ISSN: 2520-5234

*Available online at* http://www.sjomr.org

SCIENTIFIC JOURNAL
OF MEDICAL RESEARCH

Vol. 3, Issue 12, pp 139-145, Autumn 2019

ORIGINAL ARTICLE

# Cytotoxic Effect of Ink Extracted from Cephalopoda on Cancer Cell Line

**Israa H. Khudhair** [1], **Dhamia K. Suker** [2] and **Balsam A. Hanna** [3]

[1] Educational directorate of Basrah, Basrah, Iraq
[2, 3] Department of Biology, College of Science, University of Basrah, Basrah, Iraq.

**ARTICLE INFORMATIONS**

**Article History:**
Submitted: 30 October 2019
Revised version received:
22 November 2019
Accepted: 25 November 2019
Published online: 1 December 2019

**Key words:**
*Sepia* sp. Crude ink
Melanin free ink
Anticancer
Biochemical

**Corresponding author:**
Israa H. Khudair
Email: bioesraa55@gmail.com
Educational directorate of Basrah
Basrah
Iraq

**ABSTRACT**

**Objectives:** The present study describes the biochemical, antioxidant and anticancer properties of the crude and melanin free ink of *Sepia* which is rich in both organic and inorganic components.

**Methods:** The specimens of *Sepia sp.* were collected from Fao, South of Basrah /Iraq during November and December of 2018. They held the collected animals ventrally and squeezed the animal's posterior end to eject ink. The extracted ink samples both crude and melanin free ink were lyophilized, weighed and stored at 4°C until use .Then determination of the moisture, ash, total protein, lipid and carbohydrate in samples. The activity of ink as antioxidant was determination by the DPPH method. Finally, evaluation of anticancer activity by the MTT assay.

**Results:** The quantitative analysis define the presence of protein (1.1 – 1.3 mg/ml), lipid (1.38 – 1.48 mg/ml), carbohydrates (0.01 –0.9mg/ml), ash (14and5%) and moisture (91.7-83.75 %) content.. The ink revealed good antioxidant activity, thereby lowering or retarding the initiation of lipid oxidation process. The ink was screened for its anticancer activity against human breast adenocarcinoma cell line (MCF 7) and it exhibited a strong cytotoxicity by inhibiting the cell growth.

**Conclusion:** Thus the ink, especially the melanin free ink is known to possess significant potent antioxidant activity and anti-proliferative effect. The present study showed that the melanin free ink was richer in biochemical properties than the crude ink.



**Citation: Khudair I.H., Hanna B.A. and Sugar D.K.** "Cytotoxic Effect of Ink Extracted from Cephalopoda on Cancer Cell Line". Sci. J. Med. Res. 2019; 3 (12): 139-145.

## INTRODUCTION

Cancer is one of the diseases that spreads worldwide, causes morbidity and death, the new cases of cancer increase during the last two decades [1]. It is a disease which is characterized by uncontrolled cell multiplication and doesn't obey the ordinary laws of cell division. The normal cells are continuously exposed to many signals that stimuli it to the division, it will convert to another cell or die. The cancer cell usually develops and emigrates from side to another by metastasis. metastasis is responsible for 90% of cancer related deaths [2].

Breast cancer has become one of the main types of high mortality cancer [3] and due to tumor heterogeneity and the

production of multidrug resistance phenotypes, it is not immune to the latest cytotoxic agents used in chemotherapy.

Cancer can become lethal due to lack of effective drugs, expensive chemotherapeutic agents and side effects of anticancer drugs, cancer can become fatal. Till now the availability of treatments for breast cancer remains unsatisfactory [4]. Several marine derived compounds are currently extracted and synthesized by chemical process for cancer treatment.

The marine environment is a rich source for biologically active natural products with different structurally active natural products, most of which have not been detected in terrestrial sources. Aquatic species and their surrounding environment have been antitumor, antiviral, antibacterial, anticoagulant, hemolytic, analgesic, cardioinhibitory, anticonvulsant, vasopressant and other effective substances. The marine organisms have had a major impact on the development of medical science. More recent studies on marine organisms have focused mainly on their application for the treatment of human diseases. Many marine chemicals often possess quite novel structures which lead to pronounced biologically activity and novel pharmacology [5].

The Cephalopoda, the largest group of molluscs, is predominantly aquatic, intelligent and has many medicinal properties [6]. Cephalopods have long recognized cephalopods as an adaptive response to predation and physical threat through a variety of mechanisms including chemical deterrence, sensory disruption and phago-mmicry [7] .

The melanin pigment is formed in the mature cells of the ink gland, a highly specialized organ at the bottom of the ink sac. Cephalopod ink is an unsettling material that confuses predators and warns about risk specifics [8]. Sepia, commonly referred to as cuttlefish, whose pigment in a viscous colorless medium consists of melanin granules. The melanin pigment is formed in the mature cells of the ink gland, a highly specialized organ at the bottom of the ink sac. Melanin isolated from ink sac has been proposed as a standard for natural eumelanin. The cuttlefish ink finds wide application in homeopathic medicine and *Sepia.sp* ink is used to treat hormonal imbalances especially in women [9]. It is also used to treat kidney stone, gonorrhoea and it also act as anti-oxidant, anti-radiation, antiretroviral, antibacterial and anticancer agents.

Sepia ink is a multifunctional marine bioactive agent that destroys cancer cells [10, 11, 12]. The ink has potential medical use in health care and medicine as a healthy natural product.

## MATERIALS AND METHODS

**Collection and identification of experimental animal:** The specimens of *Sepia* sp. were collected from Fao, South of Basraha /Iraqa during November and Decemberof 2018and identified [13]. The animals were preserved in ice and were brought to the laboratory within 24 hours of capture.

**Preparation of ink sample:**They held the collected animals ventrally and squeezed the animal's posterior end to eject ink. The ink was collected and processed at -20 C[14]. The crude ink specimen was split into 2 parts and one part of it was centrifuged to extract melanin pigment at 13,000 rpm for 45 minutes at 4 C°to remove the melanin pigment [9]. The supernatant obtained was referred to as 'melanin-free ink' and was used for further analyses. The another part remined as crude ink. The extracted ink samples both crude and melanin free ink were lyophilized, weighed and stored at 4°C until use.

**Determination of moisture content:** Method of Association of Official Analytical Chemists (AOAC)[15] determined the moisture content in the samples. The weight difference between the wet sample and the lyophilized samples was calculated and expressed as the sample's percentage of humidity content.

**Determination of ash content:** The amount of ash was calculated by the AOAC process. Weighed the samples into clean dry beakers and put in the hot air oven at 100oC for 24 hours. The specimens were moved to the muffle furnace crucibles and incinerated until they were free of black carbon particles and turned white in colour. The temperature was kept for about 5 hours at 550 °C. The samples were refreshed and weighedand the percentage of ash content was determined.

**Determination of Proteins:** The amount of total protein in the samples were determined by the method of Bradford [16]. The samples were mixed with 5 ml of Bradford reagent. Protein standard (BSA) solution was also prepared and absorbance was read at 595 nm using Shimadzu 160 UV-VIS double beam spectrophotometer. Standard curve was prepared and the amount of protein in the ink samples of *Sepia* sp.were calculated.

**Determination of lipids:** The total quantities of lipids in the samples was measured using the sulpho-phospho-vanillin method as stated in [17] Standard olive oil was taken in different concentrations and the ink samples were heated for 10 minutes in a boiling water bath, cooled and put in a dry pipe. A blank sulphuric acid containing 6 ml of phospho-vanillin reage was applied to eachwas added 6 ml of phospho-vanillin reagent and kept at room temperature for 15 minutes. The absorbance was read at 546 nm in UV visible spectrophotometer.

**Determination of antioxidant activity:** DPPH radical scavenging activity was calculated using the Blois [18] method and updated by [19]. Ink specimens were prepared at concentrations of 200-800 µg / ml and 0.1 mM of DPPH were applied to the sample in 95% (v / v) ethanol. Ascorbic acid was used as the reference standard and was used instead of the sample in the empty deionized water. The mixture was allowed to stand 30 minutes in the dark at room temperature. A spectrophotometer was used to measure the absorbance of the resulting solution at 517 nm. The ink samples' antioxidant activity was expressed as IC50 and compared to standard.

**Evaluation of anticancer activity**: MTT Assay: The human breast adenocarcinoma cell line MCF 7 were plated in 96 well plate (1 x 105/well) with DMEM medium containing 10 % FBS. The cytotoxic effect of ink samples was assessed by MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5- diphenyltetrazolium bromide) method of [20]. The plates were sealed with a self – adhesive film. Then, they were incubated for 24-48 h. to obtain Confluent. The medium was removed and serial dilutions of maintenance medium were prepared [21, 22]. Then three replicates of cells were treated with ink sample at the following concentrations 400, 600, 800 and 1000 µg/ml according to [23], six wells were used for control seeding cells in serum free medium only . The plates were re-incubated at 37 °C for the selected exposure time 24 h.

After incubation, the medium was decanted and 28 ul. of MTT dye were added to each well and incubated for 2 h at 37 °C. Then, the excess dye was removed and then 130 µl of Dimethyl Sulfoxide Solution( DMSO) was added to each well to dissolve and extract the dye from the viable cells .Then, the plates were placed on a shaker for 15 min. Optical density(O.D.) of living cells of each well in plate (set two) was read using Enzyme Linked Immunosorbent Assay (ELISA) Reader at a transmitting wavelength of 492 nm [21, 22]. The percentage of cytotoxicity was calculated according to [24].

as: (A-B)/A X100.

where A:was the mean optical density of untreated wells

B:was the optical density of wells with ink sample

**Cytomorphological Examination:** MCF-7 cells was trypsinized, 5 ml of growth medium was added to cell suspension and was placed in the sterilized petri plate. After 2 days the monolayer of cells formed was observed under Inverted microscope and photographed.

## RESULTS

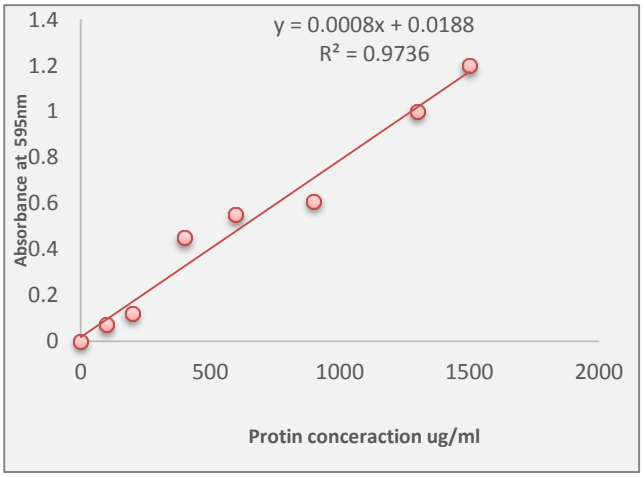
**Determination of Moisture and ash content:** The percentage of moisture present in both the ink samples with and without melanin was 83.75 and 91.7 which indicates the presence of high water content. The percentage of ash in the lyophilised crude ink and melanin free ink were 14% and 5% respectively.

**Quantitative analysis of the ink:** Table 1 offers quantitative ink analysis: the amount of protein, lipid and carbohydrate in crude ink solid (CIS), melanin free ink solid (MFS) There was a slight variation in the amount of proteins, lipids and carbohydrates within the lyophilized solid. The amount of each of the coarse and melanin-free specimens also varied. The density of the ink was lower overall.

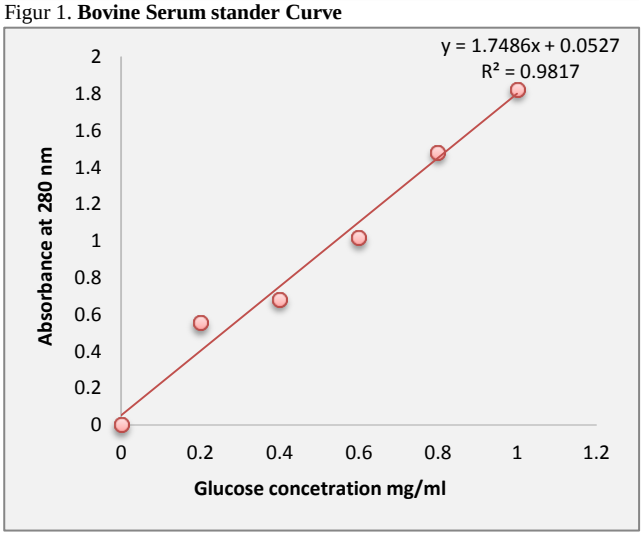
There was a slight variation in the amount of proteins, lipids and carbohydrates within the lyophilized solid. The quantity of each of the crude and melanin-free samples also differed. The ink had a lower carbohydrate concentration overall compared to the proteins and lipids. Proteins were the main components of raw and melanin-free pigmentthe results Figurs 1, 2 and 3.

Table 1: **Biochemical composition of *Sepia* sp. ink.**

| Samples | Protein mg/ml | Lipid mg/ml | Carbohydrate mg/ml |
|---|---|---|---|
| **Crude ink** | 1.1+_0.14 | 1.38+_0.09 | 0.01+_0.07 |
| **Melanin free ink** | 1.3+_0.14 | 1.48+_0.08 | 0.9+_0.09 |




Figur 1. **Bovine Serum stander Curve**




Figur 2. **Glucose Stander Curve.**

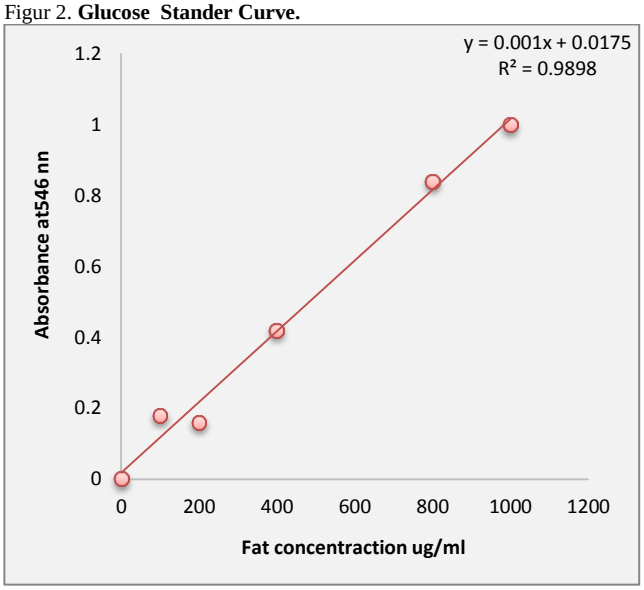



Figure 3. **Lipid stander curve**.

**Determination of antioxidant activity:** The antioxidant activity was slightly higher in melanin free ink than in crude ink sample (Table 2). The $IC_{50}$ of melanin free ink sample was (215.157) which showed that it has slightly higher antioxidant activity compared to crude ink.

Table 2: **DPPH free radical scavenging effect of *Sepia* sp. ink.**

| Concentration (µg/ml) | Crude ink %Of free radical scavenging activity | Melanin free ink | Ascorbic acid |
|---|---|---|---|
| 200 | 50.6±0.14 | 51.4±1.49 | 61.55±0.13 |
| 400 | 52.74±0.29 | 53.59±2.98 | 89.17±0.141 |
| 600 | 64.45±0.29 | 67.61±0.29 | 93.39±0.127 |
| 800 | 70.04±0.148 | 73.62±0.148 | 93.75±0.134 |
| IC50 | 222.77 | 215.157 | 46 |

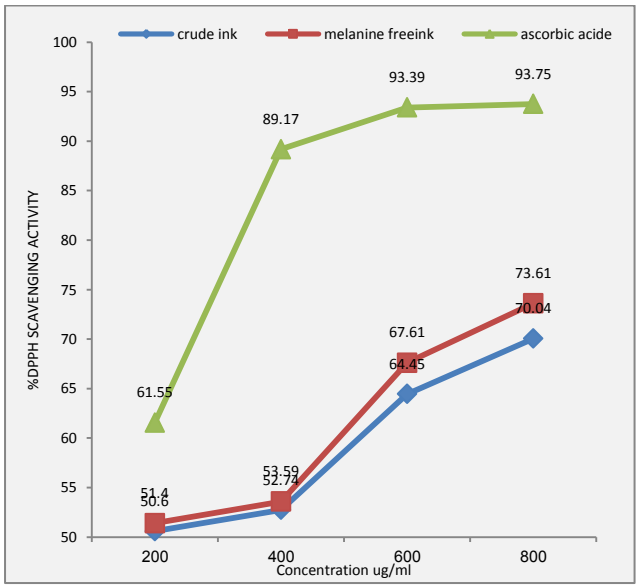
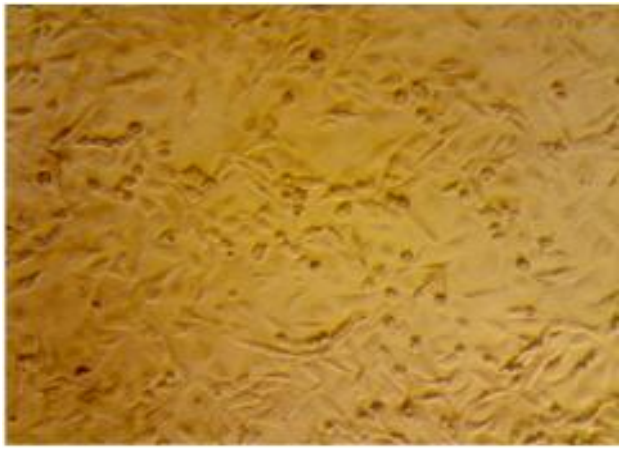
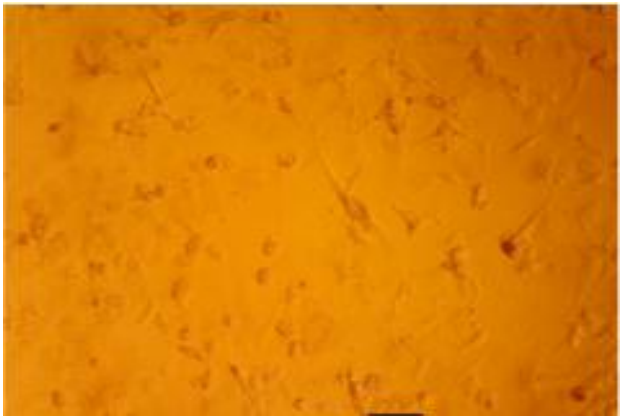



Figure 4. **DPPH radical scavenging activity of crude ink and melanin free ink of *Sepia* sp.**

**Evaluation of anticancer activity:** The cells of the MCF-7 Human breast adenocarcinoma appeared in polygonal form and the cell mat was very intact (Figure 5-a). The wells treated with the ink showed dramatic changes in cell viability. Variations are observed in the percentage of cell growth inhibition with cell shrinkage and cell breakup resulting in the cell aggregation (Figure 5-b-c-d-e).

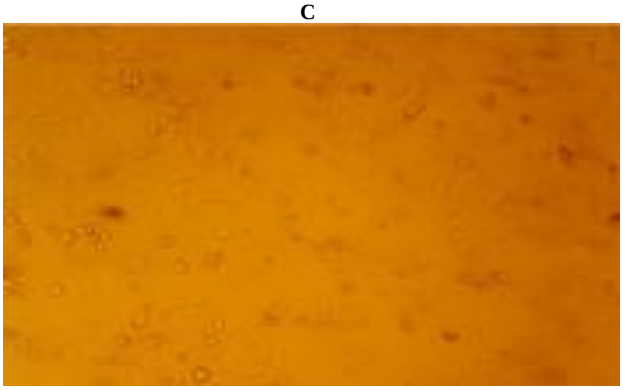


**A**



**B**

**C**



**D**

**E**

Figure 5. **Effect of Crude ink and melanin free ink of *Sepia* .sp at concentration 800,1000ug/ml and morphology of mcf 7 cancer cell for 24 hrs of treatment.**
**A:**Control cells; **BandC:** Cells treated with 800ug/ml,1000ug/ml of crude ink**. DandE:** Cells treated with melanin free ink.

The findings of the simple Sepia ink test for cell viability and toxicity were reported in (Figures 5 and 6). The percentage of cell viability varied from 64.23 to 97.37. The percentage of cell viability was decreased with increased concentration (400µg/ml 97.37%, 600µg/ml -94.14%, 800µg/ml – 85.9%,1000ug/ml64.23) of the substance and the percentage of toxicity showed variation in between 2.63 and 35.72.

Peak toxicity (35.72%) was reached at 1000 µg crude Sepia ink concentration and minimum (2.63%) at 400 µg crude ink concentration. A similar study was conducted by [11] to test the capacity for squid ink anticancer in vivo mod [11].

Effect of melanin free ink on MCF7cell was shown in Figures (7 and 8) MCF7 experienced a significant decrease in viability at low concentration (400µg/ml 57.48%) of Sepiaink, with an eventual decline at the highest concentrations (1000µg/ml – 37.83%) tested. The viability ranged from 37.83%-57.48%. The toxicity varied from 42.53-62.18 %. Maximum toxicity of 62.18% was observed at 1000µg concentration of the melanin free ink Sepia ink and minimum of 42.53% was observed at 400 µg concentration. IC50 value was obtained at a concentration of 1000 (concentration causing death of 50%).

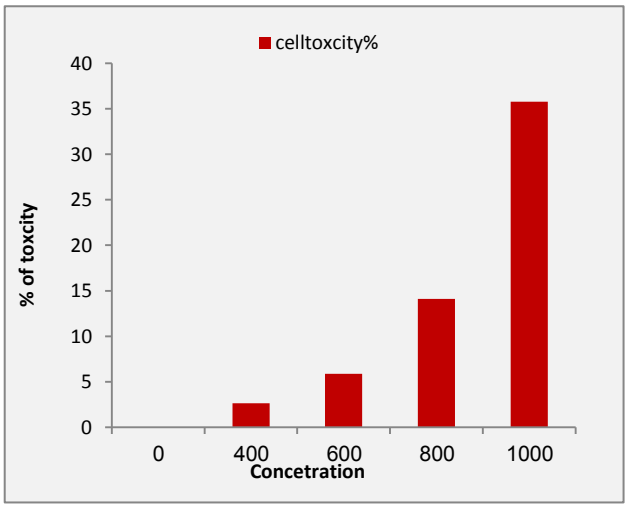



Figure 6. **Analysis of cell toxicity – crude *Sepia* ink**

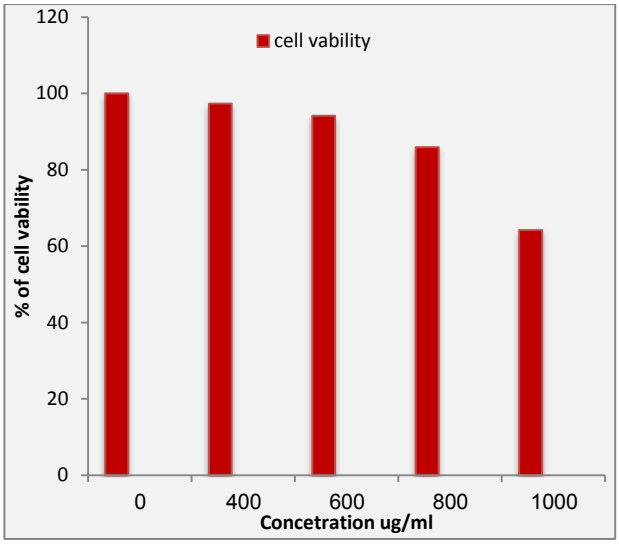



Figure 7. **Analysis of cell vability– crude *Sepia* ink**

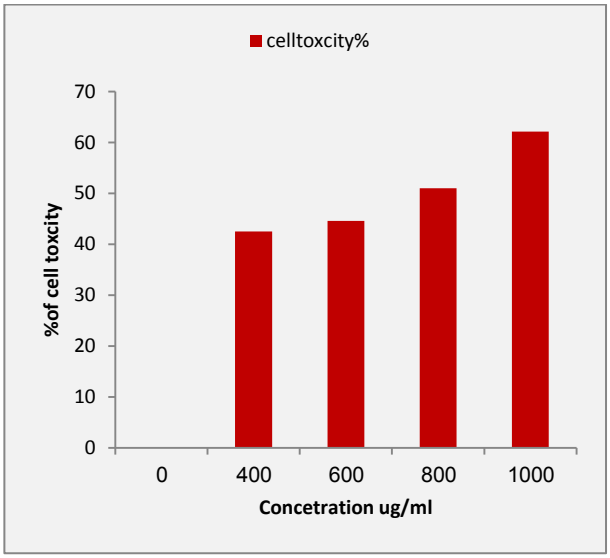



Figure 8. **Analysis of cell toxicity melanin free ink *Sepia* ink**

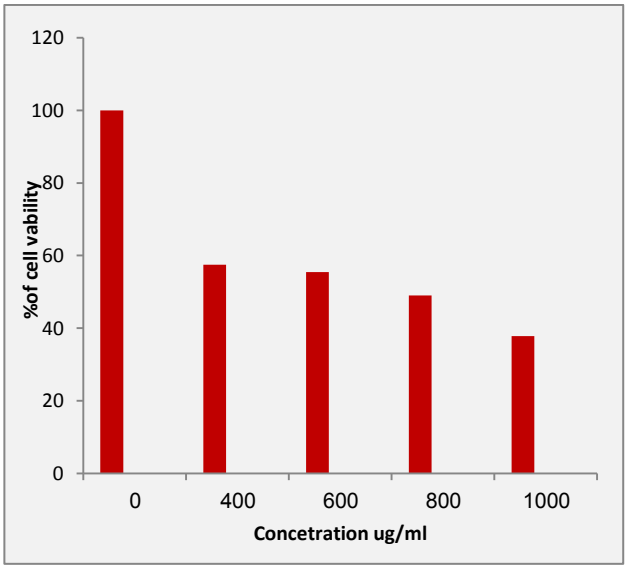



Figure 9. **Analysis of cell vability of melanin free ink *Sepia* ink**

## DISCUSSION

Marine natural products appear to be a source of bioactive metabolites that is structurally complex and most important in pharmacology. Some of them have great potential to develop new and much-needed drugs primarily in diabetic, inflammatory, cancer, etc. treatment. Sepia ink is extremely rich in important nutrients like protein, lipid and carbohydrate. The current study was designed to evaluate the biochemical properties, and to determine its antioxidant and anticancer propertiesThe ink was obtained as indicated in the [14] process. The goal of this analysis was to investigate the properties of both Sepia sp. crude ink and melanin-free ink in order to extend the application of ink. For storage purposes, the ink was lyophilized and protein degradation was prevented.

The sticky existence is due to mucus [25] presence. Sepia's ink consists not only of melanin, but also rich in organic and inorganic components, according to [26], our results were consistent with his reports. The amount of moisture in both raw and melanin-free ink was almost identical. The ash content in crude ink was higher and in melanin free ink was lower. The difference in percentage may be due the melanin pigment composed of minerals like calcium, potassium, magnesium and iron [27]. Protein content was higher in melanin free ink lyophilized samples than crude ink samples. The *S. prabahari* ink consists of a good amount of protein and this indicates the biological activity of the ink as reported earlier in other species of sepia [28]. The percentage of carbohydrate content is very less when compared to the other biochemical components of the ink, where the melanin free ink was rich in sugars than crude ink. The lyophilized ink sample had less carbohydrate but the liquid ink sample had high amount of carbohydrates. Our results were in correspondence with the reports of [29]. Determined lipid content was consistent with previous studies, increased fat content in melanin-free ink samples relative to crude ink was observed.

It indicates the Sepia biochemical composition. Go hand in hand with the findings of other sepia [30] species biochemical components. Sepia ink has acquired unique space in the biomedical application, for example high in antioxidants, which as well as all know help protect the cells and the heart from free radicals damage. This means that Sepia ink may be useful in the fight against obvious signs of aging, heart disease and various immune threats [29].

DPPH-free radical scavenging testing is a simple, fast, and sensitive method to screen animal extracts with antioxidants [31]. The radical scavenging activities of DPPH are based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them into non-radical species [32]. DPPH is a radical with an odd electron and reacts with hydrogen donated from an electron. he radical DPPH receives another electron and the absorption decreases [33]. The sepia extracts in the present study have a high capacity for DPPH scavenging, which increased with increased concentration (Table 2). The free operation of radical scavenging was high in melanin free ink relative to crude ink. Some melanin-free ink compounds may chelate pro-oxidant metals, reducing or retarding the inkof lipid oxidation process [34].

The development of novel chemotherapeutic agents would play a key role in the treatment of refractory or relapsing cancer patients. The assay of *S. prabahari* ink against MCF-7 breast cancer cell lines revealed good anticancer activity. The melanin free ink had a higher rate of antiproliferative activity than the crude ink [26].

## CONCLUSIONS

It is noted in this study that melanin-free ink consists of abundant biochemical components other than crude ink such as water, minerals, proteins, lipids and carbohydrates. The melanin-free ink is more active as antioxidants and anti-cancer agentsIt is evident that after melanin removal, a unique molecular mechanism is involved in the melanin-free ink. Since the ink is a cheap and readily available waste material from the processing industry, it will make sense to look at ink through further studies as a potential raw material for drug production.

## REFERENCES


1. Stewart B.W. and Wild C.P. "World cancer report". 2014; ISBN 978-92-832-0429-9.
2. Hejmadi M. "Introduction to Cancer Biology". Bookboon. 2010; ISBN: 978-87-7681-478-6.
3. Serag H.B. and Mason A.C. "Rising incidence of hepatocellular carcinoma in the United States". New England Journal of Medicine. 1999; 340(10): 745-750. DOI:10.1056/NEJM199903113401001.
4. Honda K., Sbisa E., Tullo A., Papeo P.A., Saccone C., Poole S. and Davies A. "p53 mutation is a poor prognostic indicator for survival in patients with hepatocellular carcinoma undergoing surgical tumour ablation". British journal of cancer. 1998; 77(5): 776-782. doi: 10.1038/bjc.1998.126.
5. Lei J. and Zhou J. "A marine natural products Database". J. Chem. Inf. Comp. Sci. 2001; 42: 742- 74.
6. Mohan J. "Studies on some aspects of landings, utilization and export of commercially important cephalopods". 2007.
7. Nair J.R., Pillai D., Joseph S.M., Gomathi P., Senan P.V. and Sherief P.M. "Cephalopod research and bioactive substances". Indian Journal of Geo-Marine Sciences. 2011; 40(1): 13-27.
8. Jereb P. and Roper C.F. "Cephalopods of the World. an Annotated and Illustrated Catalogue of Cephalopod Species Known to Date. Volume 1. Chambered Nautiluses and Sepioids (Nautilidae, Sepiidae, Sepiadariidae, Idiosepiidae and Spirulidae)". Rome, Italy: FAO. 2006.
9. Sherief P. and Nair J.R. "Anticancer property of purified fraction c2 of cuttlefish (sepia pharaonis) ink on cervical cancer cells". Indo American Journal of Pharmaceutical Research. 2013; 3: 7444-54.
10. Takai M. "Comparative studies on microbiological and chemical characteristics of" Ika-shiokara Akazukuri" and" Ika-shiokara Kurozukuri". Nippon Suisan Gakkaishi. 1992; 58(12): 2373-78. https://doi.org/10.2331/suisan.58.2373
11. Sasaki J.I., Ishita K., Takaya Y., Uchisawa H. and Matsue H. "Anti-tumor activity of squid ink". Journal of nutritional science and vitaminology. 1997; 43(4): 455-46. DOI:10.3177/jnsv.43.455.
12. Rajaganapathi J., Thyagarajan S.P. and Edward J.K. "Study on cephalopod's ink for anti-retroviral activity". Indian J Exp Biol. 2000; 38(5): 519-20.

13. Neethiselvan N. and Venkataramani V.K. "Sepia prabahari sp. nov.(Mollusca/Cephalopoda), a new species of Acanthosepion species complex from Tuticorin bay, southeast coast of India". Indian journal of marine sciences. 2002; 31(1): 45-51.
14. Fiore G., Annarita P.O.L.I., Di Cosmo A., d'ISchia M. and Palumbo A. "Dopamine in the ink defence system of Sepia officinalis: biosynthesis, vesicular compartmentation in mature ink gland cells, nitric oxide (NO)/cGMP-induced depletion and fate in secreted ink". Biochemical Journal. 2004; 378(3): 785-791.
15. AOAC. "Official Methods of Analysis of Association of Official Analytical Chemists (AOAC) International". 19th Edition, 2012.
16. Bradford M.M. "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding". Analytical biochemistry. 1976; 72(1-2): 248-254. https://doi.org/10.1016/0003-2697(76)90527-3.
17. Barnes H. and Blackstock J. "Estimation of lipids in marine animals and tissues: detailed investigation of the sulphophosphovanilun method for total lipids". Journal of Experimental Marine Biology and Ecology. 1973; 12(1): 103-118.
18. Blois M.S. "Antioxidant determinations by the use of a stable free radical". Nature. 1958; 181(4617): 1199-1200.
19. Binsan W., Benjakul S., Visessanguan W., Roytrakul S., Tanaka M. and Kishimura H. "Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shrimp (Litopenaeus vannamei)". Food Chemistry. 2008; 106(1): 185-193.
20. Mosmann T. "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays". Journal of immunological methods. 1983; 65(1-2): 55-63. DOI:10.1016/0022-1759(83)90303-4.
21. Mahony D.E., Gilliat E., Dawson S., Stockdale E. and Lee S.H. "Vero cell assay for rapid detection of Clostridium perfringens enterotoxin". Applied and Environmental Microbiology. 1989; 55(9): 2141- 2143.
22. Fresheny R.I.A.W. "Culture of animal cells. Amanual of Basic Technique". 3rd ed. A John Wiley and sons. Ins .New York. 1994.
23. Lai J.C.K ., Lai M.B., Jandhyam S., Dukhande V.V., Bhushan A. Daniels C.K. and Leung S.W. "Exposure to titanium dioxide and other metallic oxide nanoparticles induces cytotoxicity on human neural cells and fibroblasts". International Journal of Nanomedicine. 2008; 3(4): 533–545. doi: 10.2147/ijn.s3234.
24. Betancur-Galvis L., Morales G., Forero J. and Roldan J. "Cytotoxic and antiviral activity of Colombian medicinal plant extract of the Euphorbia genus". Mem. Inst. Oswaldo Cruz. Rio de Janeiro. 2002; 97(4): 541-546.
25. Senan V.P., Sherief P. and Nair J.R. "Anticancer property of purified fraction c2 of cuttlefish (sepia pharaonis) ink on cervical cancer cells". Indo American Journal of Pharmaceutical Research. 2013; 3(9): 7444-54.
26. Zheng G.L., Zhang X.Y., Zhou Y.G., Meng Q.C. and Gong W.G. "Comparison between components and trace elements in sepia and squid ink". Chinese Journal of Marine Drugs. 2002; 21(3): 12-14.
27. Magarelli M., Passamonti P. and Renieri C. "Purification, characterization and analysis of sepia melanin from commercial sepia ink (Sepia Officinalis)". Revista partial CES Medicina Veterinariay Zootecnia. 2010; 5(2): 18-28.
28. Vate N.K. and Benjakul S. "Antioxidative activity of melanin-free ink from splendid squid (Loligo formosana)". International Aquatic Research. 2013; 5(1): 9.
29. Mimura T., Maeda K., Hariyama H., Aonuma S., Sataka M. and Fujita T. "Purification of melanin from OmmastrephesbastamiLesueur and its inhibitory activity in gastric juice secretion in rats". Chem. Pharm. Bull. 1982; 30(4): 1381-1386.
30. Liu H., Luo P., Chen Sand Shang J. "Effects of Squid Ink on Growth Performance, Antioxidant function s and immunity in growing broiler chickens". Asian-Aust. J. Anim. Sci. 2011; 24(12): 1752-1756. DOI: 10.5713/ajas.2011.11128.
31. Hagerman A.E., Riedl K.M., Jones G.A., Sovik K.N., Ritchard N.T., Hartzfeld P.W. and Riechel T.L. "High molecular weight plant polyphenolics (tannins) as biological antioxidants". Journal of agricultural and food chemistry. 1998; 46(5): 1887-1892.
32. Chandrasekara A. and Shahidi F. "Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity". Journal of agricultural and food chemistry. 2010; 58(11): 6706-6714. DOI:10.1021/jf100868b.
33. Brand-Williams W., Cuvelier M.E. and Berset C.L.W.T. "Use of a free radical method to evaluate antioxidant activity". LWT-Food science and Technology. 1995; 28(1): 25-30. https://doi.org/10.1016/S0023-6438(95)80008-5.
34. Maqsood S. and Benjakul S. "Synergistic effect of tannic acid and modified atmospheric packaging on the prevention of lipid oxidation and quality losses of refrigerated striped catfish slices". Food Chemistry. 2010; 121(1): 29-38. https://doi.org/10.1016/j.foodchem.2009.11.086.