Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Asian Journal of Plant Science and Research, 2011, 1 (4):49-55

ISSN : 2249 – 7412

Effect of pharaoh's cuttlefish ink against bacterial pathogens

M. Nithya., V. Ambikapathy.* and A. Panneerselvam.

P.G. and Research Department of Botany and Microbiology, A. V. V. M Sri Pushpam College(Autonomous), Poondi, Thanjavur, Tamilnadu

---

ABSTRACT

The cuttlefish ink extracts was good antibacterial activities. Sepia pharaonis was assayed against selected human pathogens using agar well diffusion technique. Crude ink extract in hexane and column purified ink extract in diethyl ether shows maximum inhibitory effect against Pseudomonas aeruginosa, Staphylococcus epidermidis, Klebsiella pneumoniae. The minimum inhibitory concentration was found to be lower in Escherichia coli and lesser degree of inhibition was observed in the column purified fractions when compared to crude ink extract.

Keywords: *Sepia pharaonis*, ink glands, human pathogens, antibacterial activity.

INTRODUCTION

Indiscriminate use of antimicrobials for disease control in human beings and animals has increased the natural emergence bacterial resistance.[1] Emergence of multiple resistance has greatly limited the effectiveness of most of the antibiotics. It is therefore necessary to search for novel antibacterial compounds with therapeutic potential for which the pathogens may not have resistance.[2] The resistance of the organisms increased due to indiscriminate use of commercial antimicrobial drugs commonly used for the treatment of infectious diseases. Fish farming is a productive venture. Fishes were highly nutritious sources of proteins lysine, methionine, minerals and vitamins. Marine microorganisms encompass a complex and diverse assemblage of life forms and approximately 2500 new metabolites have been reported from a variety of marine organisms.[3] Many studies on bioactive compounds from molluscs exhibiting antibacterial, anti leukemic and antiviral activities have been reported.[4-5] Among the molluscs, the cephalopods are found in all the oceans on Earth and none of them can tolerate fresh water. Cuttlefish are marine animals of the order *Sepiida* belonging to the class Cephalopoda (which also includes Squid, Octopuses, Nautiluses). The presence of antimicrobial activity in molluscs has been reported from the mucus of giant snail *Achatina fulica* and from the egg mass and purple fluid of the sea hare *Dobella auricularia*.[6] Proteins and glycoproteins with antibacterial activity have been demonstrated in the digestive organs of molluscs.[7-8] Crude extract was prepared from the molluscs and fractionated by diethyl ether, hexane, methanol solvents were analysed for

antimicrobial activity using agar well diffusion technique against bacterial pathogens.[9] The Pharaoh's Cuttlefish "*Sepia pharaonis***"** is a large cuttlefish species, growing to 42cm in mantle length and 5kg in weight.[10] The melanin present in the ink sac of the fish is called as Sepiomelanin. Here an attempt was therefore made to explore the antibacterial properties of the ink of *Sepia pharaonis* against selected human pathogens and comparison done with column purified ink.

MATERIALS AND METHODS

Sample- fish collection

Fishes (Pharaoh's Cuttlefish *Sepia pharaonis*) were collected from Anna salai, Cuddalore Dt, in Tamilnadu. Fishes were identified and authenticated at Suganthi Devadason Marine Research Institute, Tuticorin, Tamilnadu.

Bacterial strains

In the present study three gram negative bacterial strains such as *Escherichia coli*, *Citrobacter* sp, *Klebsiella pneumoniae* and two gram-positive bacterial strains i.e., *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were used. The microorganisms were purchased from the Microbial Type Culture Collection(MTCC), Chandigarh, India.

Composition of medium

Tryptone soya agar (soyabean casein digest agar)

Pancreatic digest of casein	-	15.0g
Papaic digest of soyabean meal	-	5.0g
Sodium chloride	-	5.0g
Agar	-	15.0g
p^H	-	7.3±2

Nutrient broth

Beef infusion	-	3.0g
Peptone	-	5.0g
Sodium chloride	-	5.0g
Distilled water	-	1000ml
p^H	-	7.0

Sample – ink gland

Ink gland of pharaoh's cuttlefish *Sepia pharaonis.*

Extracts preparation

Fish (*Sepia pharaonis***)** was washed with sterile water and then dissections were made for removing the ink sacs. The ink was gently squeezed out of the ink sac and air-dried. The air-dried ink sample from approximately 30g ink sacs obtained from 15 animals were immersed separately in solvents like acetone, chloroform, butanol, hexane and coldsteeped for overnight at -18°C. The extracts in each solvents were filtered separately through Whatmann No.1 filter paper. The filtrate was poured in previously weighed petridishes, evaporated to dryness and the dried extract was used for the experiments.

Antibacterial activity assay

Standard agar well diffusion method

Each Tryptone soya agar (TSA) plate was inoculated with the standard inoculum suspension by soaking a swab and rotating it over the agar plate. The 18-24h old cultures were used for the experiments. The antibacterial activity of the sample was assayed by the standard Nathan's agar well diffusion technique.[11]

Drilled wells of 6mm diameter were made. A constant amount of 0.7mg of the dried extract in 50µl solvent was placed onto each well. The well at the center without the extract served as control. After 22-24 hours of incubation at 37°C, zone of inhibition around the well was measured and recorded.

Agar well diffusion- column purified ink extract

Agar well diffusion technique was done with column purified ink extract. Solvents like diethyl ether, hexane and methanol were used in different ratio as 100:0, 80:20, 60:40, 40:60, and 20:80 and then well diffusion method was performed.

Minimum inhibitory concentration (MIC) of crude ink extract

For determination of Minimum Inhibitory Concentration, nutrient broth was prepared. Then crude extract solution was prepared by measuring 2mg/ml. 2ml of extract solution in 2ml of nutrient broth in test tube was taken and shaked well and then 2ml in tube 2 and subsequently transferred in rest of tubes containing 2ml of nutrient broth except the last one that doesn't contain any crude extract solution. It is possible to calculate the concentration of crude extract solution in each tube.

In each tube with one drop of culture were inoculated and incubated at 37°C for 48hours. Turbidity was measured in terms of Optical Density (OD) by using Spectrophotometer. Then table was prepared and graph was plotted between antibiotic concentration and turbidity.[12]

Minimum inhibitory concentration (MIC) of column purified ink extract

For determination of minimum inhibitory concentration, column ink extract was used and the turbidity was measured in terms of OD by spectrophotometer with the solvents like hexane and diethyl ether was used in 0:100, 20:80, 40:60, 60:40 ratios.

Normal phase silica gel column chromatography

Partial purification of the ink extract was carried out following the method given by Wright.[13] After initial screening, the extract obtained with acetone was fractionated using normal phase silica gel column chromatography employing a step gradient solvent system from low to high polarity. The step gradient protocol were used 80 % hexane: 20 % diethyl ether; 60 % hexane: 40 % diethyl ether; 40 % hexane: 60% diethyl ether; 20 % hexane: 80 % diethyl ether; 20 % methanol; 60 % diethyl ether: 40% methanol; 40 % diethyl ether: 60 % methanol; 20 % diethyl ether: 80 % methanol.

The classical preparative chromatography column is a glass tube with a diameter from 50mm and a height of 50cm to 1m with a tap at the bottom. Here dry method was used and the column was first filled with dry stationary phase: Silica Powder (250mg), followed by the addition of mobile phase: Diethyl ether, which is flushed through the column until it is completely wet and from this point is never allowed to run dry. Care must be taken to avoid air bubbles.

A solution of the organic material i.e., acetone extract of crude ink, was pipetted on tip of the stationary phase. This layer was usually topped with a small layer of sand or with cotton or glass wool to protect the shape of the organic layer from the velocity of newly added eluent. Eluent was slowly passed through the column to advance the organic material. Often a spherical eluent reservoir or an eluent tilled stoppered separating funnel was put on top of the column.

The individual components were retained by the stationary phase differently and separate from each other while they are running at different speeds through the column with the eluent. This process was completed within 1 ½ hours. At the end of the column the elute one at a time was collected in a series of fractions.

RESULTS AND DICUSSION

Agar well diffusion method:

Out of the 4 solvents used for the crude extract of the ink of *Sepia pharaonis*, the maximum inhibition activities in the *Pseudomonas aeruginosa* (10mm) with chloroform solvent followed by *Staphylococcus epidermidis* (9mm) with the solvent of hexane. The minimum inhibitory activities of ink with respective fish *Sepia pharaonis* with acetone extracts of *K.pneumoniae* (2.5mm) was recorded. (Table: 1)

Agar well diffusion– column purified ink extract:

The various concentrations of Hexane and Diethyl ether fractions had prominent activity against *Staphylococcus epidermidis* and *E.coli* and no activity in *Klebsiella pneumoniae.* Then 100% Diethyl ether column purified fractions had prominent activity (6.5mm) against *Klebsiella pneumoniae* followed by *Staphylococcus epidermidis* (5mm), *Escherichia coli* (3.5). (Table: 2).

MIC of crude ink extract:

The Minimum Inhibitory Concentration (MIC) of Chloroform fractions found to be 0.52 OD of *Escherichia coli* followed by *Staphylococcus epidermidis*(0.51OD), *Pseudomonas aeruginosa* (0.46OD), *Klebsiella pneumoniae* (0.31OD). (Table: 3).

MIC of column purified ink extract

The MIC of various concentration of Hexane: Di ethyl ether (0:100, 20:80, 40 :60, 60 :40) fractions found to be 2.5, 2.75, 2.75, 3.0 OD value of *K.pneumoniae*, followed by *S.epidermidis* (3.0,3.0,-,-) and *S.pneumoniae* (1,1.25,1.5,1.5). (Table: 4).

Normal phase silica gell column chromatography:

During the entire chromatography process the eluent was collected in a series of fractions. The composition of the eluent flow can be monitored and each fraction was analysed for dissolved compounds.

Table: 1. Well diffusion method-Antibacterial activity of the different solvents of crude ink extracts of *Sepia pharaonis* against human pathogens

Pathogens	Zone of inhibition(mm)			
	Solvents used			
	Acetone	Chloroform	Hexane	Butanol
Pseudomonas aeruginosa	8	10	10	7.4
Staphylococcus epidermidis	5.2	10	9.3	2.5
Escherichia coli	8	10	10	6.5
***Citrobacter* sp.**	4.5	3.5	7	3.2
Klebsiella pneumoniae	2.5	5	6.5	5.4

Table: 2. Well diffusion method – Column purified ink extract

Pathogens	Hexane: Diethyl ether				
	Zone of inhibition(mm)				
	100:0	80:20	60:40	40:60	20:80
Klebsiella pneumoniae	-	-	-	-	-
Staphylococcus epidermidis	0.5	1.5	2.0	3.0	3.5
Escherichia coli	1.0	1.0	1.0	1.5	3.0
Pathogens	**Methanol : Diethyl ether**				
	100:0	80:20	60:40	40:60	20:80
Klebsiella pneumoniae	6.5	-	-	-	-
Staphylococcus epidermidis	5.0	-	-	-	-
Escherichia coli	3.5	1.5	2.0	2.5	-

Table: 3. MIC of crude ink extract (in OD)

Concentration	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus epidermidis*	*Klebsiella pneumoniae*
10^{-1}	0.2	0.20	0.24	0.25
10^{-2}	0.48	0.21	0.42	0.29
10^{-3}	0.52	0.46	0.51	0.31
10^{-4}	0.84	0.63	0.82	0.46
10^{-5}	1	0.90	1.33	0.59
Control	0	0	0	0

Table: 4. MIC of the Column-purified ink extracts (in OD)

Pathogens	Hexane : Diethyl ether			
	0:100	20:80	40:60	60:40
Klebsiella pneumoniae	250	275	275	300
Staphylococcus epidermidis	300	300	-	-
Escherichia coli	100	125	125	150

Sepiomelanin has ca^{++} and mg^{++} salts. In addition tyrosinase, dopachrome, tautomerase, dopamine Dopa and some amino acids glutamic acid, aspartic acid, taurine, lysine, alanine were present.[14]

The ink showed significant inhibitory effect on the development of the embryos. The antitumour activity of the different peptidoglycon fractions of *Sepia pharaonis* ink in Dalton's Lymphoma Ascites (DCA) bearing mice showed increased activity with increasing purification.[15]

The protective and therapeutic effects of cuttlefish ink on hemopoiesis in ^{60} co γ radiated model female ICR mice were investigated[16]

The antibacterial activity of the samples of *Sepia pharaonis* was assayed by the Standard agar well diffusion technique against the test strains on tryptone soya agar. Compared to solvents, such as hexane with a maximum inhibition of 9mmfor *Staphylococcus epidermidis.* All other extracts showed lower antibacterial activity. Generally lesser degree of inhibition by the Column-fractionated extracts in comparison to the crude may be due to degradation or modification on the active components during the fractionation process[17] and generally lesser degree of inhibition by the column fractionated extracts in comparison of the active components during the fractionation process. Among the various concentration of Hexane: Diethyl ether 40:60 had prominent MIC of *Klebsiella pneumoniae*(2.75), *Staphylococcus epidermidis*(-) *Streptococcus pneumoniae*(1.25). The differences in antibacterial activity found in the molluscan extracts may depend on the solvents used for extraction and the compounds extracted.[18]

The structure of the Ascessary Nidamental Gland (ANG) of the ripe female cuttlefish *Sepia pharaonis* has been investigated by using transmission electron microscopy. The gland has certain structural features of the secretary organ containing a number of tubules filled with dense populated. During the maturity phase of the animals ANGs exhibited antibacterial activities.[19] In the present investigation also establish the minimum inhibitory concentrations (MIC) of an antibiotic which may inhibit the growth of the particular bacterium.

Patterson Edward and Murugan (2000) and Patil *et al* (2001) reported that the ink extracts showed antibacterial activity. In the present study the ink extracts showed maximum antibacterial activity against *Pseudomonas aeruginosa, Staphylococcus epidermidis,* and *E.coli.*

Chromatography technique was studied for the separation of mixtures. This mixture dissolved in a mobile phase which separate from each and every molecules in differential retention on the changing the separation. In the present work was carried out the different solvents employed with step gradient solvent system from low to high polarity. A solution of the organic material of crude ink was pipetted on tip of the stationary phase. At the end of the column the elute one at a time and it was collected in a series of fractions. The fractions contains rich amount of sepiomelanin, dopamine and L-dopa against different human pathogens.[21]

REFERENCES

[1] Allsop, A.E., **1998**. *Curr opinions*. *Microbiol.* 1:530-534.
[2] Patil, R., Jeyasekaran, G., shanmugam, S.A., and Jeyashakila, R., **2001**. *Indian J Marine. Sci* 30: 264-267.
[3] Ireland, C., Copp, B., Foster, M., Mc Donald, L., Radisky, D., and SwerseyJ.,**1993**. *Marine Biotechnol*. 1:1-43.
[4] Faulkner, D.J., and Ghiselin M.T., **1983**. *Marine Ecol.prog*. series13:295-301.
[5] Rajaganapathy, J., S.P. Thyagarajan and J.K.P.Edward., **2000**. *J. Exp. Biol.*, 38: 519-520
[6] Ijima, R., J. Kisugi and M.Yamazaki., **2003**. *Dev. Comp. Immunol*, 27:305-311.
[7] Iguchi, S.M.M., T.Aikawa and J.J. Malsumoto., **1982**. *Comp Biochem. Physicol*., 72:571-574.
[8] Pakrashi, A., **2001**. *Indian J. Physicol.* Pharmacol., 45: 249-252.
[9] Shanthi, V., Sivakumar, V., Thilaga, R.D., Jamima Florence Borgia, V., **2011**. *Global J Pharmacology* 5(3) 130 -135.
[10] Sheu T & Chou C (**1990**) *J.Chinese Agri soc* 28:59-68.
[11] Perez, C., Paul, M., Bazerque, P., **2010**. *Acta Bio Med Exp*, 15, 113-115.
[12] Dubey, R.C and D.K. Maheshwari., **2005**. Determination of minimum inhibitory concentration (MIC) of an Antibiotic. *Textbook of Microbiology*. 2nd ed. S.Chand &Co.Ltd, Ram nagar. New Delhi. Pp.175.
[13] Wright A.E (**1998**) Isolation of marine natural products. In: Methods in biotechnology, vol4: Natural products isolation (Canell R) Human press inc, New Jersey, USA, Pp 365-408
[14] Sherief P M, Senan V P, George M C, Nair J R, Devika P and Joseph S M., **2004**. Antitumour activity of peptidoglycon isolated from the ink of the cuttlefish *Sepia pharaonis* Ehrenberg papaer presented to the biology in Asia International Conference, Singapore, Abstract, P. 93.
[15] Lei M, Wang J, Wang Y, Pang L, Wang Y, Xu W, and Xue C., **2007**. *Asia Pac. J. Clin.Nutr*, 16(1) 239-243.
[16] Charles., D.Derby., **2007**. Marine mollusks avoid predators through a rich array of chemicals and mechanisms. Escape by inking and secreting. P. 1121-1135.

[17] Annamalai. N., R. Anburaj, S. Jayalakshmi and R.Thavasi., **2007**. *Research J. Microbiol*., 2 : 978-987.
[18] Cannel, R.J.P., **1998**. How to approach the isolation of a natural product. In : Methods in Microbiology. Vol: 4. Natural products isolation (Canell, R.J.P ed.) Human press. New Jersey .A. Pp.1-51.
[19] Ehrenberg., **1982**. Animalia invertebrate exclusis insects. Symbiosis physicae. Seu 1 cones at descriptions corporum nauralium novorum aut cognitorum. Vol: 4. Pp. 1-14.
[20] Patterson Edward, and Murugan, **2000**. *Indian J Marine*. *Sci* 26, Pp. 206-208.
[21] Still, W.C., Kahn, M., Mitra, A.J., **1998**. *Org. Chem.* 43(14). 2923-2925.