Original Article

A SURGE IN ANTI-BACTERIAL AND ANTI-INFLAMMATORY ACTIVITY OF SEPIA PHARAONIS INK EXTRACT IN DIFFERENT SOLVENTS-AN IN-VITRO STUDY

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Abstract

Aim & Objective: To analyse the anti-bacterial and anti-inflammatory action of Sepia pharaonis ink extract using various solvents. To test the anti-bacterial activity of SPIE using microbial culture. To evaluate the anti-inflammatory action of SPIE using Egg albumin denaturation assay.

Methodology: The Sepia pharaonis fish was procured from kasimedu coastal area. The SPIE was lyophilized into a black powder. One gram of lyophilized SPIE powder was mixed individually with DMSO, Ethanol and water respectively in an Eppendorf tube. The anti-bacterial action of SPIE was performed using Culture plate coated with Mullein Hinton agar. E. faecalis, S. Mutans, S. Aureus, Lactobacillus were the different bacteria used in the study. The Anti-Inflammatory action was performed using the Egg albumin denaturation assay technique and different concentrations of SPIE mixed solvents were used.

Result: The Anti-inflammatory and anti-bacterial action of Sepia pharaonis ink extract proved to be very effective in DMSO solvent followed by ethanol solvent and at last with water solvent. The anti-inflammatory action of SPIE mixed with DMSO was effective in 30 µL,40 µL and 50 µL Concentration compared to the standard drug. The anti-bacterial action of S.Mutans was very effective followed by E.faecalis and Lacto-bacillus and the action against S.Aureus was found to be minimal.

Conclusion: This study proves that SPIE can be used in the anti-inflammatory drugs in the treatment of patients with tumours. Antibacterial activity holds good in the formation of ointments, soaps and various cosmetics

Keywords: Sepia pharaonis ink extract, Dimethyl sulfoxide, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*.

Introduction:

The pharaoh's cuttle fish is a nutrition rich sea food got from the sea food fisheries and rich in bioactive nutrients and health benefits. This cuttle fish is normally distributed in the Mediterranean and Asian sea coasts (Paulose,S.K and Chakraborty,K., 2021). The sepia pharaonis ink is normally discarded from the fish sellers as a

waste product. In few places it is used as food colouring agents in pastas, as natural dyes in dying industries. The cuttle fish bones are normally used as a food for various pet birds to increase the calcium levels.

The important source exhibiting anti-oxidant, anti-inflammatory and anti-tumour activity are derived from the marine source especially mollusks that are stuffed with bioactive metabolites (Anbuselvi, S et al.,2009). The sepia secretes ink continuously from the ink glands that has melanin granules. The sepia or squid ink has anti-bacterial activity (Nair, J. R et al.,2011). The sepia melanin has various size of smaller grains that are rounded in shape with diameter that varies from 100-200nm (Mbonyiryivuze, A et al.,2015). The microorganism forms biofilm and attaches to the biological tissue that represent phylogenetic state in the form of gene expression and rate of growth (Pozo J L D., 2018).

The S.Mutans is one of the main causative agent causing dental caries producing glucans, a polysaccharide that forms a biofilm on the tooth surface(Teughels ,W et al.,2014).The staphylococcus aureus is isolated from oral cavity in routine clinical practice and accounts for 24% -36% of staphylococcus species. The S.Aureus is isolated from apical abscesses, mucosities, endodontic infection , mandibular osteomyelitis and as dental implant complication in postoperative cases(Oliveira ,JRD et al.,2014). The dental caries is also caused by lactobacillus acidophilus that can ferment carbohydrates (Kouidhi, B et al.,2015). Enteroccocus faecalis is responsible for secondary root canal infection and act as a pathogen. The root canal and persistent endodontic infection is loaded with bacteria's and retreatment is needed (Yim ,N-H et al., 2013).

The oral pathogens are treated with various antibiotics like Erythromycin, Ampicillin, Vancomycin etc and tolerance has developed against microorganism and multidrug resistance strains has led to explore biodegradable anti-microbial compounds that are specific and safe (Giriraju,A and Yunus G.,2013). Antibacterial activity of sepia pharaonis purified with diethyl ether, hexane extract inhibited the activity of E. coli, Staphylococcus epidermidis, Klebsiella pneumoniae and Pseudomonas aeruginosa (Nithya, M et al.,2011)

Triterpenoids and steroids are present in melanin from squid ink shows antibacterial activity against E.Coli and L.Monocytogenes from low to medium level(Sari, R. C et al.,2019). The ink has the ability to chelate metals due to the melanin content and Aeromonas species, a fish microbe is affected by the cyto-toxic activity of melanin ink (Fitrial ,Y and Khotimah,.T K.,2021).

Reduced dose of sepia pharaonis liver oil enhanced the immune response and decreased the inflammatory mechanism of formalin-induced and carrageenan-induced paw oedema in rats (Joseph,.,SM et al.,2005)

Materials & Methodology:

Sepia pharoanis ink extract:

The Sepia pharaonis was purchased fresh from the chennai sea coast. The species identification was authenticated by the zoological society of India, Adyar. The ventral aspect of the fish was given a neat incision and the ink sac was removed and cleaned with the spirit. The ink was collected in a sterilised glass bottle and kept under -40℃. The glass bottle was transferred to TANUVAS, Vepary and lyophilised into fine powder. The lyophilised powder was used for further study.

Stock solution:

1 gm of lyophilised sepia pharaonis ink extract was mixed in three different solvents namely water, ethanol and dimethyl sulfoxide individually. The ultra-sonicator was used to mix SPIE with the solvents.

Microbial culture:

Staphylococcus aureus, Streptococcus mutans, Lactobacillus and Enterococcus faecalis were the gram positive, four bacterial species used in the study. Staphylococcus aureus is an aerobic organism and Staphylococcus aureus, Streptococcus mutans and Lactobacillus are the anaerobic organisms. The bacterial isolates were procured from Pune in the form of liquid bacterial broth. The nutrient broth was sterilised for 20 minutes. The petri dishes are sterilised and the Mueller Hinton Agar was poured and sterilised in the incubator for 37℃. The wells were created in the petri dishes using glass tubes and sterile cotton swabs were used to inoculate the bacterial broth in the petri dishes.

Five petri dishes were prepared and three wells were created. Four petri dishes were used to inoculate the bacterial broth and one well was used as a control. The different solvents were dropped in the wells inoculated with the bacterial broth. The procedure was carried in the laminar air flow chamber for an hour. The petri dishes were incubated in an Incubator for 24 hours at 37 ˚C. The inhibition zone was measured and recorded.

Egg albumin denaturation assay

A solution containing 2.8ml of freshly prepared phosphate buffered saline of pH - 6.3, 0.2 ml of egg albumin extracted from hen's egg. Specific concentrations were prepared separately for SPIE (DMSO, ETHANOL, AQUEOUS) as (10µL,20µL,30µL,40µL,50µL). Diclofenac sodium was used as a positive control. The mixtures were heated in a water bath at 37ºC for 15 minutes. cool down to room temperature absorption was measured at 660 nm.

Results:

Table-I showing Zone of Inhibition of SPIE mixed Aqueous, Ethanol and DMSO in microbial culture in mm

	Aqueous	ETHANOL	DMSO
E. FAECALIS	9	10	17
S. AUREUS	10	10	13
LACTOBACILLUS SP	11	15	17
S. MUTANS	9	12	18

FIGURE I- ANTI-BACTERIAL ACTIVITY OF SEPIA PHARAONIS INK EXTRACT

SPIE mixed with Water, Ethanol and Dimethyl sulfoxide

A- Streptococcus mutans
B- Lacto-bacillus
C- Staphylococcus aureus
D- Enterococcus faecalis

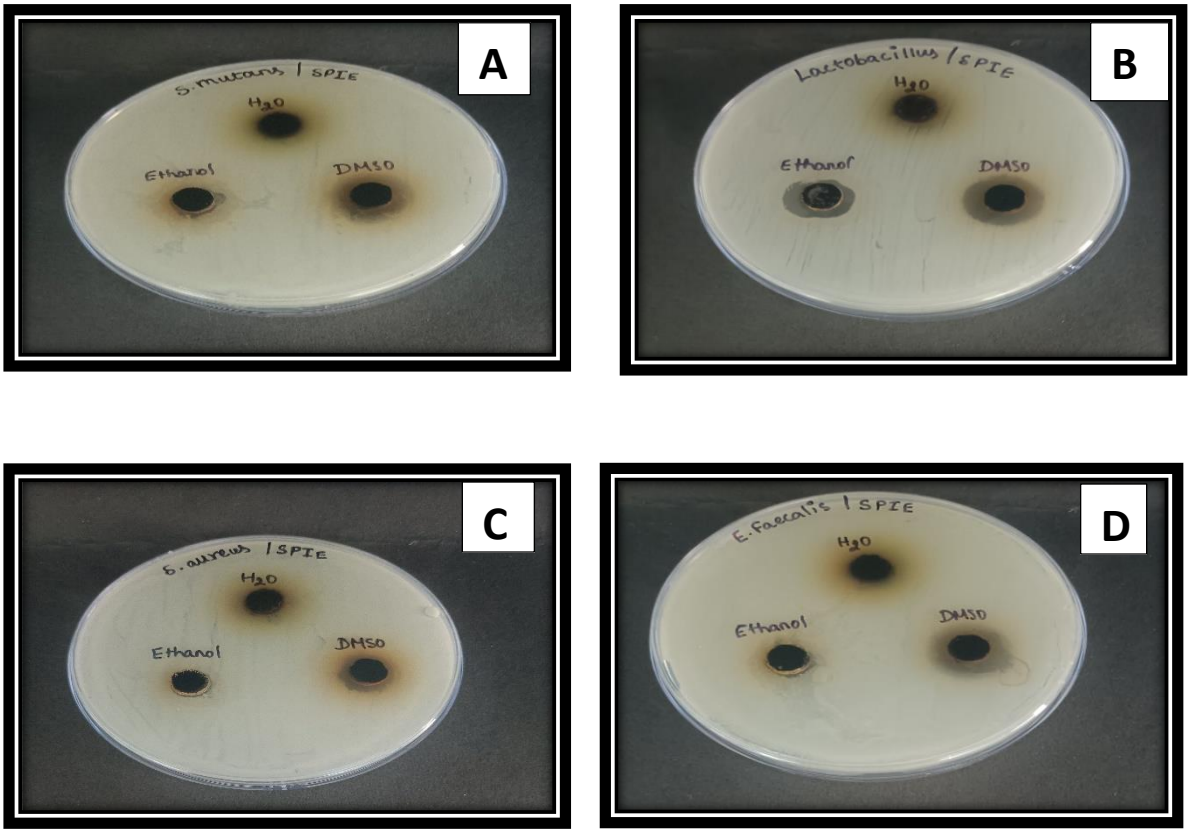

FIGURE II- ANTI-INFLAMMATORY ACTIVITY OF SEPIA PHARAONIS INK EXTRACT USING EGG ALBUMIN DENATURATION ASSAY WITH DMSO SOLVENT

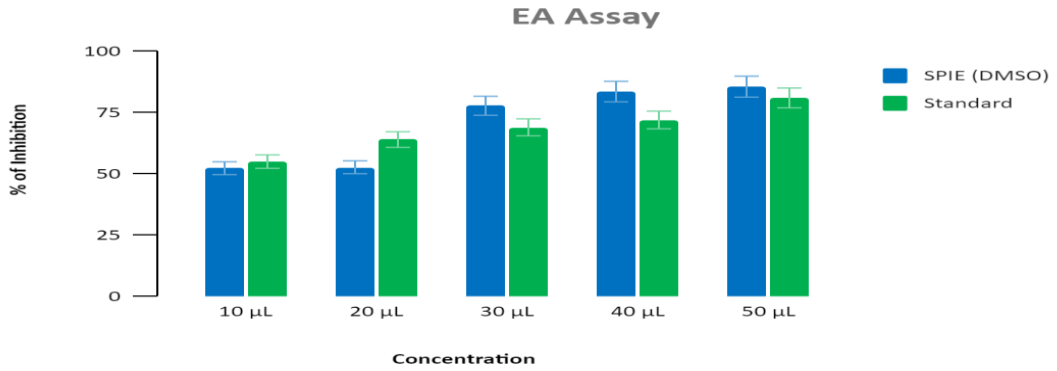

Table 2: Anti-Inflammatory action of SPIE mixed with DMSO Solvent in different concentration

	10 µL	20 µL	30 µL	40 µL	50 µL

SPIE (DMSO)	52.3	52.7	77.8	83.6	85.6
Standard	55	64	69	72	81

FIGURE III- ANTI-INFLAMMATORY ACTIVITY OF SEPIA PHARAONIS INK EXTRACT USING EGG ALBUMIN DENATURATION ASSAY WITH ETHANOL SOLVENT

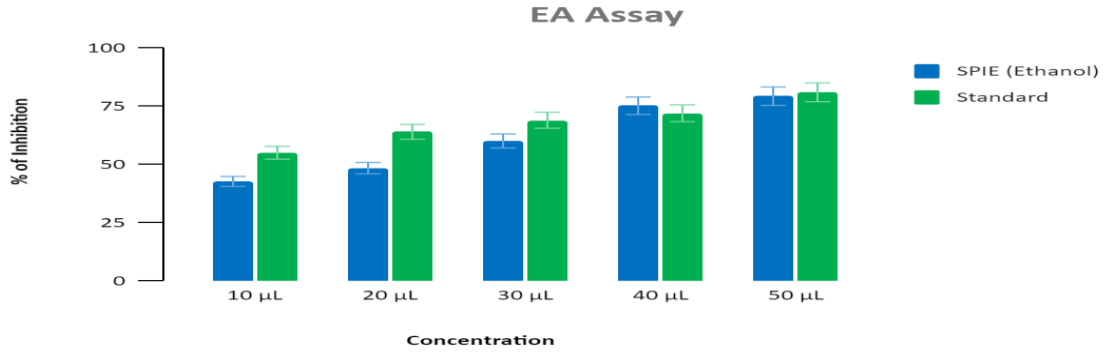

Table 3: Anti-Inflammatory action of SPIE mixed with Ethanol Solvent in different concentration

	10 µL	20 µL	30 µL	40 µL	50 µL
SPIE (Ethanol)	42.7	48.4	60.1	75.2	79.3
Standard	55	64	69	72	81

FIGURE IV- ANTI-INFLAMMATORY ACTIVITY OF SEPIA PHARAONIS INK EXTRACT USING EGG ALBUMIN DENATURATION ASSAY WITH AQUEOUS SOLVENT

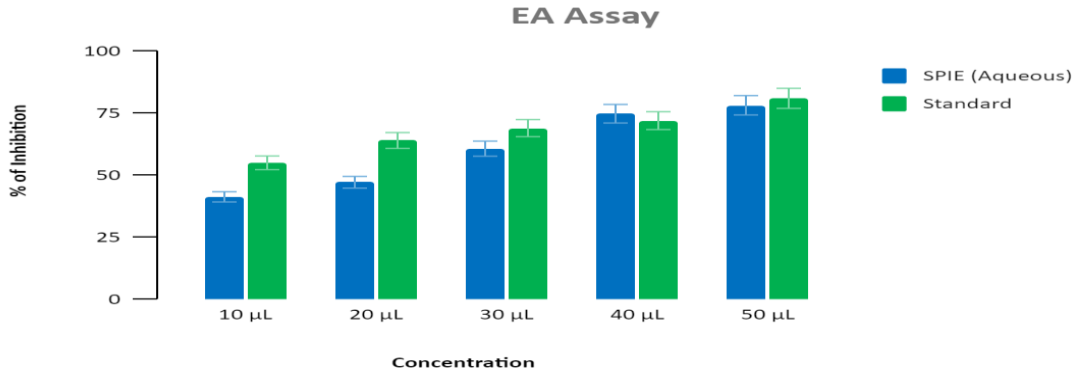

Table 4: Anti-Inflammatory action of SPIE mixed with Aqueous Solvent in different concentration

	10 µL	20 µL	30 µL	40 µL	50 µL
SPIE (Aqueous)	41.27	47.15	60.7	74.8	78.2
Standard	55	64	69	72	81

Discussion:

The important constituent of squid ink is melanin and it has been identified to be a tyrosinase (Derby, C. D.,2014). Squid ink contains eumelanin granules that has been observed in a colourless medium. Eumelanin is an heterogenous compound and a polymer normally insoluble that is derived from the oxidation of tyrosine, an amino acid. The melanosomes are an organelle that has pigment cell responsible for the generation of eumelanin (Magarelli, M et al.,2010). This melanin has revealed itself to inhibit the proliferation of the bacteria (Mackintosh, J. A.,2001).

The bacteria have an important metal magnesium that is having a role in cell wall orders (Matthews, T. H et al.,1979). The melanin has anti-bacterial activity that might be due to the metal ion required by bacteria for cell wall ordering (Aisiah, S et al.,2020). The ordering of bacterial cell wall by melanin elevated carbon and nitrogen elements, after purification due to the release of ions (Sari, R. C et al.,2019). The anti-bacterial activity is enhanced by the carbon and nitrogen element (Poernomo, A. T et al.,2020)

Many types of cations are present in the bacterial wall, namely K +, Na+, Ca2+ and Mg2+. These ions are responsible for maintaining the cell outer layer integrity, enzymatic activity and regulation of metabolism. The stability of the external layer is dependent on Ca2+ and Mg2+ ion concentration (Chen, S et al.,2009). The external membrane of the bacterial cell will not allow the entry of enzymes, hydrophobic and bacteriocin compounds. The cations are adsorbed by the melanin, the bacteria cell metabolic system as well as the growth of the bacteria is also disturbed (Hong, L. and Simon, J. D., 2007). There is elevated optical density after melanin extract administration on growth of E.Coli (Fitrial, Y. and Khotimah, I. K.,2017). The optical density elevation is associated with the presence of bacteria and the melanin inhibits the growth of bacteria by degrading the bacterial cell wall (Zhang, X et al.,2015)

Sepia secreted melanin showed reduced activity of growth of Aeromonas species and resulted in irregular cell shape and decreased cell size (Sari, R. C et al.,2019)). The spirolactone, spiropharanone is isolated from S.Pharoanis organic extract on chromatographic fractionization. The anti-oxidant property of spiropharanone has dipeptidyl peptidase-4,insulin secretion regulating enzyme and pro-inflammatory 5-lipoxygenase. This naturally derived marine food proved to be anti-inflammatory and anti-diabetic that can anticipate the product to be used in pharmaceutical formulation in glucose homoeostasis and inflammation (Paulose,S.K and Chakraborty,K., 2021).

The anti-bacterial activity in the present study was done in gram positive bacteria like E. Faecalis, S. Mutans, Lactobacillus and S. Aureus with SPIE mixed solvents (figure 1 & Table 1) showing promising results in all the three (DMSO, Aqueous and Ethanol) solvents. The SPIE mixed with DMSO showed good results compared to ethanol solvent and aqueous solvent.

In a study conducted by Senam et al the crude SPIE was delipidated with acetone and extracted with Tris -HCL followed by lyophilization and trypan blue was used to perform the cell viability assay. The staining was done with acridine orange and Ethidium bromide and study with chick embryo fibroblast cells revealed anti-proliferative capability and a good therapeutic potential in anti-cancer approach (Senan, V.P et al.,2013). The egg albumin denaturation assay resulted in higher anti-inflammatory activity of SPIE mixed with DMSO (figure2 & Table 2) compared to other solvents and control. SPIE mixed with Ethanol (figure3 & Table 3) and water (figure 4 & Table 4) showed the anti-inflammatory action very close to control. The anti-bacterial and anti-inflammatory action of SPIE can be taken into animal study and in human trials.

Conclusion:

The Sepia pharaonis ink extract showed promising anti-bacterial activity against common oral pathogens like S. mutans, Lactobacillus, S. aureus, and E. faecalis. Thus, SPIE can help to prevail against the occurrence of dental caries, abscess formation, root canal infection and mucositis. The anti-inflammatory action of SPIE can be used

to control various tumours both benign and malignant in a harmless way. The clinical trials are in need to bring out the hidden mechanism of action in controlling tumours.

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