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ANTITUMORAL ACTIVITY IN INKS OF *SEPIA OFFICINALIS* AND *OCTOPUS VULGARIS* (CEPHALOPODA) FROM THE NORTHERN TUNISIAN COAST (CENTRAL MEDITERRANEAN SEA)

Emna SOUFI-KECHAOU & Ichrak SARIYA
Department of Animal Sciences, Halieutics and Food Technology, National Institute of Agronomy of Tunis, 43 avenue Charles Nicolle, 1082-Tunis-Mahrajène,Tunisia
e-mail: esoufi@gmail.com

Amine BEZAA, Neziha MARRAKCHI & Mohammed EL AYEB
Laboratory of Venoms and Toxins, Pasteur Institute of Tunis. 13, place Pasteur, B.P. 74. 1002Tunis-Belvédère, Tunisia

ABSTRACT

In the present study, authors investigate the antitumor effects of peptides from the ink of two cephalopod species: the common cuttlefish, Sepia officinalis *(Linnaeus, 1758), and the common octopus,* Octopus vulgaris *(Cuvier, 1797), from specimens sampled in the northern Tunisian coasts (central Mediterranean). The results indicate that the crude ink show anti-adhesion properties of the IGR39 cells depending on the Extra Cellular Matrixes (ECM) tested. The partially purified fractions with a molecular weight inferior to 10 kDa for* Sepia officinalis *(F_{inf10}) and the superior to 10 kDa for* Octopus vulgaris *(F_{sup10}) revealed anti-invasion, anti-migration and anti-adhesive activities on the U87 glioma cell lines, with a dose-dependent response. No antiproliferative activity was found for both of the partially purified fractions and the MTT assay showed toxicity effect only for high ink fraction concentrations.*

Key words: Cephalopoda, *Sepia* ink, *Octopus* ink, antitumor, enzymatic hydrolysis, oligopeptide, Tunisia, central Mediterranean

ATTIVITÀ ANTITUMORALE DI INCHIOSTRI DI *SEPIA OFFICINALIS* E *OCTOPUS VULGARIS* (CEFALOPODA) PROVENIENTI DALLA COSTA SETTENTRIONALE DELLA TUNISIA (MEDITERRANEO CENTRALE)

SINTESI

Nel presente studio gli autori analizzano gli effetti antitumorali dei peptidi ricavati dall'inchiostro di due specie di cefalopodi: la seppia comune, Sepia officinalis *(Linnaeus, 1758), e il polpo comune,* Octopus vulgaris *(Cuvier, 1797), provenienti da campioni prelevati lungo la costa settentrionale della Tunisia (Mediterraneo centrale). I risultati indicano che l'inchiostro grezzo mostra proprietà di anti-adesione delle cellule IGR39, in dipendenza delle matrici extra cellulari (ECM) analizzate. Le frazioni parzialmente purificate, con un peso molecolare inferiore a 10 kDa per* Sepia officinalis *(Finf10), e superiore a 10 kDa per* Octopus vulgaris *(Fsup10), hanno evidenziato attività anti-invasione, anti-migrazione e anti-adesività sulle linee cellulari degli gliomi U87, con una risposta dose-dipendente. Nessuna attività antiproliferativa è stata trovata per entrambe le frazioni parzialmente purificate, e il dosaggio MTT ha dimostrato l'effetto tossicità solo per elevate concentrazioni di frazioni di inchiostro.*

Parole chiave: Cefalopoda, inchiostro di seppia, inchiostro di polpo, antitumorale, idrolisi enzimatica, oligopeptide, Tunisia, Mediterraneo centrale

INTRODUCTION

In recent years, researchers have focused on identifying novel natural products as anticancer drugs. Anticancer peptides have characteristics of multi-function, high sensitivity and stability (Leng *et al*., 2005; Simmons *et al*., 2005).

Molluscan species, such as sea hares show a wide range of uses in pharmacology as they produce bioactive metabolites used in the treatment of cancerous tumors (Chakraborty & Ghosh, 2010). Peptides as antitumor drugs can improve immune response, inhibit the tumor angiogenesis and metastasis of tumor cells, directly eradicate tumor cells and induce the apoptosis of tumor cells and stop the cell cycle (Shen *et al*., 2000; Aneiros & Garateix, 2004; Zheng *et al*., 2007). The most studied, *Dolastatins* is a family of cytotoxic peptides isolated from the mollusk *Dollabella auricularia*, where the linear pentapeptide *Dolastatin*-10 and the depsipeptide *Dolastatin* 15 have had the most promising antiproliferative activity reported (Pettit *et al*., 1995; Garteiz *et al*., 1998; Pettit *et al*., 1998). The pentapeptide Dolastatin-10 is characterized by four of the residues being structurally unique but with many side effects. Also, the Keenamide A is a cytotoxic cyclic hexapeptide isolated from the mollusk *Pleurobranchus forskalii*, elicits antitumor activity via unknown mechanisms. This compound exhibited significant activity against the P388, A549, MEL-20 and HT-29 tumor cell lines (Wesson *et al*., 1996). Strong anticancer peptides were also found from *Meretix meretrix* with IC_{50} of 10 μg·mL^{-1} (Liu & Qiu, 2004).

Cephalopoda ink had been used in the treatment of hemostasis for centuries in Chinese traditional medicine (Zhong *et al*., 2009). As early as 1982, it was reported that *Sepia* ink could regulate gastric juice secretion and had antiulceration activity (Andersen & Roepstorff, 1982). Researchers in Japan found that the peptidoglycan extracted from *Sepia* ink had higher antitumor activity than the other fractions (Takaya *et al*., 1996, 1997). Other research works reported antitumor activity of cephalopoda ink (Naraoka *et al*., 2000; Palumbo *et al*., 2000). For example, it has been reported that *Sepia* ink has antitumor activity against Meth-A fibrosarcoma in BALB/c mice and its fraction containing peptidoglycan showed higher antitumor activity than the other fractions (Tetsushi *et al*., 2000; Mayer *et al*., 2010). Nowadays, none of the currently available anticancer drugs acts solely on carcinoma cells. Anticancer drugs are usually extremely toxic and kill both malignant and normal cells. However, despite its wide spectrum of clinical uses, they are known to cause several adverse effects. These limits on the use of chemotherapeutic agents thus constrain their use in effective therapy.

Protein hydrolysates formed by the enzymatic digestion of aquatic and marine by-products are an important source of bioactive peptides. Purified peptides from these sources show cytotoxic effect on several human cancer cell lines such as HeLa, AGS, and DLD-1 (Wang *et al*., 2010). These characteristics imply that the use of peptides from marine sources has a great potential for the prevention and treatment of cancer, and that they might also be useful as molecular models in anticancer drug research.

In this paper, the inks from *Sepia officinalis* and *Octopus vulgaris* had been used for *in vitro* antitumor activities. These two marine species have been chosen because of their widespread geographical distribution in Tunisia and also because of the popularity of this seafood. The cephalopoda ink, which is the natural substance released for defence purpuses against predators is composed mainly of melanin and proteoglycans (Shen *et al*., 2007; Mayer *et al*., 2013). It is produced by the ink gland, a by-product of marine-product processing, generated after gutting procedures. The objective of this research work was the characterization and the evaluation of anticancer potential from the ink of *S. officinalis* and *O. vulgaris* through the antiproliferative effect, inhibition on invasion, migration of tumor cells, as well as cytotoxicity. A characterization of the nature the peptide has also been carried out after an enzymatic hydrolysis process.

MATERIAL AND METHODS

Biological material

Like all the cephalopoda species, *S. officinalis* and *O. vulgaris* (Fig. 1) are positioned in a high level in the marine food web and are carnivorous since egg hatchling *S. officinalis* is a demersal and neritic species present in the infra and circalittoral zones, on sandy or muddy-sandy bottoms and phanerogam meadows, from the coast up to 150 meters. The cuttlefish specimens are present in the coastal waters from April till October. In the winter, they migrate to deeper zones searching for abundant food and more adequate temperatures.

O. vulgaris is a benthic, neritic species occurring from the coastline to the outer edge of the continental shelf (in depths from 0 to 200 m), where it is found in a variety of habitats, such as rocks, coral reefs, and grass beds. Throughout its distribution range, this species is known to undertake limited seasonal migrations, usually overwintering in deeper waters and occurring in shallower waters during summer. In the western Mediterranean, large mature or maturing individuals migrate inshore in early spring, followed later on by smaller, immature individuals. These two groups begin their retreat into deeper waters by August/September and November/December respectively.

Specimens of *S. officinalis* and *O. vulgaris* were captured off Bizerte coasts (Fig. 1), (North of Tunisia, Mediterranean Sea) by deep-sea trawling for the Cuttlefish and by traditional coast-fishing for *Octopus*, during April 2010. The specimens were then transported to the

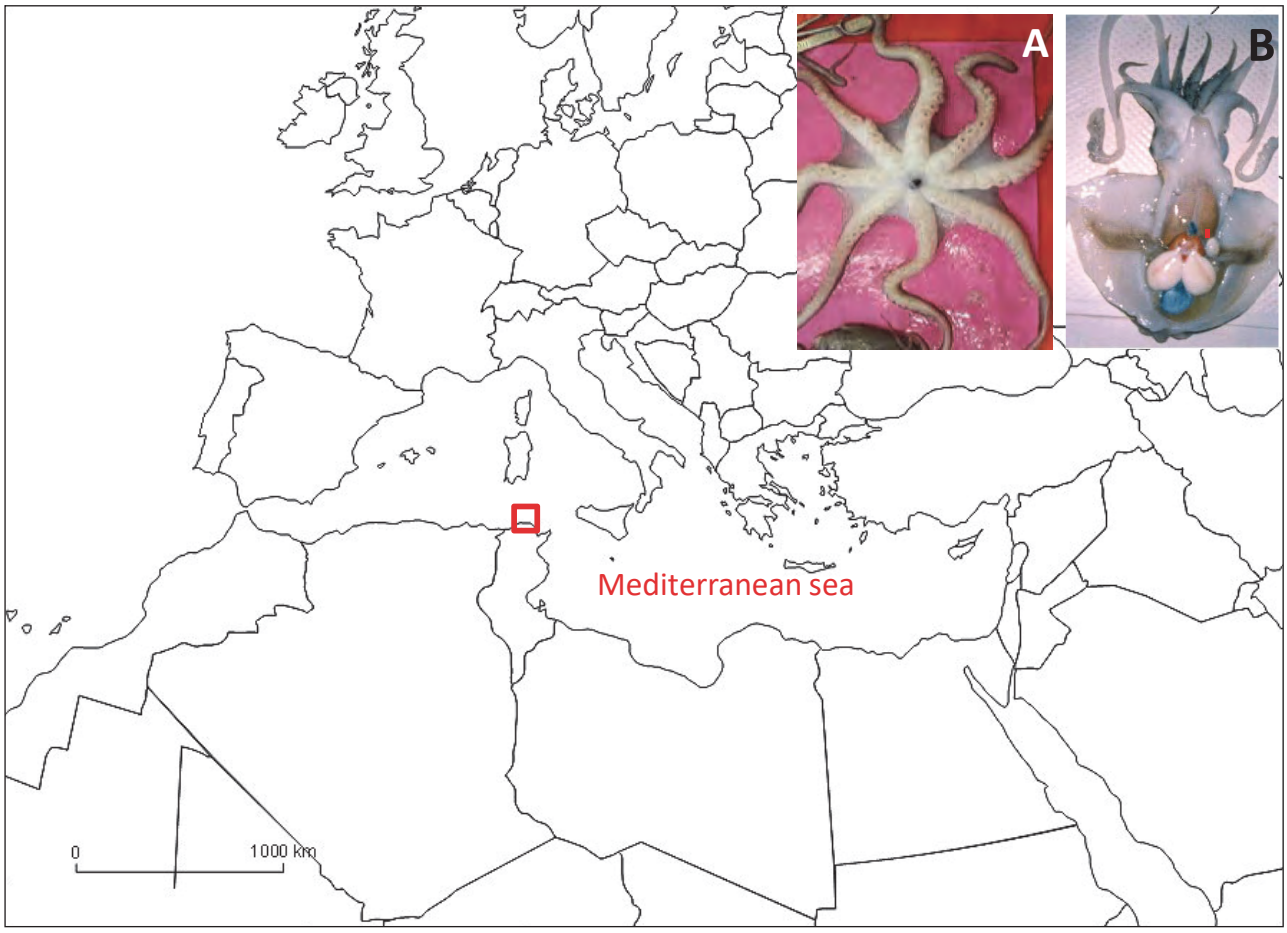

Fig. 1: Location of the capture sites (FAO fisheries department 2005) of the biological material. A. *Sepia officinalis* ***and B.*** *Octopus vulgaris***.**
Sl. 1: Lokalitete, kjer je bil nabran biološki material (FAO fisheries department 2005). A. *Sepia officinalis* ***in B.*** *Octopus vulgaris***.**

laboratory packed in ice and the ink gland was extracted. In order to avoid biochemical variation due to the physiological state of the animals, inks were homogenized by triturator and stored at -20°C before use.

Ink extraction and preparation

At a first time, the crude *cuttlefish* or *Octopus* inks was dissolved in PBS buffer (500 mL). After centrifugation at 1000 *g* during 10 min., the supernatants were collected for the antitumoral tests on tumor cell lines deriving from human melanoma IGR39. The sediment is stored at -80°C for further analyses. In a second time, the crude *cuttlefish* or *Octopus* inks were homogenized with acetone at -30°C (4V), according to the method of Takaya *et al*. (1994). The supernatant was collected after centrifugation at 1000 *g* during 15 min. and lyophilised. The sediment was stored at -80°C for further analyses. The lyophilised extracts were dissolved in a 0.1 M Tris-HCl solution (PH = 6.8) (40 v) for 72 hours at 4°C and then centrifuged at 11.000 *g* during 30 min (Mikro 200 R, Hettich Zentrifuger), in Amicon centrifuging cells (YM10) in order to separate the ink extracts according to their molecular weight. Two fractions were obtained for each cephalopoda species: the $F_{sup\ 10}$ molecular compounds with a molecular weight higher then 10kDa and another fraction denoted $F_{inf\ 10}$ with the molecular weight inferior to 10 kDa. For Cuttlefish, as well as Octopus, these two fractions were lyophilised under vacuum (LABCONCO, 2.5 ($_{Plus}$) Freezone). Finally, the freeze-dried fractions, $F_{inf\ 10}$ and the $F_{sup\ 10}$ of *Sepia* and *Octopus* were dissolved in PBS (v/v) according to the method of Naraoka *et al*. (2000). Aliquots were stored at -80°C.

Enzymatic hydrolysis

The Pepsin (EC 3.4.23.1) was provided by DSM. The enzymatic hydrolysis conditions were: a temperature of

45°C, pH2, an E/S ratio of 0.1% and an hydrolysis time of 10 hours. The reaction was stopped by heating the solution to 85°C to inactivate the enzyme. The resulting hydrolysate was centrifuged at 20,000 *g* for 20 min.

Isolation and purification of anticancer peptide

Sepia ink hydrolysates were fractionated into a high and low molecular weight fractions and by ultrafiltration at 4°C by PM-10 membrane (MWCO = 3000 Da) and kept for use in gel filtration. Prior to use, the membrane was washed with 10 mL of distilled water. The ultrafiltrate was filtered again through a Millipore membrane filter (0.45 µm) and applied to a (2.8 cm × 90 cm) column saturated in Sephadex G-25 resin. The Sephadex G-25 column was eluted with distilled water and fractions were collected every 4 minutes with a fraction collector. The absorbance was measured at 280 nm. The hydrolysate was fractionated into five fractions by gel filtration chromatography. Each fraction was tested for anticancer activities.

The fraction showing the highest anticancer activity was further purified using reverse-phase HPLC on a Primesphere 10 C_{18} column (10 mm × 250 mm) with a linear gradient of acetonitrile (0-50% for 20min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 $mL \cdot min^{-1}$. The absorbance of the eluent was monitored at 280 nm. Active peak representing anticancer activity was pooled and freeze-dried immediately for a future analysis of the bioactive fraction.

The yield of *Sepia* ink oligopeptides

The fraction provided by the G-25 gel chromatography was concentrated in vacuum under 25°C and concentrated liquid was dried under 70°C by vacuum freeze-drying. Then the powder of *Sepia* ink oligopeptides was collected and weighed.

Cell line and cell culture

Human glioma cell lines U87 and melanoma cell lines IGR 39 were used for anticancer activity tests with non purified ink fractions. The human prostate cancer cell lines PC-3 were used later for the purified and isolated active peptide. The cell lines were grown at 37°C in a 5% CO_2, 95% air humidified atmosphere, in MEM medium for U87 and DMEM-Ham's F12 medium for IGR39. The medium was supplemented with 10% FCS + 5% heat inactivated horse serum to which streptomycin (100 µg/mL) and penicillin (100 U/mL) had been added. The cells grown in flasks were washed with PBS, trypsinized (3 mL of trypsine-EDTA, 500 µg/ml), centrifuged at 800 rpm for 5 min and dissolved in fresh culture medium at 104 cells/90 µL. Subsequently, a 90 µL volume of suspension cells was added to each well of a 96-well microplate and incubated at 37°C for 24 hours.

Cell migration and invasion assay in vitro

The assays were achieved in Boyden Chambers (Becton Dickinson). A 24-well transwell (Corning, NY, USA) was used to evaluate the motility and invasive ability of U87 and IGR29 cells in vitro. The upper surface of polycarbonate filters with 8 µm pores (0,3 cm^2) was coated with 5 mg of Matrigel, fibrinogen (Sigma-Aldrich) at 50 µg/mL of PBS and incubated during 2 hours at 37°C . The lower chambers were filled with the medium (MEM/BSA 0,1%) (Sigma-Aldrich) (500 µl in the lower well, and 200 µl in the upper one). The glioma cells U87 were pre-incubated with different doses of the molecular fractions $F_{inf\ 10}$ and the $F_{sup\ 10}$ of *Sepia* and *Octopus* or 1% BSA (negative control) for 24 hours at 37 °C in a CO_2 incubator and then washed with PBS, detached with the versene (Gibco) and resuspended in serum-free MEM. A suspension of cells (2 $x10^5$ cells/200 mL) was placed in the upper chambers. After 5 h of incubation at 37°C under optimal conditions, the supernatant was completely removed and the upper and lower faces of the membranes are washed with PBS. Cells on the upper surface of the filter (that did not migrate) were completely removed by wiping them with a cotton swab. Cells that invaded the Matrigel and were fixed during 10 min with glutaraldehyde 1% and then stained with crystal violet at 0.5 % for 30 min. The cellular migration was quantified by counting the number of cells that migrated using a microscope (Leica) with 5 mm^2 fields at a magnification of x 400, or by measuring the absorbance at 560 nm after solubilization of the colorant in SDS 1%.

Antiproliferative activity assay

This assay aims to evaluate the effect of the molecular fractions, $F_{inf\ 10}$ and the $F_{sup\ 10}$ of *Sepia* and *Octopus* on the multiplication of tumor cells. U87 cells are incubated in the wells of a microplate (5 x 10^3 cells/well) in 50 µl MEM/10% FCS (Fœtal Calf Serum/ 5% horse serum. After one hour incubation, the medium is renewed in presence of the fractions to test. Each day, 3 wells are washed with PBS and the cells are fixed with glutaraldehyde 1% then fixed with PBS. At the end of the week, the cells are stained with crystal violet 0.1% and quantified by measuring the absorbance at 560 nm.

Cytotoxicity assay

The MTT assay (Mosmann, 1983) allows the evaluation of the effect of the molecular fractions $F_{inf\ 10}$ and the $F_{sup\ 10}$ on the cell viability of cancer cells U87. The peptidic ink fractions from Cuttlefish and Octopus, at a concentration of 10 mg/mL was prepared in PBS 0.1 M (pH 7.4), and diluted 10-fold in cell culture medium containing the cells. The microplate was then incubated at 37 °C for 24, 48 and 72 h, changing the culture medium every 24 h and adding the ink fractions at a

final concentration of 1 mg/mL. At the end of every incubation period, 15 µL of 5 mg/mL tetrazolium salt (MTT) solution was added to each well, and the plate was incubated for 3 h. To stop succinate-tetrazolium reductase activity and solubilize formazan crystals, 200 µL of dimethyl sulfoxide (DMSO) was then added to each well and kept at 37 °C for 1 h. Absorbance was read on a plate reader at 570 nm.

Cellular adhesion assay

The aim of this assay was to analyze the ability of ink fractions to inhibit the adhesion of tumor cells IGR39 on the proteins of the extracellular matrix (ECM). Four proteins were assyed: fibrinogen, fibronectin, collagen type I and polylysin. The substrates of adhesion were prepared by coating the wells of a microplate (Nunc) by 50 µL of a proteic solution : fibrinogen at 50 µg/mL, la polylysine (PL) at 20 µg/mL, the collagen type I (Coll I) at 10µg/mL and fibronectine (Fn) at 5µg/mL and incubating it during 2 hours at 37°C. The wells are then saturated with 50 µL PBS/BSA 0.5 % during 1 hour at 37°C. During saturation, the cells were detached by PBS/EDTA and washed twice with adhesion buffer (MEM, NaHCO3 1,2 g/l, HEPES 15 mM pH 7.3 and BSA 0,2%). For the assay of the ink fractions, the cells were pre-incubated during 30 min at room temperature with stirring and then deposited in the wells where 50 µL cellular suspension (10^6 cells/mL adhesion buffer) were added and incubated during 1 hour at 37°C. After incubation, the non adherent cells are eliminated by washing with an adhesion buffer. The adherent cells are fixed with glutaraldehyde 1% during 10 min at room temperature and are washed twice with distilled water and stained during 30 min by 100 µL of a crystal violet solution at 0.5%. Cellular adhesion was quantified by measuring the absorbance at 560 using a microplate reader (Σ960 Metertech). All the data were analyzed by the software of SPSS.

RESULTS AND DISCUSSION

Effect of the crude ink on the adhesion of IGR23 to the proteins of the ECM

Cephalopoda ink had been studied for its antimicrobial and antiviral activities, but also for its toxicity for some cell lines (Derby *et al*., 2007). To this context, we investigated the antitumor potential of *S. officinalis* and *O. vulgaris* inks. At a first time, the crude inks were diluted in PBS and then centrifuged. We evaluated the anti-adhesive effect of the supernatants on IGR39 cell lines deriving from human melanoma, on 3 different ECM: fibrinogen, fibronectin and collagen type I. The results showed that cuttlefish ink - at a concentration of 5,28 µg/ml - significantly ($p < 0.05$) inhibits the adhesion to fibrinogen, with an inhibition percentage of 60%. This inhibition was 25% on fibronectin. There was no inhibition of the adhesion of the IGR 39 cells on the collagen type I (Fig. 2). Concerning Octopus ink, we noticed that the adhesion of IGR 39 cells on fibronectin is significantly ($p < 0.05$) decreased by 40% (concentration of 8,75 µg/mL), but there was no inhibition for fibrinogen and collagen type I.

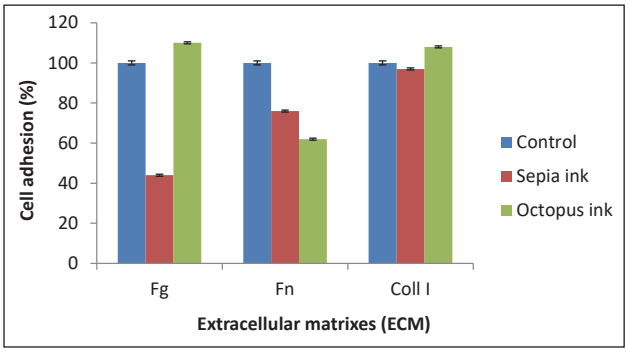

Fig. 2: Effect of crude** Sepia **and** Octopus **ink on the adhesion of the melanoma IGR 39 cells to fibrinogen, fibronectin and collagen type I.
Sl. 2: Učinek surovega črnila sipe in hobotnice na lepljenje celic ***melanoma IGR 39 na fibrinogen, fibronectin in kolagen tipa I.***

Effect of ink fractions on the adhesion of tumor cells U87 to the proteins of the ECM

After the fractionation of the inks in Amicon cells, two fractions were obtained for each cephalopoda specie, the $F_{sup\ 10}$ (MW > 10 kDa) and the $F_{inf\ 10}$ (MW < 10 kDa). With a concentration of 30 µg/mL, the cuttlefish $F_{sup\ 10}$ poorly inhibits the adhesion of U87 cells on fibrinogen (Fig. 3.A), whereas the $F_{inf\ 10}$ fraction inhibits cell adhesion on fibrinogen in a dose-dependent manner, with an IC_{50} of 25µg/ml (Fig. 3.B). In the same way, *Octopus* $F_{sup\ 10}$ inhibits cell adhesion with an IC_{50} of 75 µg/mL (Fig. 4.A). However, the fraction $F_{inf\ 10}$ assayed at dose 100µg/mL did not show a significant antitumor effect at the level of 5%. (Fig. 4.B). The adhesion assays of the U87 cells on Polylysine-L showed an inhibition that did not exceed 20 % with cuttlefish $F_{inf\ 10}$ and *Octopus* $F_{sup\ 10}$. At that point we cannot conclude yet that these inhibitions are integrin-dependent (Fig. 5.A and B).

Effect of ink fractions on the migration of U87 cells

The cellular migration plays a very important role in the metastatic dissemination and requires cellular adhesion to the proteins of the extracellular matrixes (ECM). Because the extracts of the active fractions, $F_{inf\ 10}$ of *Sepia* ink and $F_{sup\ 10}$ of *Octopus* ink exhibited an inhibiting potential of the adhesion of U-87 tumoral cells, we have essayed the effect of these extracts on their migration.

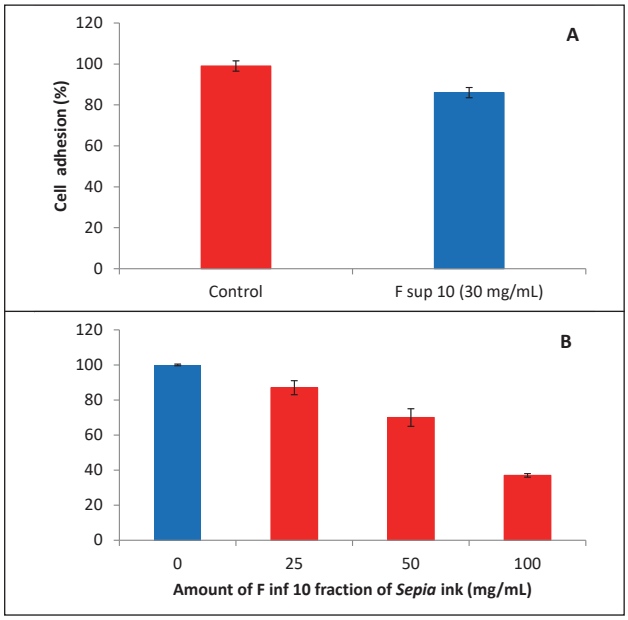

Fig. 3: Effect of the** Sepia **ink fractions on the adhesion of the glioma U87 cells on fibrinogen. A: $F_{sup\ 10}$ (30µg/ml), B: Amount of $F_{inf\ 10}$ fraction.
Sl. 3: Učinek frakcije surovega črnila sipe na lepljenje celic glioma U87 na fibrinogen. A: $F_{sup\ 10}$ (30µg/ml), B: Količina $F_{inf\ 10}$ frakcije.

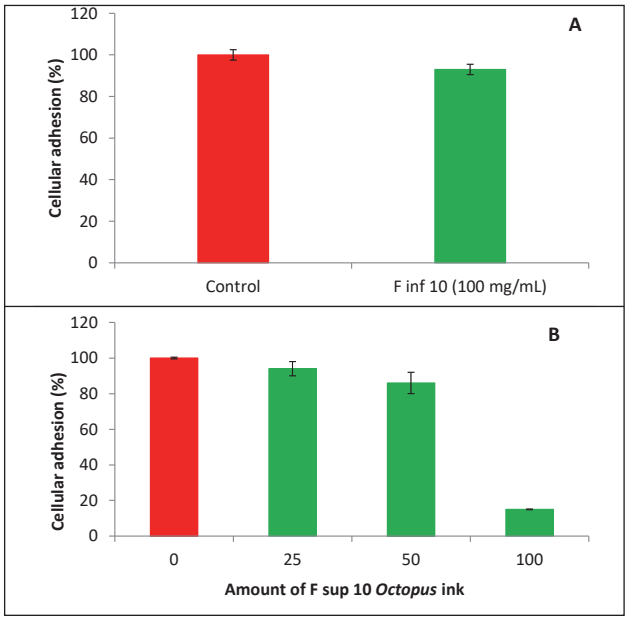

Fig. 4: Effect of the** Octopus **ink fractions on the adhesion of the glioma U87 cells on fibrinogen. A: $F_{inf\ 10}$ (100µg/mL); B: Amount of $F_{sup\ 10}$ fraction.
Sl. 4: Učinek frakcije surovega črnila hobotnice na lepljenje celic glioma U87 na fibrinogen. A: $F_{sup\ 10}$ (30µg/ml), B: Količina $F_{sup\ 10}$ frakcije.

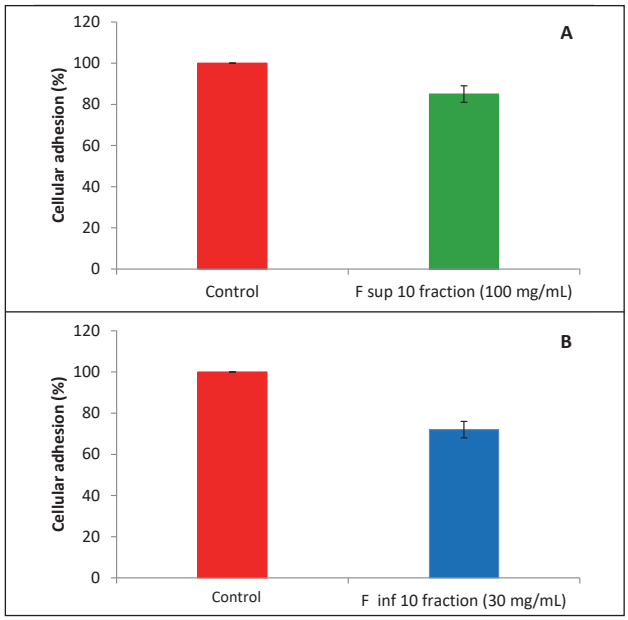

Fig. 5: Dose-response effect of ink fractions on the adhesion of U87 cells to Polylysin-L. A:** Sepia **$F_{sup\ 10}$, B:** Octopus **$F_{inf\ 10}$
Sl. 5: Učinek frakcij črnila na lepljenje U87 celic na polilizin-L v odvisnosti od doziranja. A:** Sepia **$F_{sup\ 10}$, B:** Octopus **$F_{inf\ 10.}$

The U87 cells treated with increasing concentrations of cuttlefish F_{inf10} or *Octopus* $F_{sup\ 10}$ stopped the migration of the cells and their adhesion to fibrinogen, with a dose-dependence (IC_{50} =15µg/ml) for *Sepia* $F_{inf\ 10}$ and (IC_{50} = 40µg/ml) for *Octopus* $F_{sup\ 10}$ (Fig. 6 and Fig. 7).

Antiproliferative effect of ink fractions on the U87 cells

Because the ink fractions *Sepia* $F_{inf\ 10}$ and *Octopus* $F_{sup\ 10}$ showed interesting antitumor activities (inhibits adhesion and migration of U87), their antiproliferative potential was assayed. Our results showed that the ink fractions did not show any significant inhibition of cell proliferation during 4 days (Fig. 8 A and B).

Isolation and purification of the *Sepia* ink peptide

Because the peptidic fraction inferior to 10 kDa of *Sepia* ink ($F_{inf\ 10}$) is the one who was the most active, and because biologically active peptides have low molecular weight in general, we decided to concentrate our study on *Sepia* ink anticancer peptides. After ultrafiltration using a 3000 Da MWCO membrane the permeates of Pepsin hydrolysates were loaded on a gel filtration column (Sephadex G-25). Five anticancer peptide fractions with the highest activity were collected. The purity was

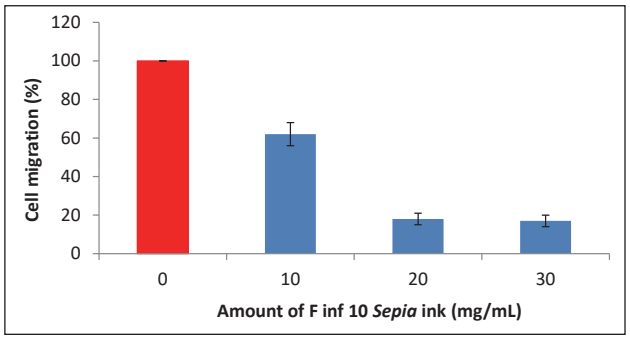

Fig. 6: Effect of the** Sepia* **$F_{inf\ 10}$ ink fraction on the U87 cell migration and morphological changes. (I): Microscopic observation. A-D: Cells were incubated with F $_{inf\ 10}$ Sepia ink fractions (0, 10, 20, 30 µg/mL) during 5 hours at 37°C. (II): U87 cellular migration (%) according to the concentration of Sepia ink fraction F_{inf10} (0, 10, 20, 30 µg/mL).

Sl. 6: Učinek frakcije črnila sipe ($F_{inf\ 10}$) na migracijo celic U87 in morfološke spremembe. (I): Mikroskopska opazovanja. A-D: Celice inkubirane s frakcijami $F_{inf\ 10}$ črnila sipe (0, 10, 20, 30 µg/mL) v peturnem obdobju pri 37°C. (II): U87 celična migracija (%) glede na koncentracijo frakcij črnila sipe F_{inf10} (0, 10, 20, 30 µg/mL).

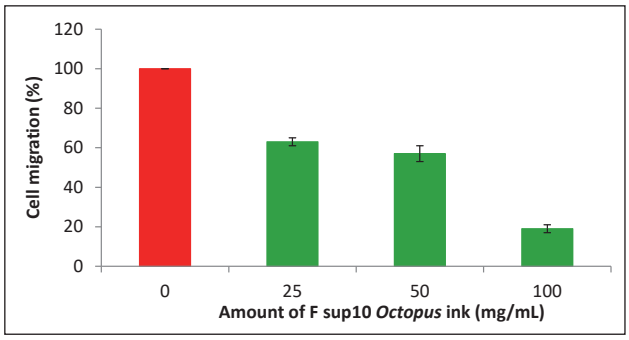

Fig. 7: Effect of the** Octopus* **$F_{sup\ 10}$ ink fraction on the U87 cell migration and morphological changes. (I): Microscopic observation. A-D: Cells were incubated with $F_{sup\ 10}$ Octopus ink fractions (0, 25, 50, 100 µg/mL) during 5 hours at 37°C. (II): U87 cellular migration (%) according to the concentration of Octopus ink fraction $F_{sup\ 10}$ (0, 25, 50, 100 µg/mL).

Sl. 7: Učinek frakcije črnila hobotnice ($F_{sup\ 10}$) na migracijo celic U87 in morfološke spremembe. (I): Mikroskopska opazovanja. A-D: Celice inkubirane s frakcijami $F_{sup\ 10}$ črnila sipe (0, 10, 20, 30 µg/mL) v peturnem obdobju pri 37°C. (II): U87 celična migracija (%) glede na koncentracijo frakcij črnila hobotnice $F_{sup\ 10}$ (0, 25, 50, 100 µg/mL).

then detected by HPLC. The sample was divided into five peaks and the peak 2 had the highest anticancer activity. After a further reverse-phase HPLC, the second peak had a molecular weight of 340.6. After freeze drying, 1.74 g *Sepia* ink oligopeptide was obtained from 100 g *Sepia* ink. So the yield was 1.74 %.

Effect of *Sepia* ink oligopeptides on cell viability

The PC-3 cells (human prostate cancer cell lines) were treated with 2-10 µg·mL^{-1} of *Sepia* ink oligopeptides for 24-72 h. PC-3 cells displayed dose-dependent decreases in viability, detectable as early as 24 h. At 24 h, the threshold concentration which caused a decrease in PC-3 cell viability was 1.89 µg·mL^{-1} (89 % of control, $P > 0.05$). The *Sepia* ink oligopeptide concentration that produced the maximal effect was 10 µg/mL (26% of control, $P < 0.05$), and the half inhibitory concentration (IC_{50}) was 7.45 µg·mL^{-1}.

At 48 h, the threshold concentration was 2,2 µg·mL^{-1} (33 % of control, $P < 0.05$), the maximal effect was 10 µg·mL^{-1} (0.24% of control, $P < 0.05$), and the IC_{50} was 1.23 µg·mL^{-1}. At 72 h, the threshold concentration was again 2 µg·mL^{-1} (43 % of control, $P < 0.05$). The maximal effect was 10 µg·mL^{-1} (0% of control, $P < 0.05$), and the IC_{50} was 1.64 mg·mL^{-1}. The threshold concentration at 24, 48 and 72 h were the same (2 µg·mL^{-1}), even though the level of the significance increased from day 1 to days 2 and 3. The IC_{50} decreased from 7.45 µg·mL^{-1} at 24 h to 1.23 µg·mL^{-1} at 48 h. These results suggest that *Sepia* ink oligopeptides had a dose-dependent deleterious effect on PC-3 cell viability.

Biologically active antitumor compounds have been isolated from different marine sources. Recently research has been focused on peptides from marine animal sources, since they have been found as secondary metabolites from sponges, ascidians, tunicates, and mollusks. The structural characteristics of these peptides include various unusual amino acid residues which may be responsible for their bioactivity. However, many side effects had been observed in clinical trials and the complexity and low yield of chemical synthesis, together with low water solubility, have been significant obstacles to broader clinical evaluation, triggering the development of analog compounds (De Arruda *et al.*, 1995; Pitot *et al.*, 1999; Tamura *et al.*, 2007).

Even if the bioactive peptides from marine mollusks had been well documented, there had been a few publications on anticancer peptides from cephalopoda, specifically the species *O. vulgaris* and *S. officinalis* ink wastes.

Interestingly in our study, the crude *Sepia* and *Octopus* inks assayed on tumor cells IGR39, showed a selective inhibition according to the cellular matrix used. In order to refine this investigation, we adopted an acetone fractioning of the ink and interested particularly to the supernatant. The two fractions obtained

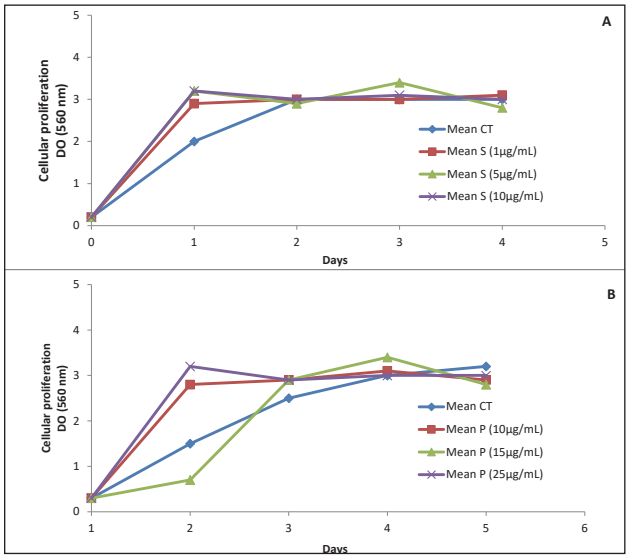

Fig. 8: Anti-proliferative effect of the active fractions on U87 cells. (A): *Sepia* ***$F_{inf\ 10}$, (B):*** *Octopus* ***$F_{sup\ 10}$.***
Sl. 8: Anti-proliferativni učinek aktivnih frakcij na U87 celice. A): *Sepia* ***$F_{inf\ 10}$, (B):*** *Octopus* ***$F_{sup\ 10}$.***

$F_{inf\ 10}$ (MW< 10kDa) and $F_{sup\ 10}$ (MW> 10kDa) were then assayed *in vitro* on glioma cell lines U87. The in anti-adhesive activities of the ink fractions belonging to the two cephalopoda species are not comparable. *Sepia* $F_{inf\ 10}$ and *Octopus* $F_{sup\ 10}$ inhibit the adhesion of U87 cells on fibrinogen, according to their concentrations (dose-dependent) with an IC_{50} = 25µg/mL for cuttlefish ink fraction and 75µg/mL for *Octopus*. However, both of the ink extracts slightly inhibit the non-specific adhesion of U87 cells on Polylysin-L. This result suggests that the fractions would own an inhibition mechanism through one or more membrane receptors. It is also important to mention that the anti-adhesive effect requires a high concentration of the *Octopus* $F_{sup\ 10}$ (~50µg/mL), unlike *Sepia* $F_{inf\ 10}$ fraction (~10 µg/mL). We can thus emit the hypothesis that these ink fractions may contain antagonistic activities. According to the literature, the antitumor effect is correlated to a synergy between different chemical ink compounds. This action is related to the tyrosinase activity and peptidoglycans (Naraoka *et al.*, 2000). We also showed that with concentrations of 10 µg/mL of *Sepia* ink fraction, the cell migration is reduced and is completely stopped with a concentration of 30 µg/ml. However, concerning the *Octopus* ink fraction, we observe an inhibition of cell migration starting from a concentration of 25 µg/mL. This inhibition is complete at 100 µg/mL. Somehow, there was no inhibition of cell proliferation. Our results are in concordance with the research work on squid (*Ommastrephes bartrami*) ink where the authors did not detect evident antiprolifertive activity on tumor cells Hep G2, but induces a suppression of cell invasion and cell migration, according to the concentrations of ink fractions (Chen *et al.*, 2010). The cytotoxicity assays of the $F_{inf\ 10}$ and $F_{sup\ 10}$ of Sepia and *Octopus* during 5 hours showed that these fractions are toxic only at very high concentrations. It had previously been reported that the tyrosinase (MW=94 kDa) is responsible of the toxic effect of cephalopoda ink (Prota et *al.*, 1981, Palumbo et *al.*, 1985, 1994, Takaya et *al.*, 1994, Naraoka et *al.*, 2000, 2003). At this point, we can only hypothesize that the cytotoxicity of the *Octopus* F_{sup10} is also due to the enzymatic effect of tyrosinase, but this is to be confirmed.

The *Sepia* ink oligopeptides extracted using the protease Pepsin also inhibited the growth of PC-3 cells. In U-87 cells, *Sepia* ink oligopeptides caused a linear decrease of cell viability in a dose-dependent manner. However, the mechanism of the anticancer activity is unclear. Therefore, further studies are needed to identify the mechanism of the potent antitumor activity.

Finally we can deduce that the fractions $F_{inf\ 10}$ et $F_{sup\ 10}$, respectively from *S. officinalis* and *O. vulgaris*, do not have antiproliferative but are responsible of antiadhesive and anti-migration activity. However, we still have to investigate whether these antitumor activities are due to one or more chemical components and to determine their chemical nature and molecular mechanisms that are implied. The results of our study also demonstrated the effect of *Sepia* ink oligopeptides on growth inhibition and could be a potentially useful adjunct in the treatment of cancer. Hence, since the cephalopod species *S. officinalis* and *O. vulgaris* are easily accessible Tunisian marine resources, their ink protein wastes are attractive as a protein source for the future industrial production of functional peptides.

PROTITUMORSKA AKTIVNOST ČRNILA PRI SIPI *SEPIA OFFICINALIS* IN HOBOTNICI *OCTOPUS VULGARIS* (CEPHALOPODA) IZ SEVERNE TUNIZIJSKE OBALE (OSREDNJE SREDOZEMSKO MORJE)

POVZETEK

Avtorji poročajo o protitumorskih učinkih peptidov iz črnila dveh glavonožcev in sicer sipe, Sepia officinalis *(Linnaeus, 1758), in hobotnice,* Octopus vulgaris *(Cuvier, 1797), dobljenih na primerkih, ujetih ob severnotunizijskih obalah (osrednje Sredozemsko morje). Rezultati prikazujejo, da učinkovine iz surovega črnila kažejo protiadhezijsko aktivnost na celice IGR39 v odvisnosti od testiranih izvenceličnih matriksov. Delno prečiščena frakcija z molekulsko maso, manjšo od 10 kDa pri vrsti* Sepia officinalis *(F_{inf10}) in višjo od 10 kDa pri vrsti* Octopus vulgaris *(F_{sup10}) sta pokazali koncentracijsko odvisno protiinvazivno, protimigracijsko in protiadhezivno aktivnost na celičnih linijah glioma U87. Delno prečiščene frakcije niso pokazale nobenih protiproliferativnih aktivnosti, MTT protokol pa je pokazal toksični učinek le v primeru visoke koncentracije frakcije črnila.*

Ključne besede: Cephalopoda, črnilo sipe**,** črnilo hobotnice, protitumorska aktivnost, encimatska hidroliza, oligopeptidi, Tunizija, osrednje Sredozemsko morje

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