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Original Article

In vitro anti-microbial and anticancer properties of Ink gland from *Kalinga ornata* (Alder & Hancock 1864).

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ABSTRACT

The present study investigated the anticancer and antimicrobial properties of ink gland from *Kalinga ornata* using three different solvents such as acetone (AIG), ethyl acetate (EIG) and methanol (MIG). The ink gland sample was extracted with three different solvents and subjected to anti-microbial activities and then purified through CM-Sephadex, the pooled fraction was screened for anticancer activity and characterization was done using High-Performance Liquid Chromatography (HPLC) and Fourier-Transform Infrared Spectroscopy (FT-IR) respectively. The antimicrobial activity was conducted to determine the zone of inhibition against the human pathogen. Preliminarily, fractionations of the extracts were performed by CM-Sephadex (C-50) column chromatography. The purified fractions were pooled based on their high absorbance by UV-visible spectroscopy. Among the purified fractions, MIG showed significant anti-proliferative activity (41.06%) than AIG (30.44%) and EIG (14.35%) against colon cancer cell line (SW620). It is concluded that bioactive components from marine natural products pave way for finding a potential drug candidate.

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1. Introduction

The molecular architecture study and its role in biological activity have led to the discovery of bioactive molecules. The compounds obtained from various sources of living organisms are of great significance [1]. Such compounds are called as natural products, which account for one-third of the world's best-selling medicines in the pharmaceutical industry [2]. Among 80% of the world's population, 85% of the treatment regimens are natural products based on traditional knowledge or modern practices. The ocean has more fauna and flora when compared to the terrestrial ecosystem [3]. Approximately, 14,000 natural products have been isolated so far, i.e. 25% from algae, 33% from sponges, 18% from coelenterates and 24% from other invertebrates such as ascidians, nudibranch, echinoderms, bryozoans, etc [4]. Nudibranchs are not as much of known gastropod mollusc, which shows worldwide circulation from polar-regions to the tropical shores and even in the deep sea [5]. Nudibranch or sea slugs are one of the most diverse groups of mollusc. The colorful nudibranchs are not protected by a shell, unlike other molluscan species. Hence, they release various colored fluids as chemical defenses against predators [6]. Over a decade, the sessile bodied nudibranch has gained more attention in the drug discovery research. For instance, a variety of bioactive compounds have been isolated from *Aplysia*

sp such as 1-deacetoxyalgaone, 1-deacetoxy-8-deoxyalgaone and ibhayinol [7]. Doridosine, a pharmacologically potent purine derivative has been isolated from the digestive glands of the California dorid, *Anisodoris nobilis* [8]. Likewise, the Dolastatin 15, a cytotoxic depsipeptide from the Indian Ocean sea hare, *Dolabella auricularia* has successfully entered the clinical trials [9]. Another drug in the clinical trial evaluation is the Kahalalide F, from the Hawaiian sacoglossan, *Elysia rufescens* and its feed *Bryopsis* sp [10]. *K. ornata*, the only species in the genus *Kalinga* is a colorful nudibranch, commonly found in the Indian coast. The anti-bacterial and α -amylase activities of polar extracts of *K. ornata* have been already illustrated [11]. Since, *K. ornata* has been shown to possess vast bio-potency and due to its availability, it is one of the promising candidates in drug development. Thus, the present work exploring the antimicrobial and anticancer potential of *K. ornata*.

2. Materials and Methods

2.1 Sample Collection

The nudibranch, *K. ornata* was collected from Pazhayar landing center (Lat. 11° 21' N and Long. 79° 49' E) and was shifted to the laboratory in cold condition immediately. Further, it was thoroughly rinsed with D.H₂O and froze at -40 °C for future purposes. The whole tissues were dissected and the ink gland was removed aseptically. The pooled ink glands were used for further analysis.

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2.2 Preparation of extracts using different solvents

The standard method described was followed for the solvent extraction. 50 g of ink gland tissues were homogenized with 150 mL of methanol, acetone and ethyl acetate respectively. The homogenate was incubated for 24 h at 4 °C and was centrifuged (6000xg, 15 min). The residue was recovered and was subjected to re-extraction. The pooled supernatant was concentrated to dryness in a rotary evaporator under reduced pressure. Further, the crude samples were lyophilized for further use [12].

2.3 Fractionation by Ion exchange chromatography

Ion exchange chromatography was performed on the three ink gland extracts using the methodology [13] with slight modifications. Approximately, 100 mg of lyophilized crude samples were dissolved in 1 ml of 0.1 M NaCl and centrifuged at 10,000x g at 4 °C for 10 min. The upper layer was loaded onto a CM-Sephadex C-50 column (1.5 x 18 cm) and eluted using a linear gradient of 20 mL NaCl (0.1 M – 0.3 M) at a flow rate of 1 mL/min. The 2 ml fractions were collected and pooled together based on their bio-activity. All these pooled fractions were dialyzed, lyophilized and stored at -20 °C for further analysis.

2.4 Antimicrobial assay

Human bacterial strains including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Cryptococcus neoformans* were obtained from Microbial Culture Laboratory, Department of Medical Microbiology, Rajah Muthaiah Medical College and Hospital (RMMCH), Annamalai University, Tamilnadu, India. The 18 h old fresh microbial pathogens were suspended in 5ml of sterile saline (105 CFU/ml fresh cultures). The culture solution was thoroughly mixed by using a vortex and the turbidity was adjusted to yield 2×10^6 cells/ml (0.5 McFarland standards). The standard method was employed to screen the antimicrobial potential of the pooled fractions of *K. ornata* as described by [14]. A 100 µL of pathogenic strain was spread on Muller Hinton Agar plates using a sterile cotton swab under aseptic conditions. The sterile filter paper disc was saturated with 10 µL of the extract and the antibiotic Ampicillin, 100 µg (control). Then, the disc was placed on culture plates and was incubated at 37 °C for 24 h. The diameter of zone of inhibition was determined and expressed in mm.

2.5 Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

The purified fraction was dissolved in HPLC grade water and centrifuged at 9469x g for 2 min. The clear solution was injected in the analytical HPLC Shimadzu C18 column (Luna 5µ C18 (2) 100Å 250 mm x 4.60 mm) attached with UV-Vis detector. The fractions purity were checked by eluting for first 5 min with 95% water and 5% methanol in a linear gradient to 100% methanol for 30 min and the absorbance was monitored at 254nm.

FIGURE II : SEX OF PATIENT

2.6 FT-IR spectrum

The infrared spectra were recorded on Shimadzu IR-470 model in the range of spectra 400–4000 cm⁻¹. The samples were subjected to FT-IR analysis through pressed KBr pellet technique. The spectra were plotted as intensity versus wave number.

2.7 Anti-cancer activity using MTT assay:

The anti-cancer work was carried out at Pondicherry Centre for Biological Sciences (PCBS). The colon cancer cell line (SW620) were plated separately using 96 well plates with the concentration of 1×10^5 cells/well in DMEM media in 1X Antibiotic Solution and 10% fetal bovine serum (Himedia, India) kept in the CO2 incubator at 37 °C with 5% CO2. The cells were washed with 200 µL of 1X PBS, and then the cells were treated with various test concentration of the compound in serum-free media and incubated at 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5mg/ml MTT prepared with 1X PBS was added and incubated at 37 °C for 4 h using the CO2 incubator. After the incubation period, the medium containing MTT was discarded from the cells and washed using 200 µL of PBS. The formed crystals were dissolved in 100 µL of DMSO and thoroughly mixed. The development of color intensity was evaluated at 570 nm [15].

$$\% \text{ cell inhibition} = \frac{A_{570} \text{ of treated cells} - A_{570} \text{ of control cells}}{A_{570} \text{ of control cells}} \times 100\%.$$

2.8 Statistical Analysis

Data were expressed as Mean ± S.D obtained from three independent experiments. The data were analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range test. The level of significance was set at $p \leq 0.05$ using SPSS statistics software version 19.

3. Results and discussion

3.1 Yield

The pharmacological properties of marine natural products have led to the discovery of many viable active molecules that are clinically significant. The morphology and feeding behavior [16, 17] of the *K. ornata* has been extensively studied. Such studies may be substantial in understanding the complex chemical structure of numerous potent metabolites. As there is limited data on chemical characterization of *K. ornata*, this work was focused on analyzing its molecular composition and biological activities. The yield of the lyophilized samples was estimated and was expressed in percentage as shown in Table 1.

Table 1. The yield of crude extracts of *K. ornata*.

Extracts	AIG	EIG	MIG
Yield (%)	9.868	7.092	5.526

AIG-crude acetone extracts of ink gland; EIG- crude ethyl acetate extracts of ink gland; MIG-crude methanol extracts of ink gland.

3.2 Fractionation by Ion Exchange Chromatography

The extracts were subjected to a 2-step purification using Column chromatography and dialysis. The collected fractions were pooled based on their bioactivity. From AIG (F4-F8) fraction, EIG (F3-F6) fractions and MIG (F4-F8) fractions were pooled.

3.3 Antimicrobial susceptibility test

The initial screening of antimicrobial activity was performed for fraction of methanol, acetone and ethyl acetate extracts of ink gland at the concentration of 100µg/mL. The antibacterial potential of AIG, EIG and MIG of ink gland showed moderate inhibition of *E. coli* and *S. aureus*, whereas *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were found to be resistant. Earlier, reported that methanol and acetone extracts of *Bursatella leachii*, *K. ornata* and *Aplysia* sp. exhibited significant activity against seven bacterial fish pathogens [11]. The glycoprotein contents of the ink fluid of the sea hare *A. kurodai* also inhibited *S. aureus*, *E. coli* and *Salmonella enteric* [18]. Hence, the present study suggested that extracts of *K. ornata* are not only significant against fish pathogens but are also effective against the human pathogens. The activity variation of these extracts towards the resistant or less susceptible pathogens could be due to the ineffective concentration of active molecules. The antifungal susceptibility test of AIG, EIG and MIG shows significant activity against *C. neoformans*. Similarly, the ink of *A. oculifera* and *A. fasciata* inhibited the growth of *Fusarium oxysporum* [19]. The methanolic whole body extract of *B. leachii* inhibited the growth of *Fusarium* sp. and *Aspergillus fumigates* [20]. Thus the extracts of AIG, EIG and MIG exhibited promising activity against the fungi *C. neoformans* when compared to the bacterial pathogens. The potency of extracts against other fungal pathogens has to be considered in this study. Such a comparison will help in the evaluation of antifungal molecules in the extract of *K. ornata*. The antimicrobial potential of the three different extracts methanol, acetone and ethyl acetate of IG were summarized in Table 2. All the three extracts exhibited positive inhibition against *E. coli*. The MIG and EIG showed inhibition against *S. aureus*, whereas the extracts were not effective against *K. pneumonia* and *P. aeruginosa*. In addition, the extracts possessed inhibitory activity against the fungi, *C. neoformans*.

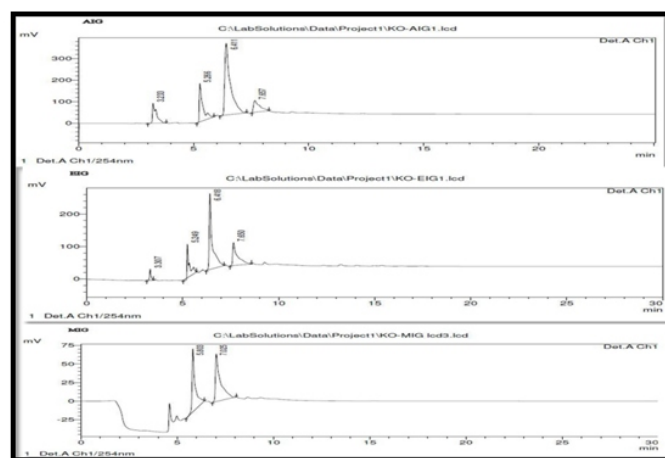
Table 2. Anti-microbial activity of crude extracts for AIG, EIG and MIG.

Human pathogens	Samples (mm)			
	AIG	EIG	MIG	STD
Anti-bacterial				
<i>Escherichia coli</i>	9 [±] 0.20	8 [±] 0.15	8 [±] 0.15	18 [±] 0.13
<i>Staphylococcus aureus</i>	-	7 [±] 0.11	7 [±] 0.15	12 [±] 0.15
<i>Klebsiella pneumonia</i>	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-
Anti-fungal				
<i>Cryptococcus neoformans</i>	11 [±] 0.15	11 [±] 0.57	11 [±] 0.52	16 [±] 0.12

The values are given as mean ± SD of three experiments (n=3) in each group.

3.3 RP-HPLC

Subsequently, the hydrophobic and hydrophilic properties of protein or compound play a significant role in their retention time on an RP-HPLC column and the separation of molecules depends on the ratio of polar (H₂O) and nonpolar (CH₃OH and CH₃CN). From this, we could interpret that nonpolar solvents were capable of extracting similar nature of compounds. Furthermore, MIG sample showed two sharp peaks at 6.3 and 7.5. Hence, through the HPLC chromatogram, it was observed that there was the presence of various polar and nonpolar compounds. The pooled fractions were analyzed using RP-HPLC on a Shimadzu C18 column and the absorbance of the eluted fractions was measured at 254 nm "Fig 1". Moreover, the purified fractions of AIG, EIG and MIG showed prominent peaks with a closer retention time of 5.26, 6.41 and 7.793; 5.29, 7.167 and 7.850; 6.36 and 7.56 respectively.

**Fig 1. HPLC chromatogram peak profile of AIG, EIG and MIG.**

3.4 FT-IR

FTIR spectrum for the purified fraction of AIG and MIG showed 8 peaks "Fig 2". Whereas, EIG fraction were recorded the 6 major peaks and 4 minor peaks. To identify the functional group of bioactive molecules in the fractions, the FT-IR analysis was performed. Accordingly, AIG, EIG and MIG spectrum showed peaks at 3332.99 cm⁻¹, 3331.07 cm⁻¹ and 3344.57 cm⁻¹, which is responsible for the presence of aliphatic primary amine (N-H stretch) group. Whereas, in EIG, a peak at 2943.37 cm⁻¹ marked the CH₂ and CH₃ groups contained mainly in the lipid acryl chains. Likewise, the amino acid side chains and fatty acids signals were indicated at the frequency of 1450 cm⁻¹ while the peak at 1024 cm⁻¹ shows the presence of primary amine C-N stretch and glycogen vibrations. The peaks at 1639.49 cm⁻¹ and 1635.64 cm⁻¹ for AIG along with MIG fraction denotes C=O stretching, which occurs for the similar wavelength of polyamides and protein whereas, the disulfide C-S stretch was found at a wave number of 659.66 cm⁻¹. The peaks found at 553.57 cm⁻¹ and 597.93 cm⁻¹ revealed the aliphatic iodo compounds in AIG and MIG samples respectively. In addition, three peaks were observed at 449.4 cm⁻¹, 445.56 cm⁻¹ and 416.62 cm⁻¹ respectively in AIG, EIG and MIG. These peaks revealed the presence of aryl sulfide groups in the respective bioactive fractions. The FT-IR result resembled merely with the tissue extracts from *Conus inscriptus*[21] and *C. betulinus*[22]. Hence, the FTIR spectrum exposed the presence of various functional groups that could play a vital role in the structure and activity of these compounds in *K. ornata*.

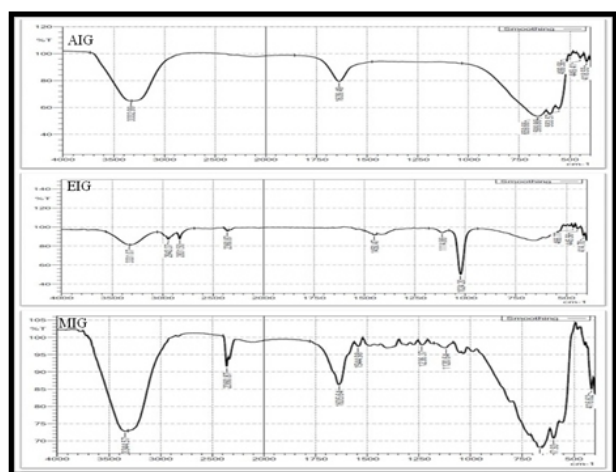


Fig 2. FTIR spectrum for AIG, EIG and MIG.

3.5 Anti-cancer study

The effect of ink gland fractions on cell proliferation strategies was calculated by MTT assay. The SW620 cell line proliferation was extensively inhibited by all the three fractions after 24 h of incubation. The AIG fractions showed higher anti-cancer activity in comparison with the EIG and MIG. The percentage of anti-proliferative activity was found to be dose-dependent. The cytotoxicity and anti-cancer activity of AIG, EIG and MIG in Vero cells

as well as colon (SW620) cancer cells recorded at 25µg-500µg/mL "Fig 3a & 3b" indicated variability in the cell viability percentage of both control and treated cells. The dose dependent studies of cytotoxicity on normal Vero cell lines revealed that the AIG, EIG, and MIG had a LC50 Value of 745 µg/mL, 725 µg/mL, and 735 µg/mL. The 25µg/ml of AIG and EIG did not show any significant inhibition whereas, the 25µg/ml MIG showed minimal proliferation on colon cancer cell line (9%). At 250µg/mL concentration, AIG exhibited moderate inhibitory activity (38.63%), whereas EIG and MIG exhibited lower inhibition (18.44% and 14.35%) on SW620 cells. Subsequently, AIG and EIG showed moderate inhibition on SW620 cells (41.06% and 30.36%) at the concentration of 500µg/mL when compared to other MIG shows lesser inhibition (15.23%) at the same concentration. Hence, efforts have been made in this work to identify the anticancer potential of *K. ornata* ink gland extracts. The highest anticancer activity of *K. ornata* extract (AIG) was found to be 41.06% at 500 µg/ml concentration against the colon cancer cell line (SW620). The fractions of *K. ornata* showed higher inhibition than three different hydrolysates (AH, TH, PH) of *Styela clava*, on human colon cancer line (DLD-1) as 8%-22%, stomach (AGS) as 22%-31.6% and cervical (HeLa) cells as 15-31.9% at 1000 µg/ml [23]. In addition, protein hydrolysate of *Saccostrea cucullata* has been found to exhibit 72% cytotoxicity activity at 100 µg/ml concentration against (HT-29) colon cancer cell line in a dose-dependent manner, which was higher than the activity of ink gland extracts of *K. ornata*[24]. The acetone extracts of *K. ornata* were found to have a more anti-proliferative effect than EIG and MIG. This could be potential due to the presence of fatty acid moieties as found in the GC-MS analysis. Various other cancer studies have been conducted for methanol extracts of marine species. Likewise, the methanolic extract of marine sponge *Spongia tosta* (73.24% at 1000 µg/ml) against human breast carcinoma MCF-7 cell line has been reported [25].

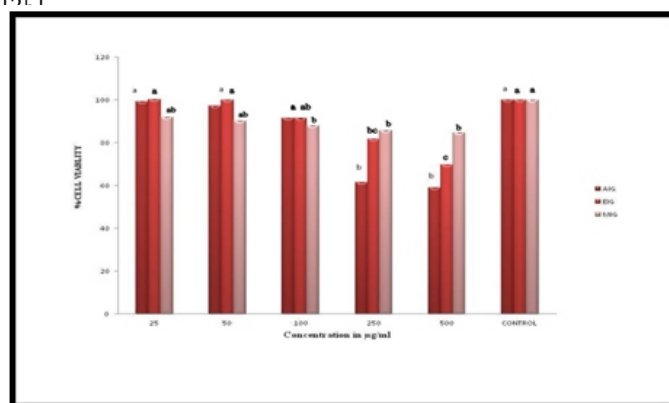


Fig 3a. Anti-cancer activity of AIG, EIG and MIG on colon cancer (SW620) cell line.

The values are given as mean ± SD of three experiments (n=3) in each group. Mean values followed by different superscript letters in the same column are significantly (p<0.05) different.

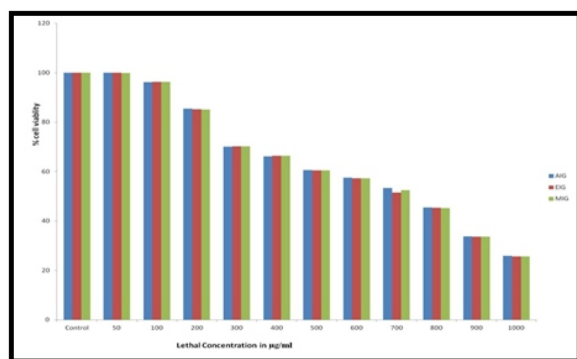


Figure 3b. LC50 determination of AIG, EIG and MIG on Vero cell line.

4. Conclusion

Antimicrobial and anticancer drugs are essential for human health concern. The contribution of marine chemical diversity to explore the potent drug candidate is an emerging trend for various diseases. In the present study, the acetone fractions of *K. ornata* exert a significant anti-proliferative activity on human colon cancer cell line. The compositional characterization of the fractions suggests that molecules could be a potent source of anticancer or antimicrobial agents. The study also demonstrates that acetone fractions are more effective as anti-carcinogenic agents than acetone and ethyl acetate fractions. Hence, we hypothesize that the ester compound present in the AIG fractions could be dominant in the expression of the anticancer activity. Subsequently, studies will focus on evaluating the molecular mechanisms of these fractions against the targeted diseases.

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Conflict of interest:

The authors declare that there are no conflict(s) of interests.

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