., 2007).

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