



Original Research Article

A novel study on the antibacterial effect of the crude squid ink extracts from the Indian squid against four bacterial pathogens isolated from carious dentine

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ABSTRACT

Keywords

Dental caries, squid ink, caries pathogens, anti-caries agents, antimicrobial, *Loligo* sp.,

Caries is a multifactorial disease and is considered in terms of the dynamic relationship between the dental plaque microbiota and host factors. Antimicrobial resistance by the oral pathogens against the oral antiseptics create an emergency to screen for novel, safer and more effective agents that can be used to control and manage caries pathogens. This study is thus designed to explore the antimicrobial activity of crude solvent extracts from the ink gland of the squid *Loligo duvauceli* against *Lactobacillus acidophilus*, *Streptococcus mutans*, *Actinomyces viscosus* and *Candida albicans*. Squid ink extracts were obtained from five solvents by crude solvent extraction method and were rotary evaporated. The crude extracts thus obtained were checked for its antimicrobial efficacy by agar well diffusion method at a concentration of 100 mg/ml against the potent caries pathogens *S.mutans*, *L.acidophilus*, *A.viscosus* and *C.albicans*. The mean zone of inhibition was measured and recorded. Further serial dilutions of the extract with maximum activity were checked by microbroth dilution method and the MBC value was deduced. Statistical analysis was done by Non-parametric Mann-Whitney test to compare the antibacterial activity exhibited by various extracts. Among the crude extracts hexane extract showed a promising activity against the dental caries pathogens. The MBC value of the hexane extract was deduced as 5 mg/ml for *L.acidophilus* and 2.5 mg/ml for *C.albicans*, *A.viscosus* and *St.mutans*. This study is the first report to suggest the antibacterial potential of squid ink extracts against the dental caries pathogens. This minor research work is concluded by stating that squid ink could be employed as a novel therapeutic anti-caries agent in near future.

Introduction

Caries remains as one of the commonest disease throughout the world. It is a

bacterial damage of hard tooth enamel which requires four main factors like tooth

enamel, acid producing bacteria, fermentable carbohydrates and time (Kidd *et al.*, 2004). The microbial presentation of caries is highly variable and it is a disease that has been associated with *Streptococcus* sp., mainly *S.mutans* and *S.sobrinus* in case of superficial caries (Hardie, 1982), along with *Lactobacillus* sp (Kneist *et al.*, 2010) in deep caries and *A.viscosus* (Komiyama *et al.*, 1992) and *C.albicans* (Shen *et al.*, 2002) in root caries. Mechanical removal of the dental plaque is the most efficient procedure in caries prevention but this is not completely perfect in most of the population (Elkholany *et al.*, 2009). Moreover, dental treatment is often very expensive and is not readily accessible, especially in developing countries (Pack, 1998). Extensive efforts have been made by many microbiologists towards the search for anti-cariogenic compounds from various natural sources to remove the biofilms and to reduce the incidence of caries in humans (Badria *et al.*, 2004).

Recent studies have demonstrated the great importance of natural products, both plant extracts and isolated compounds, as natural antibacterial agents in oral care products (Fabricant *et al.*, 2001). These secondary metabolites from plants continue to provide new and important pharmacological targets (Balunas *et al.*, 2005). Apart from these natural therapeutic metabolites, the marine habitat encompassing a complex assemblage of life forms has created a vital interest among the researches to discover novel therapeutic agents. The biochemical metabolism of various marine organisms such as blue green algae, sponges and squids are inspiring new ideas for drug development (Mc Connell *et al.*, 1994). Under the phylum Mollusca, hails the squids, gastropods and cephalopods which

secrete a black pigmented ink as a defensive ploy to escape from predation. The squid ink has proved to play various primary roles in the world of alternative medicine and has widest range of therapeutic applications (Takai *et al.*, 1993). The potential antibacterial activity of the ink from the South Indian squid *Loligo duvauceli* has already been reported against various clinical pathogens (Smiline Girija *et al.*, 2008). Squid ink has also been reported to possess antimicrobial activity against biofilm bacteria (Santhana Ramasamy *et al.*, 2005) and *Staphylococcus aureus* (Atushi Mochizuki, 1979). It has also been used as a preservative agent in canned preparations (Saloua Sadok *et al.*, 2004). It has proved to play a key role in the microbial defense with potent antioxidant values (Lei *et al.*, 2007) and for its anti-retroviral activity (Rajaganapathy *et al.*, 2000). With this background this study has been undertaken to explore the antimicrobial activity of the ink extracts from the south Indian squid *Loligo duvauceli* against the four caries pathogens *S.mutans*, *L.acidophilus*, *A.viscosus* and *C.albicans*.

Materials and Methods

Isolation and identification of caries pathogens

Cariou dentine was collected from 100 patients with visible tooth decay visiting the Department of Endodontics of Meenakshi Ammal Dental College, Chennai, India for a period of six months from Jan 2009 to June 2009 after obtaining the informed consents. Adult patients elected to have extractions for unrestored anterior, premolar and molar teeth that presented with large coronal dentine caries lesions by macroscopic examination were

included for the study. A proforma was recorded for each study case to analyze their personal and clinical history. The collection of carious dentine was as follows. The carious zone of decalcified and partially decalcified dentine was washed with sterile saline. After removal of superficial plaque and debris overlying the lesion, the carious dentine was then excavated with sterile dental explorers and was transferred in to vials of sterile Thioglycollate broth and Brain Heart Infusion broth to a concentration of approximately 10 mg (wet weight) of dentine per ml prior to processing. The samples were brought to the laboratory and were cultured within 3 hrs of collection. Samples were dispersed in the transport medium by using a vortex mixer and the broth was incubated at 37°C / 10% CO₂ for 2 hrs. After incubation the broth was inoculated onto sterile Trypticase soy blood agar, Thioglycollate agar and Saboraud's Dextrose agar (SDA). Incubation was performed at 37°C / 10% CO₂ for 48 hrs and 37°C/anaerobically for 48 hrs. SDA was incubated at 37°C/24 hrs aerobically. After incubation the pathogens were identified and characterized biochemically.

Standard ATCC and MTCC control strains were used to screen the antimicrobial activity of the squid ink extracts. The control strains include *Streptococcus mutans* (MTCC 890), *Lactobacillus acidophilus* (MTCC 447), *Actinomyces viscosus* (ATCC 19246) and *Candida albicans* (ATCC 10231).

Preparation of inoculum

A minimum of four colonies of the test organisms were touched with a sterile loop and transferred into Sterile Trypticase soy broth (for *St.mutans*), Thioglycollate broth

(for *L.acidophilus*), Actinomyces selection broth (for *A.viscosus*) and into Sabouraud's dextrose broth (for *C.albicans*) under aseptic conditions. The inoculated broths were then incubated for two hours at 37°C / 10% CO₂ for the bacterial pathogens and 37°C / aerobically for the yeast (*C.albicans*). After incubation the density of each microbial suspension was adjusted equal to that of 10⁶ c.f.u/ml (standardized by 0.5 MacFarland standards) and was used as the inoculum.

Preparation of crude extracts from squid ink gland

The crude extraction of the active biomolecules from the South Indian squid *Loligo duvauceli* was done using solvents (Ditty Chacko *et al.*, 2005). The solvents employed for the study include hexane, ethyl acetate, acetone, diethyl ether and chloroform. Collection of squids and the dissection of ink sac were done as per our previous reports (Smiline Girija *et al.*, 2008). The ink gland was then squeezed gently to excrete the ink and the crude extraction was done using solvents. The extracts thus obtained were collected and weighed.

Sterility check of the extracts

The crude extracts were sterilized by exposure under UV light for 2 hrs. 5 mgs of each extract was mixed in sterile nutrient broth and was incubated for 2 hrs which was then plated onto Nutrient agar for checking the sterility of the extracts. The extracts were then stored at 4°C in brown glass bottles.

Antimicrobial bioassay

The antimicrobial potential of the crude extracts was studied using agar well

diffusion method (Nathan *et al.*, 1978). 100 µl of the prepared inoculum of the organisms was spread as lawn cultures onto sterile Mueller Hinton agar plates for *L.acidophilus* and *C.albicans* using L-rods to achieve a confluent growth. Sterile Trypticase soy agar plates were used for the sensitivity assay with *A.viscosis* and *St.mutans*. The agar plates were allowed to dry and wells or cups of 8 mm were made with a sterile agar borer in the inoculated agar plates. 100 mgs of each crude extract were reconstituted in Dimethyl sulphoxide (DMSO) in sterile brown glass bottles for the antimicrobial bioassay. A 50µl volume of each extract was propelled directly into the wells of the inoculated agar plates for each test organism.

The plates were allowed to stand for 10 minutes for diffusion of the extract to take place and incubated at 37°C for 24 hrs. DMSO served as Negative control. Erythromycin 30µg (for bacteria) and Amphotericin B 100U (for yeast) were included as positive controls. After incubation the plates were observed for the zone of inhibition around the wells and the zone size was measured using an antibiotic sensitivity measuring scale (Himedia). The antimicrobial efficacy was graded based on the zone diameter as high activity (> 15 mm), moderately active (10-14 mm), trace activity (5-9 mm) and no activity (< 4 mm) (Rios *et al.*, 1988).

Statistical analysis

Non parametric Mann-Whitney test was employed to analyze the mean value of the zone of inhibition obtained for three consecutive test procedures for all the test pathogens. The analysis employed the statistical package of SPSS 17.0 version.

Determination of Minimum Bactericidal Concentration (MBC) value

Determination of MBC value for hexane extract (showed high activity) was determined by Microbroth dilution method (McGinnis *et al.*, 1996). Serial dilutions of the hexane extract were done in a 96 well microtitre plate with DMSO. The dilution factor was 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml. To each dilution 100 µl of the culture broths of the test strains and control strains were added in their respective wells and the plate was incubated at 37°C for 24 hrs. After incubation the spectrophotometric analysis was performed and the OD values were recorded. The MBC value was also confirmed by Microbial Spot Checker board method [Nkere *et al.*, 2005]. Briefly, 3 µl of each dilution was spotted onto Mueller Hinton agar plates and incubated at 37°C for 24 hrs. After incubation the spot showing the complete absence of microbial growth indicates the bactericidal dose or the minimum bactericidal concentration value. The dilution factor that showed the visible decrease in the growth was determined as the minimum inhibitory concentration [MIC] value.

Results and Discussion

The efficacy of the five crude solvent extracts of squid ink was evaluated against the dental caries pathogens. The solvent extracts showing the zone of inhibition for each test organism were recorded and the mean value of three experiments was calculated. Hexane extract showed a high antimicrobial activity against all the test organisms with a mean zone of 18.33 mm for *L.acidophilus*, 18.23 mm for *C.albicans*, 14.46 mm for *A.viscosus* and 15.2 mm for *St.mutans* (Figure 1).

Positive control showed a zone of inhibition of 26 mm. Negative control (DMSO) did not show any zone of inhibition in any of the test organism. All the other extracts showed a trace activity or no activity against the tested caries pathogens (Table 1). The MBC value of the hexane extract was determined as 5 mg/ml for *L.acidophilus* and 2.5 mg/ml for *C.albicans*, *A.viscosus* and *St.mutans* (Table 1). The previous dilution that showed the visible decrease in the number of colonies was determined as the MIC value and was thus deduced as 2.5 mg/ml for *L.acidophilus* and 1.25 mg/ml for *C.albicans*, *A.viscosus* and *St.mutans*. Dental caries is considered to be the most common chronic disease of childhood and is the biggest unmet health care need among the human population.

Socioeconomic disparities in both rates of disease and treatment are a major public health issue (Keyes, 2002). It is also steadily increasing due to indulging in different variety of diets and improper oral hygiene. Formation of carious tooth is concerned with the metabolism of the bio-film of microorganisms present on the tooth surface. It is a well known fact that this disease has been associated with polymicrobial flora such as *Streptococcus* spp., mainly *St.mutans* and *S.sobrinus* that have revealed their major role in the initiation and development of caries (Llena-Puy *et al.*, 2000). Further progression of caries towards the dentinal layers is caused by *L.acidophilus* which results in deep carious lesions (Roy Byun *et al.*, 2004). Finally cariogenic pathogens like *A.viscosus* and *C.albicans* colonizes the root of the tooth resulting in root caries (Howell *et al.*, 1962). In view with this, the present study was done with these four major pathogens that cause caries at different layers of the tooth structures. Surgical excavation being the major

treatment procedure in caries, the use of antimicrobials or other antifungal agents is also in use to kill a broad spectrum of organisms (Anderson *et al.*, 2006). They may be semi selective in that medicaments can be prescribed that preferentially affect the etiological agents but they still kill an array of organisms. Thus it is concluded that broad spectrum antibiotics or antimicrobials are not effective long-term unless their application is periodically repeated. Thus there is an immediate necessity of alternative therapeutic agents in the eradication of caries pathogens and also to prevent the emergence of drug resistant strains.

The reports on the antimicrobial activity of natural products against the pathogens responsible for caries diseases are scarce. However, no reports are available for the antimicrobial studies concerned with the squid ink and its extracts against the caries pathogens. In the present work different solvent extracts of squid ink were prepared using five solvents like hexane, chloroform, acetone, ethyl acetate and di-ethyl ether. Successful prediction of natural compounds from natural sources is largely dependent on the type of solvent used in the extraction procedure. Traditional healers use primarily water as the solvent but in many studies it was found that extracts in organic solvents provided more consistent antimicrobial activity compared to those extracted in water (Parekh *et al.*, 2005). These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay (Nair *et al.*, 2007). Also the choice of DMSO as a solvent is due to its solvency for a wide range of chemicals, its low antibacterial activity at concentrations less than 2% and its low toxicity (Hemaiswarya *et al.*, 2009).

Table.1 Antibacterial effect of the crude solvent extracts of the Squid ink gland against the caries pathogens

Organisms under study	Mean zone of Inhibition (in mm) for the crude solvent extracts					MBC Value (in mg/ml)
	Hex	EthAc	Chl	A	DEthE	
Reference/control organisms						
<i>Lactobacillus acidophilus</i> [MTCC 447]	20	NA	14	NA	12	5
<i>Streptococcus mutans</i> [MTCC 890]	15	NA	NA	NA	NA	2.5
<i>Actinomyces viscosus</i> [ATCC 19246]	18	NA	NA	NA	NA	2.5
<i>Candida albicans</i> [ATCC 10231]	18	NA	14	14	12	2.5
Test organisms isolated from carious dentine						
<i>Lactobacillus acidophilus</i>	18.33	NA	12.26	NA	12	5
<i>Streptococcus mutans</i>	15.21	NA	NA	NA	NA	2.5
<i>Actinomyces viscosus</i>	14.46	NA	NA	NA	NA	2.5
<i>Candida albicans</i>	18.23	NA	14	12.53	12	2.5

[Hex-Hexane, EthAc – Ethyl Acetate, Chl – Chloroform, DEthE – Diethyl Ether, NA – No Activity]

A promising antimicrobial activity displayed by the hexane extract of squid ink in this study makes it to be potentially employed in the development of natural anti-caries agents. The hexane extract has already been reported to possess a high antimicrobial activity against various clinical isolates and pathogenic yeast in our earlier report (Smiline Girija *et al.*, 2008). Results showed that the mean zone of inhibition for the hexane extract was higher for all the test organisms under study and the difference was found to be statistically significant ($p < 0.05$) when compared with the chloroform extract which showed a medium activity against *L.acidophilus* and *C.albicans* but no activity against *St.mutans* and *A.viscosus*. The mean value for the zone of inhibition between the acetone extract and chloroform extract was similar and they showed a medium activity against

C.albicans but did not act against the other pathogens under study. Extract from diethyl ether showed trace activity with a significant statistical difference ($p < 0.05$) in comparison with the other extracts against *L.acidophilus* and *C.albicans* but no activity against *St.mutans* and *A.viscosus*. Ethyl acetate extracts did not show any activity against any of the test pathogens under study. Thus this minor research study has highlighted the importance of evaluating novel therapeutic biomolecules from squid ink as anti-caries agents.

To conclude, it is evident that research involving natural products is complex. Therefore a step wise research plan in a multidisciplinary fashion is required. However the structural elucidation of the active molecules responsible for the antimicrobial activity in the squid ink and

a multi angled study for its confirmed activity has been made and has been deduced in our other research works with patent registration for the bioactive molecules. Despite limited studies, the available evidence indicates that there is potential for the discovery of novel and effective anticaries/antiplaque therapy using squid ink. There is a clear need to implement more concrete drug protocol and to conduct additional studies to evaluate the clinical efficacy and safety of these substances (Grosso *et al.*, 2007). This study recommends the use of squid ink as a valuable biopharmaceutical product that can be effectively used against the caries pathogens that might result in pain-free tooth in near future.

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