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PRO-ANGIOGENIC PROPERTY IN SELECTED CEPHALOPOD EXTRACTS

Veena Desai and Gayathri N.*

Department of Zoology, the D.G. Ruparel College of Arts, Science and Commerce, Mumbai-16.

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***Corresponding Author**
Gayathri N.
Department of Zoology, the D.G. Ruparel College of Arts, Science and Commerce, Mumbai-16.

ABSTRACT

Marine chemicals often possess unique structures which confer significant biological activity and novel pharmacology. Molluscs are one of the phyla of invertebrate organisms which are known for their biological activities. This study was carried out to compare the pro-angiogenic activities of the cephalopods - *Cistopus indicus, Loligo duvauceli, Sepia aculeata and Sepiella inermis.* The digestive system of these organisms, including the digestive glands was isolated and processed for extraction in four different solvents viz, methanol, chloroform: methanol, n-hexane and distilled water. Estimation of protein, carbohydrates and lipids were carried out. Comparison of the activity of extracts on the angiogenesis was done using CAM assay. The highest pro-angiogenic activity was noted in the chloroform: methanol extract of the digestive system of *Loligo duvauceli.* Anti-angiogenic activity was noted in the aqueous extract of the same organism. The authors thank Department of Biotechnology, Government of India for the financial grants provided to carry out this work.

KEYWORDS: Bioactivity, Chick chorioallantoic membrane, Octopus, Squid.

INTRODUCTION

Bioprospecting of marine organisms has led to the discovery of several chemically diverse compounds. More than 25,000 novel compounds have been extracted and purified from marine organisms and more are under investigation.[1] Molluscs manifest interesting pharmacological activities with unique chemical structures. Less than 1% of molluscs have been studied for secondary metabolites and the quest for bioactive components in mollusc extracts are gaining momentum.[2] Molluscs are known for their secondary metabolites

exhibiting anticancer, cytotoxic and anti-tumor activities. Over the past decades, many molluscs species, especially gastropods, bivalves and cephalopods have been reported containing bioactive compounds. Cephalopods comprises of a major group under the phylum mollusca and are very good sources of bioactive compounds and have been used in medicine traditionally. Some of the compounds extracted, isolated & purified from cephalopods include Tetrodoxin from *Octopus maculosus,* SE-Cephalotoxin from *Sepia esculenta,* Octopamine from *Octopus vulgaris* etc.[3] Digestive system is primary organs in cephalopods. It is important in secretion of digestive enzymes and absorption. In this study, the effect of the extracts of the digestive system of common cephalopods on neovascularization is explored. The pro-angiogenic potential of the digestive gland extracts of cephalopods is investigated by *in-vivo* CAM assay.

MATERIALS AND METHODS

Cephalopods *Cistopus indicus* (Old woman octopus), *Loligo duvauceli* / *Uroteuthis duvauceli* (Indian squid), *Sepiella inermis* (Spineless cuttlefish) and *Sepia aculeata* (Needle cuttlefish) were procured fresh from the fish landing centres at Sassoon Dock, Mumbai. Identification was done using keys in FAO Species Catalogue, confirmed with Fishery Survey of India, Mumbai and molecular identification was done by DNA barcoding. They were dissected immediately and the digestive system was isolated, pooled individually and extracted in four solvents. Extraction was done in methanol, chloroform: methanol (2:1), n-hexane & distilled water.[4-7] The extracts were evaporated using rotary evaporator and the crude extract was obtained. Biochemical estimation of carbohydrates, proteins and lipids was carried out by standard methods.[8-10]

Chorio-allontoic Membrane (CAM) assay

Five days old fertilized eggs were obtained from Central Poultry Developmental Organization, Mumbai. The eggs were candled and window of size 1 sq.cm was cut aseptically. Sterile discs were used to apply the samples on the CAM vessel. Window was sealed with glass coverslip. Ten eggs were treated with each of the samples. The eggs were incubated at 37ºC and observed after 24 and 48 hours. The number of blood vessels branching out in the control and treated eggs were counted to determine percentage increase / decrease in angiogenesis. The length of the branches was measured to quantify the percentage increase.[11,12]

RESULTS

The aqueous extract of *S. aculeata* contained 0.769 mg/ml protein, 0.012 mg/ml carbohydrates and 61.5% lipids. Maximum carbohydrate content was estimated to be 0.017 mg/ml in the extract of *S.inermis*. Chloroform: methanol extract of *C. indicus* contained 46.5% of lipid and 0.716 mg/ml of protein. The highest protein content of 0.506 mg/ml in methanol extract was found in *C. indicus*. The carbohydrate content was 0.07 mg/ml in methanol extract of *C. indicus.* The lipid content was 39.5% in methanol extract of *L.duvauceli.* Highest protein content was 0.054 mg/ml in hexane extract of *S.inermis*. The lipid content of 19.5% was found in hexane extract of *S.aculeata.* The carbohydrate content in hexane extract ranged from 0 to 0.002 mg/ml. The PBS treated control and the Sham control showed no significant difference from normal CAM vasculature **(Table 1).** The chloroform: methanol extract of *L.duvauceli* showed the maximum pro-angiogenic activity. It showed 57.14% increase in the number of blood vessels **(Fig.1a).** Aqueous extract of *S. aculeata* promoted angiogenesis upto 42.85% **(Fig.1b).** The chloroform: methanol and aqueous extracts of *S.inermis* showed similar effect of 14.28% increase **(Fig.1c&d).** Morphometric study of length of blood vessels in the control and treated eggs showed that the average length of the blood vessel in the eggs treated with aqueous extract of *S. inermis* was 2.6 cm, the longest was 3.0 cm and the shortest was 2.4 cm - both more than that of control **(Table 2, Fig.2).** The aqueous extract of digestive system of *L. duvauceli* (Sample No. 19) showed inhibitory effect of 42.85% in the number of blood vessels **(Fig.1e).**

Table 1: Number of main branches of blood vessels.

Sample No.	Name of the organism	Type of extract	No. main branches	% increase
Control	-	-	**7**	-
Untreated	-	-	**6**	-
9	***Sepia aculeata***	**Aqueous**	**10**	**42.85%**
12	***Sepiella inermis***	**Chloroform: Methanol**	**8**	**14.28%**
14	***Sepiella inermis***	**Aqueous**	**8**	**14.28%**
17	***Loligo duvauceli***	**Chloroform: Methanol**	**11**	**57.14%**

Table 2: Length of main branches of blood vessels.

Sample No.	No. of Branches	Average Length (in cm)	Longest (in cm)	Shortest (in cm)
Control	7	2.2	2.8	1.8
Sham Control	6	2.1	2.9	1.8
9	10	1.6	2	0.5
12	8	1.6	2.2	0.5
14	8	2.6	3	2.4
17	11	1.7	2.5	0.9
`19	4	2	2.5	1.5

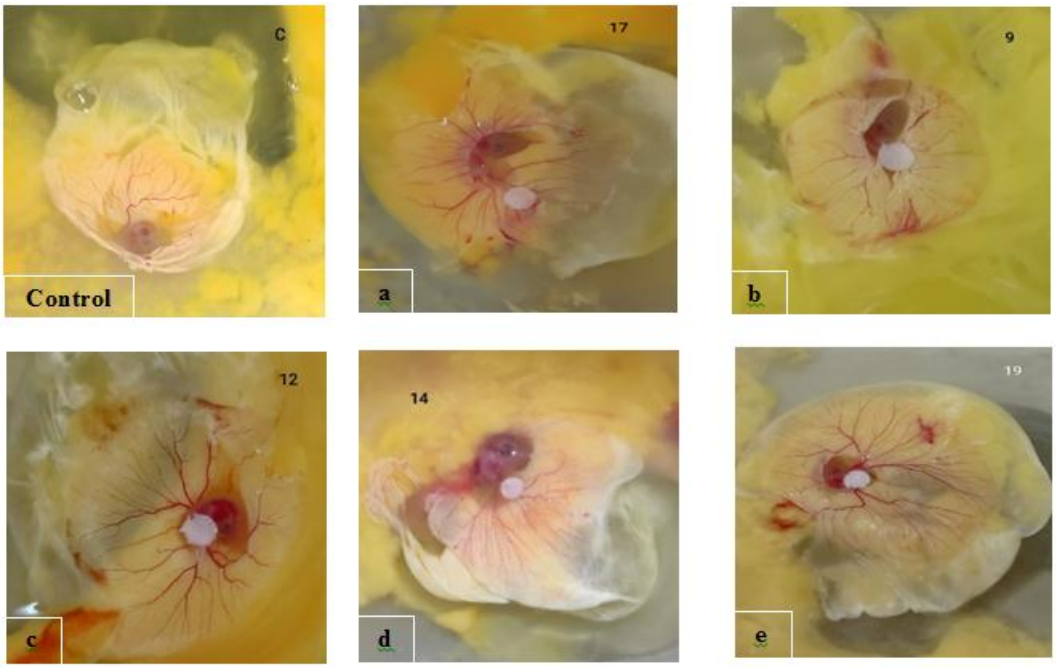

Fig. 1: Effect of the extracts on CAM angiogenesis.

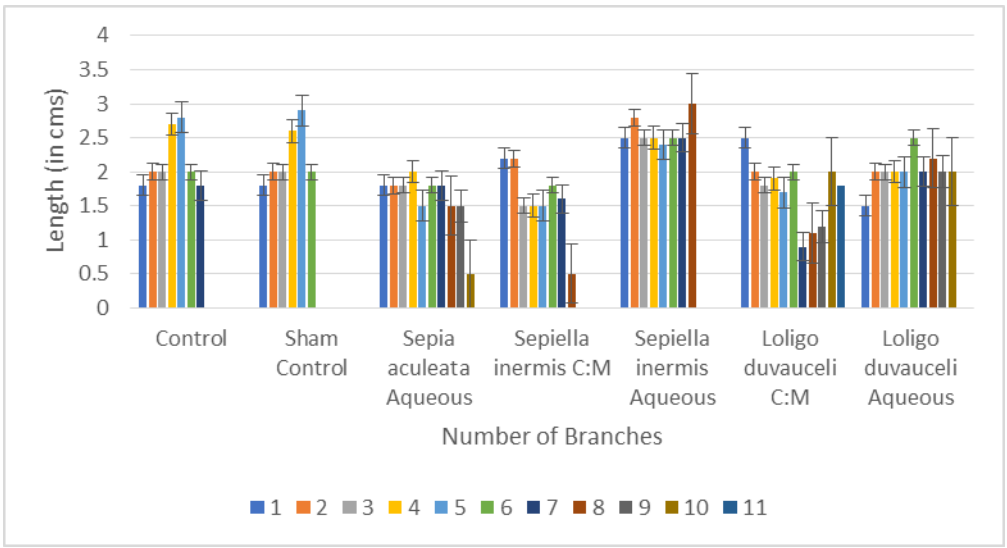

Fig. 2: Morphometric measurements of the blood vessels.

DISCUSSION

Angiogenesis is the physiological process of formation of new blood vessels. It is very important factor for growth & development of organs, tissue regeneration and wound healing.[13] Wound healing process occurs due to the sprouting of new capillaries from the old blood vessels to form granulation tissue. The wound clot is invaded by group of new blood vessels and is organized all over the granulation tissue into a microvascular network.[14] The proliferating blood vessels provide oxygen and nutrients to the tissues in that area thereby promoting faster healing and regeneration.

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane formed by fusion of the chorion and the allantois on day four of incubation. CAM model is an inexpensive model for the study and quantification of angiogenesis which can be used without previous stimulation of angiogenesis.[15,16] Due to its dense vascularization, easy availability and short growth period, CAM is considered to be suitable model for study of angiogenic activity. CAM can be used to assess the role and potential of pro and anti-angiogenic substances. Angiogenesis can be quantified by calculating the total length and density of the vessel.[17]

The extracts of the digestive system which showed potential activity in promoting angiogenesis are the aqueous extracts of *Sepia aculeata and Sepiella inermis* and chloroform: methanol extract of *Sepiella inermis and Loligo duvauceli*. The increase in the vascular density ranged from 14.28% to 57.14% in these extracts. The presence of soluble factors effecting the increase in vascularization is observed maximum (57.14%) in the chloroform: methanol extract of *Loligo duvauceli*. Pro-angiogenic factors have great applications in the process of promoting wound healing. Angiogenic stimulants can promote, repair and tissue regeneration. Impaired angiogenesis may cause further tissue damage due to lack of proper supply of oxygen and nutrients. Patients with diabetes show abnormal angiogenic activities. There is a direct role of high glucose levels in decreased angiogenesis in diabetic patients.[18] The increased pro-angiogenic activity of the extracts may exert important effects on wound healing and diabetic ulcers by improved angiogenesis. Secretions of snail *Helix aspersa* muller has been reported to have healing agents for the human skin.[19] Extracts of snails are used for their cell proliferating activity.[20] The mucus of *Phyllocaulis boraceiensis* is an inducer of angiogenesis in the endothelial cells and fibroblast. The fibroblasts migrate to the site of injury and play a major role in wound healing.[21] There is very little scientific

literature to support and compare the pro angiogenic effects in the digestive extracts of cephalopods.

The aqueous extract of *Loligo duvauceli* is the only extract in the study which showed anti-angiogenic activity. It inhibited the development of blood vessels by 42.85%. The number of branches from the main blood vessels were 4. The average length of the blood vessels in the embryo treated with this extract was 2 cm. The alcoholic shell extract of *chiton lamyi* showed antiangiogenic activity on CAM assay. There was a significant reduction in the number and length of the blood vessels in the embryos treated with this extract.[22]

CONCLUSION

The Chloroform: Methanol extract of the digestive system of *Loligo duvauceli* showed pro-angiogenic activity. It showed 57.14% increase in number of blood vessels. Further investigation of the active components of the same is being carried out.

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