

Full Length Research Paper

***In vitro* antioxidant, analgesic and cytotoxic activities of *Sepia officinalis* ink and *Coelatura aegyptiaca* extracts**

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The present study aims to evaluate the antitumor, antioxidant and anti-inflammatory activities of two molluscan extracts, *Sepia officinalis*, ink extract (IE) and *Coelatura aegyptiaca* extract (CE). The antioxidant activities of both extracts were evaluated using 1,1- diphenyl-2-picrylhydrazyl (DPPH) scavenging and lipid peroxidation assays. The analgesic effects were evaluated using the writhing, hot plate and formalin tests. Cytotoxicity assay was performed using sulphorhodamine B (SRB) method on hepatocellular carcinoma (HepG2) cell lines. The IE extract exhibited dose dependent radical scavenging activity. The two tested extracts showed inhibition of thiobarbituric acid-reactive substances (TBARAS) at all concentrations, with an IC₅₀ value of 176.77 and 177.23 (µg/ml), respectively. IE and CE extracts showed analgesic action by inhibiting the acetic acid-induced writhing. IE exhibited significant anti-nociceptive actions in mice by increasing the latency period in the hot-plate test. Both extracts significantly decreased the time of paw lickings in both early and late phases. Furthermore, IE and CE showed cytotoxic activities HepG2 cell lines with IC₅₀ value of 67 and 49.24 µg/ml, respectively. In conclusion, IE and CE extracts have antioxidant, anti-inflammatory and cytotoxic properties and can be considered as promising anticancer drugs.

Key words: *Sepia* ink, *Coelatura aegyptiaca*, antioxidant, anti-inflammatory, cytotoxicity.

INTRODUCTION

Nature is an attractive source of new therapeutic candidate compounds as a tremendous chemical diversity is found in millions of plants and animals species. Natural products continue to be a major source of pharmaceuticals and for the discovery of new molecular structures (Schaufelberger et al., 1991). Products from freshwater and marine sources have recently become attractive as nutraceutical and functional foods and as a source material for the development of drugs and specific health foods (Koyama et al., 2006). Emerging evidence suggests that marine natural products, especially the secondary metabolites from marine organisms, are far more likely to yield anticancer drugs than terrestrial sources (Hong et al., 2009).

In nature, animals are provided with their own protective response against their predators, likewise freshwater and marine mollusks are protected by their shells,

but many of them are not fully protected by shells. Chemical defenses are used extensively by both shelled and shell-less mollusks. Caldwell (2005) has proposed that the ink of cephalopods contain compounds that are capable of disrupting predator's chemical senses, but evidences are not fully recorded.

Ink gland cells of the digestive tract in the mantle cavity degenerate and shed their contents into the ink sac, acting as a reservoir of the exhausted material. Ejection of dark ink from the sac is a defensive means that cuttlefish employed to avoid dangers and risks (Liu et al., 2011). Squid ink is a multifunctional marine bioactive-material which promotes thromboxane production, kills cancer cells, and elevates leukocyte number (Sasaki et al., 1997). Moreover, it has anti-oxidant (Liu et al., 2011), anti-radiation, anti-retrovirus and anti-bacterial properties (Zhong et al., 2009; Nithya et al., 2011; Vennila et al.,

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2011). Egyptian freshwater mussel (*Coelatura aegyptiaca*) is a Molluscan bivalve that belongs to Unionoidae common in the Egypt along the River Nile from Assiut (Upper Egypt) to Damietta branches (Lower Egypt) (Moloukhia and Sleem, 2011).

Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality worldwide (Yang and Roberts, 2010). It contributes to 14.8% of all cancer mortality in Egypt, with a higher incidence in males (17.3%) than in females (11.5%) (Aleem et al., 2012). It is the second most frequent cancer type in Egyptian males after bladder cancer and the eighth most frequent in Egyptian females (Anwar et al., 2008). The primary risk factors for HCC are hepatitis B virus (HBV), hepatitis C virus (HCV), dietary aflatoxin exposure, and chronic alcohol consumption (Bosch et al., 2005).

Oxidative stress is closely related to all aspects of cancer, from carcinogenesis to the tumor-bearing state, and from treatment to prevention (Noda and Wakasugi, 2001). Epidemiologic studies have suggested that some antioxidants agent as well dietary constituents with antioxidant properties may be acting as naturally occurring cancer preventing agents and may explain some of the differences in cancer incidence seen in populations with varying dietary intake (Greenwald et al., 2001). Many cancer patients who are undergoing therapy take antioxidant supplements in an effort to alleviate treatment toxicity and improve long-term outcome.

It has long been recognized that infections and inflammation are related to cancer, and where there is a strong correlations between the presence of inflammation and the development of pre-cancerous lesions at various anatomic sites (Rayburn et al., 2009). During chronic inflammation, pro-inflammatory molecules, such as cytokines, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), and nuclear factor-kappa B (NF- κ B) are upregulated (Sarkar and Fisher, 2006). Epidemiological data suggest that the incidence of breast, colorectal, and lung cancers is inversely related to the use of aspirin and non-steroidal anti-inflammatory drugs (Arun and Goss, 2004).

Taking into consideration the relation between cancer and oxidative stress on one hand and the relation between cancer and inflammation on the other hand, the present investigation aimed to evaluate the antitumor, antioxidant and anti-inflammatory activities of two molluscan extracts, the first from the ink of the marine cephalopods cuttlefish (*Sepia officinalis*) and the second from the Egyptian freshwater mussel (*C. aegyptiaca*).

MATERIALS AND METHODS

Preparation of cuttlefish ink extract (IE)

Fresh cuttlefish (*S. officinalis*) were purchased directly from a fishmonger and rapidly transferred to the laboratory where they were dissected and the ink was collected and diluted immediately with an equal volume of distilled water and mixed sufficiently. The

admixture collected immediately, concentrated and lyophilized to a black residue using a lyophilizer (LABCONCO lyophilizer, shell freeze system, USA).

Preparation of crude freshwater *Coelatura* extract (CE)

Freshwater mussel, *C. aegyptiaca* were collected from the River Nile at Giza Governorate, Egypt. The crude extract was prepared as follows: 1 kg of fresh mussel was extracted in a boiler with 1 L of distilled water for 30 min 3 times. After filtration, the filtrate obtained was then concentrated and dried using a lyophilizer (LABCONCO lyophilizer, shell freeze system, USA).

Animals

Male white Swiss mice aged 9 to 12 weeks were used in all experiments. The animals were obtained from a closed random-bred colony at the animal's house, National Research Center. The used mice for any one experiment were selected from mice of similar age (± 1 week) and weight (± 2 g). Animals were housed in polycarbonate boxes with steel-wire tops (not more than five animals per cage) and bedded with wood shavings. Ambient temperature was controlled at $22 \pm 3^\circ\text{C}$ with a relative humidity of $50 \pm 15\%$ and a 12-h light/dark photoperiod. Food and water were provided ad libitum. All the animals received human care in accordance with the guidelines of the Cairo University, Faculty of Science, Zoology Department for ethical treatment of laboratory animals.

Drugs and chemicals

All drugs, chemicals and solvents were purchased from local firms (Egypt) and they were of highest purity and analytical grade.

Acute toxicity study

Swiss albino male mice weighing (18 to 26 g) were used for acute toxicity study. Acute toxicity was conducted according to OECD guidelines 420 (Fixed dose method) (Vanden et al., 1990; Whitehead and Curnow, 1992). The animals were divided into control and test groups containing six animals each. The control group received the distilled water whereas the test groups received cuttlefish ink extract (IE) and freshwater mussel extract (CE) (2000 mg/kg) intraperitoneally. After administration, the animals were observed for behavior changes and their mortality was noted after 48 h (acute) and at 14 days (chronic).

Antioxidant activity

Free radical scavenging activity

The free radical scavenging activities of each extract and ascorbic acid were analyzed by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Sanchez-Moreno et al., 1998). A 1.0 ml of the test extract, at gradient final concentrations of 10 to 50 mg/ml of extract and ascorbic acid as reference standards, was mixed with 2 ml of 0.3 mM DPPH solution in methanol in 5 ml test tubes. The mixtures were incubated for 20 min in the dark at room temperature then the absorbance was taken at 517 nm. The experiment was done in triplicates. The percentage antioxidant activity was calculated as follows:

$$\text{Antioxidant activity, AA (\%)} = 100 - \left[\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}}} \right] \times 100$$

where Abs_{sample} was the absorbance of sample solution (1.0 ml) + DPPH solution (2.0 ml, 0.3 mM), Abs_{blank} was the absorbance of methanol (2.0 ml) + sample solution (1.0 ml), Abs_{control} was the absorbance of DPPH solution (0.3 mM).

Lipid peroxidation assay

The degree of lipid peroxidation was assessed by estimation of the thiobarbituric acid-reactive substances (TBARS) (Okhawa et al., 1979; Tripathi and Sharma, 1998). Briefly, different concentrations of extracts (50 to 250 µg/ml) were added to the 10% liver homogenate. Lipid peroxidation was initiated by addition of 100 µl of 15 mM FeSO₄ solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 0.67% TBA in 50% acetic acid. Samples were incubated at 37°C for 1 h, and then lipid peroxidation was measured using the reaction with TBA. The absorbance of the organic layer was measured at 532 nm. All reactions were carried out in duplicates. Vitamin C was used as the positive control. The percentage of inhibition of lipid peroxidation was calculated as:

Inhibition (%) = (Abs_{control} – Abs_{test}) × 100/Abs_{control}.

Inhibitory concentration (IC₅₀) values were calculated from the plotted graph of percent inhibition against the concentrations of the samples. IC₅₀ was calculated for all the extracts and vitamin C which was used as the reference compound (positive control) with concentrations 50 to 250 µg/ml.

Determination of analgesic activity

Analgesic effects were evaluated using three different models: the writhing, and hot plate and formalin tests.

Acetic acid-induced writhing test

Acetic acid induced writhing test in mice was carried out according to the procedure earlier described by Mbagwu et al. (2007) and Nwafor et al. (2007). The animals were divided into 4 groups of 5 mice per group. Group 1 served as negative control and received 10 ml/kg of normal saline, while groups 2 and 3 were pre-treated with 200 mg/kg intraperitoneally (i.p) of cuttlefish ink extract (IE) and freshwater mussel extract (CE), respectively and group 4: received 5 mg/kg i.p of indomethacin as standard non-steroidal anti-inflammatory drug. Both extracts and indomethacin were dissolved in normal saline. After 30 min, 0.2 ml of 2% acetic acid was administered i.p. The number of writhing movements was counted for 10 min. Antinociception (analgesia) was expressed as the reduction of the number of abdominal constrictions between control animals and mice pretreated with extracts. Inhibition (%) was calculated.

Hot plate latency assay

The method of Eddy et al. (1950) was used for this study. The animals were fasted for 16 h then divided into 4 groups of 5 mice per group. Group 1 served as negative control and received 10 ml/kg of normal saline, while groups 2 and 3 were pre-treated with 200 mg/kg i.p of cuttlefish ink extract (IE) and freshwater mussel extract (CE), respectively and group 4 received 5 mg/kg of indomethacin. The animals were placed on the hot plate (maintained at 55°C). The reaction time (characterized by jumping off the animals from the hot plate) to the thermal stimuli was noted

at 30 min post extract administration. The mean of the latencies of the animals on the hot plate was determined.

Formalin induced paw licking in mice

In this study animals were grouped as earlier stated in the writhing and hot plate tests. The study was performed according to the method described by Hunskaar and Hole (1997), 0.2 ml of 3% formalin was injected into the dorsal surface of the left hind paw of mice for 30 min. The test was carried out in a transparent plastic chamber (15 × 16 × 20 cm). Each animal was allowed to explore the chamber 5 min before receiving an injection of formalin. The time which the mice spent in licking the injected paws was measured as an index of pain or nociception. The tested animal was given all treatments intraperitoneally before the administration of formalin. The 1st phase (initial nociceptive response) was 5 min after formalin injection (0 to 5 min). The 2nd phase (second nociceptive response) was between 15 and 30 min, post injection.

In vitro assay of cytotoxicity using sulphorhodamine B (SRB) method

The cytotoxicity assay was performed, following the method of Skehan et al. (1990), at the National Cancer Institute of Egypt on the cuttlefish ink extract (IE) and freshwater mussel extract (CE) against HepG2 (Liver cancer cell line). The cells were plated in a 96-multiwell plate (104 cells/well), for 24 h, before treatment of both extracts to allow attachment of cells to the wall of the plate. Different concentrations of both IE and CE extracts (10, 20, 30, 40 and 50 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each concentration. Monolayer cells were incubated with the tested samples for 48 h at 37°C, in an atmosphere of 5% CO₂. After 48 h, the cells were fixed, washed and stained with sulphorhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader at 515 nm. The relation between surviving fraction and the IE and CE extracts concentration is plotted to get the survival curve of each tumor cell line after treatment with the extracts. Data fitting and graphics were performed by means of the Prism 3.1 computer program (Graph Pad software, USA).

Statistical analysis

Values were expressed as mean ± Standard error of mean (SEM). To evaluate differences between the studied groups, one way analysis of variance (ANOVA) with Least significant difference (LSD) post hoc test was used to compare the group means and P<0.05 was considered statistically significant. Statistical Package for Social Sciences (SPSS), for Windows (version 15.0, Chicago, IL, USA) was used for statistical analysis.

RESULTS

Acute toxicity

None of the 6 mice died or showed any sign of toxicity at the limit dose of 2000 mg/kg i.p for both cuttlefish ink extract (IE) and freshwater mussel extract (CE), in the first 48 h. No evidence of toxicity was noted during the period of observation. The LD₅₀ in mice was therefore taken as above 2000 mg/kg i.p. The median effective

dose (ED_{50}) of IE and CE was selected based on the proposed LD_{50} obtained from the acute toxicity study. This dose was considered one tenth of the proposed LD_{50} , that is, 200 mg/kg body weight.

Antioxidant activity

The principle of antioxidant activity is the availability of electrons to neutralize any so called free radicals. Since, antioxidant mechanisms are diverse; a variety of in vitro techniques has been developed. It is better to use different assays based on different mechanisms to evaluate the antioxidant capacity. In this study, the antioxidant activity of both cuttlefish ink extract (IE) and freshwater mussel extract (CE) were evaluated using DPPH scavenging and lipid peroxidation assays.

Free radical scavenging activity

The results of DPPH scavenging activity of both extracts and ascorbic acid are shown in the Figure 1. The radical-scavenging activities were estimated by comparing the percentage of inhibition of DPPH radicals by the tested extracts and the ascorbic acid. Effect of water extract of both cuttlefish ink extract (IE) and freshwater mussel extract (CE) on DPPH free radical scavenging activity has been checked at various concentrations (10, 20, 30, 40 and 50 mg/ml) in three replications. The data were displayed with mean \pm SEM of three replications. The present results showed that IE produced dose dependent inhibition of DPPH radicals ranging from (86.14 to 95.19%), while CE does not exert dose-dependent inhibition of DPPH radicals.

Anti-lipid peroxidation activity

TBA test determined the content of TBAR substances at the end of lipid peroxidation. The mean TBAR substances inhibition for the IE, CE and vitamin C were 44.38, 48.17, and 39.17%, respectively (Figure 2). In addition, the results showed that IC₅₀ for IE, CE and vitamin C were 176.77, 177.23 and 245.45, respectively.

Analgesic activity

Acetic acid-induced writhing

Table 1 shows the effects of both cuttlefish ink extract (IE) and freshwater mussel extract (CE) on acetic acid-induced writhing in mice. The present results indicated that administration of both extracts at a dose of 200 mg/kg i.p showed significant ($P < 0.05$) inhibition of writhing

induced by the acetic acid as compared to the control animals. The obtained results also, showed the more potent effects of both extracts as compared to the reference drug indomethacin where the percent inhibition of writhing induced by IE and CE injections were 83.06 and 73.14%, respectively, while that of indomethacin was 38.43%.

Hot plate latency

Pretreatment with both cuttlefish ink extract (IE) and freshwater mussel extract (CE) and indomethacin (5 mg/kg, i.p) increased the response latency in the hot plate test (Figure 3). The present results showed a significant increase in the latency period ($P < 0.05$) was induced only by IE extract as compared to control group.

Formalin induced paw licking in mice

In the formalin test, both IE and CE extracts (200 mg/kg, i.p) caused a significant inhibition of both phases of formalin-induced pain (Figure 4). The recorded results also showed that IE extract showed more potent effect than the standard reference drug, indomethacin (5 mg/kg, i.p).

Antiproliferative activity

Cytotoxicity of both IE and CE extracts on hepatocellular carcinoma (HepG2) cell lines was assessed using sulphorhodamine B (SRB) (Figures 5 and 6). HepG2 cells were treated with graded concentration (10, 20, 30, 40 and 50 μ g/ml) of both IE and CE extracts for 24 h. Treatment of HepG2 cells with IE or CE extracts resulted in loss of cell viability. However, this inhibition was found to be steady and not affected by increment of dose. The recorded data showed that, the IC₅₀ (concentration causing death of 50% of HepG2 cells) of both IE and CE extracts were 76 and 49.24 μ g/ml, respectively (Figures 5 and 6).

DISCUSSION

Chemotherapy is currently the primary treatment modality in many tumors. However, the development of multidrug resistance (MDR) to chemotherapeutic drugs is a main obstacle for the successful treatment of malignant tumors (Tao et al., 2010). Therefore, the development of novel chemotherapeutic agents would play a key role in the treatment of refractory or relapsing cancer patients. Marine organisms are rich source for natural products. Many compounds that are derived from these organisms have generated interest both as challenging problems for

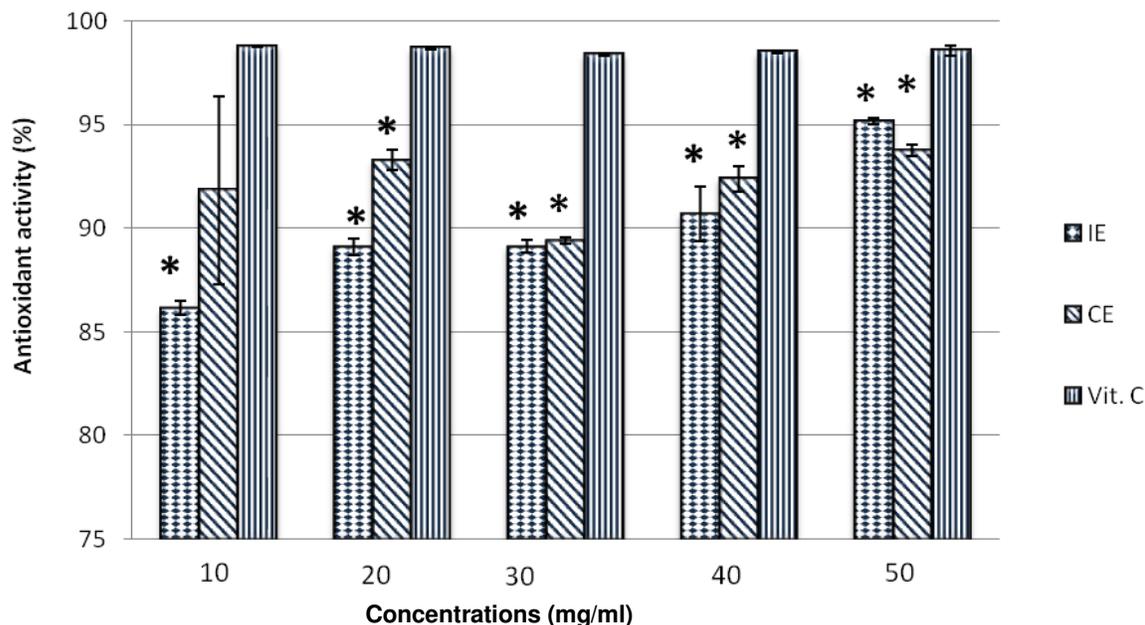


Figure 1. Antioxidant activity of both cuttlefish ink extract (IE) and freshwater clam extract (CE) and vitamin C. Each vertical column represents the mean \pm SEM of change of 6 mice. Both extracts were effective compared to vitamin

Table 1. Effect of both cuttlefish ink extract (IE) and freshwater clam extract (CE) on acetic acid-induced writhing in mice. Each vertical column represents the mean \pm SEM of change of 6 mice. Both extracts and indomethacin were effective compared to control.

Group	Mean number of writhings	Inhibition (%)
Control	48.40 \pm 5.00	-
IE	8.20 \pm 3.79*	83.06
CE	13.00 \pm 1.82*	73.14
Indomethacin	29.80 \pm 2.06*	38.43

*Significantly different from control group at $p < 0.05$.

structure elucidation and synthesis as well as for their cytotoxicity (Schwartzmann, 2000; Schwartzmann et al., 2001). It is believed that, a rich source of anticancer drug candidates could be obtained from marine organisms or their metabolites. But, we have shown in the present study that anticancer drug candidates can be also obtained from freshwater organisms.

Given the relation between oxidative stress and cancer, it has been assumed that ingestion of antioxidants is useful in preventing carcinogenesis (Terry et al., 2000). Moreover, inhibition of inflammation using antioxidants has also been studied in relation to the risk of carcinogenesis (Kimura et al., 1998). So, if the novel cancer therapeutic drug has both antioxidant and anti-inflammatory properties at the same time, it can be a promising anticancer drug.

Cephalopod inks are chemical secretions produced by and released from the ink sac (Roseghini et al., 1996). Furthermore, freshwater bivalves occurring in Egypt represent a neglected animal group and little is known about them or their diversity; perhaps due to the fact that they have no apparent economic or medical importance (Sleem and Ali, 2008). But, recent attention has been focused upon supplements derived from freshwater foods in Egypt and their utilization as hepatoprotective agents (Fahmy et al., 2009; Fahmy and Hamdi, 2011; Soliman, 2011).

During normal metabolic processes or due to the exogenous factors and agents, reactive oxygen species (ROS) in the forms of superoxide anion radical ($\bullet\text{O}_2^-$), hydroxyl radical ($\bullet\text{OH}$), hydrogen peroxide (H_2O_2) may be generated. Formation of ROS can cause oxidative damage to human cells, leading to various diseases such as diabetes, cardiovascular diseases, inflammatory conditions, cancer and ageing (Joyce, 1987; Velioglu et al., 1998).

In the present study, we attempted to evaluate the antioxidant and prooxidant effects of the cuttlefish ink extract (IE) and freshwater mussel extract (CE). Antioxidant tests could be based on the evaluation of lipid peroxidation or on the measurement of free radical scavenging potency. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers (Roginsky and Lissi, 2005). DPPH assay is a stable free radical widely used to evaluate the free radical-scavenging activity (RSA) of various natural products and some synthetic pure compounds. The DPPH antioxidant assay is based on

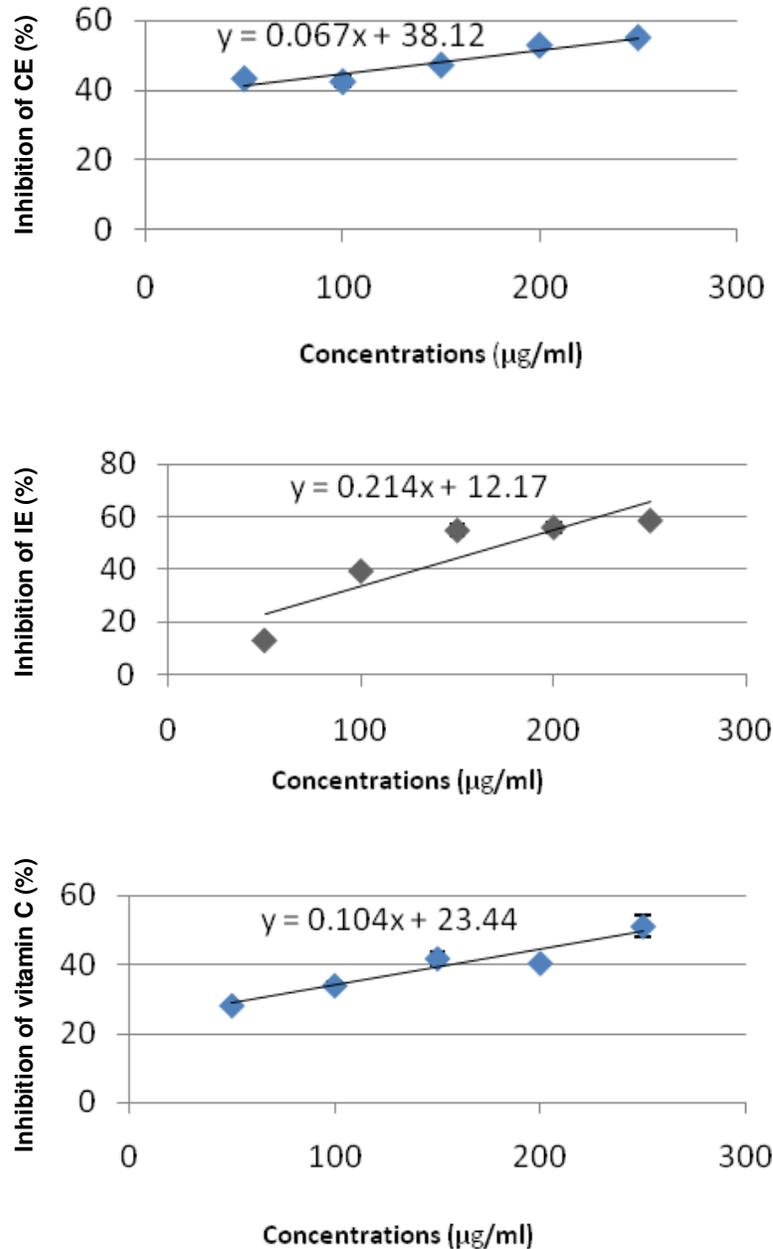


Figure 2. Effect of cuttlefish ink extract (IE), freshwater clam extract (CE) and vitamin C on TBARS in liver homogenates of male Swiss albino mice.

the ability of DPPH, a stable free radical, to be decolorized in the presence of antioxidants (Kumarasamy et al., 2007). The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 to 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. Therefore, in this study, the selected IE and CE extracts were screened for their possible antioxidant and radical scavenging activity by DPPH technique and their IC50 values were calculated. The data recorded from this study

showed that, the IE extract exhibited dose dependent radical scavenging activity. Moreover, during lipid peroxidation, low molecular weight end products, generally malondialdehyde, are formed by oxidation of polyunsaturated fatty acids that may react with two molecules of TBA to give a pinkish red chromogen (Okhawa et al., 1979). In the lipid peroxidation assay, the two tested extracts showed inhibition of peroxidation effect at all concentrations, with an IC50 value of 176.77 and 177.23 mg/ml, respectively.

Squid ink is a mixture containing melanin, protein, carbohydrate and lipid (Liu et al., 2011). Melanins are

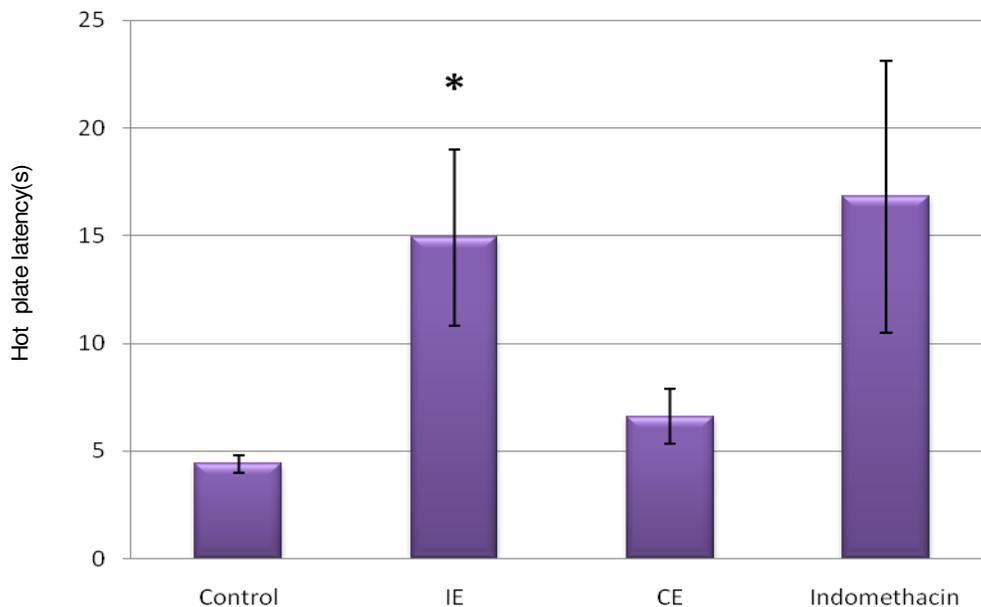


Figure 3. Effect of both cuttlefish ink extract (IE) and freshwater clam extract (CE) on hot-plate latency (sec) in mice. Each vertical column represents the mean \pm SEM of change of 6 mice. Both extracts and indomethacin were effective compared to the control.

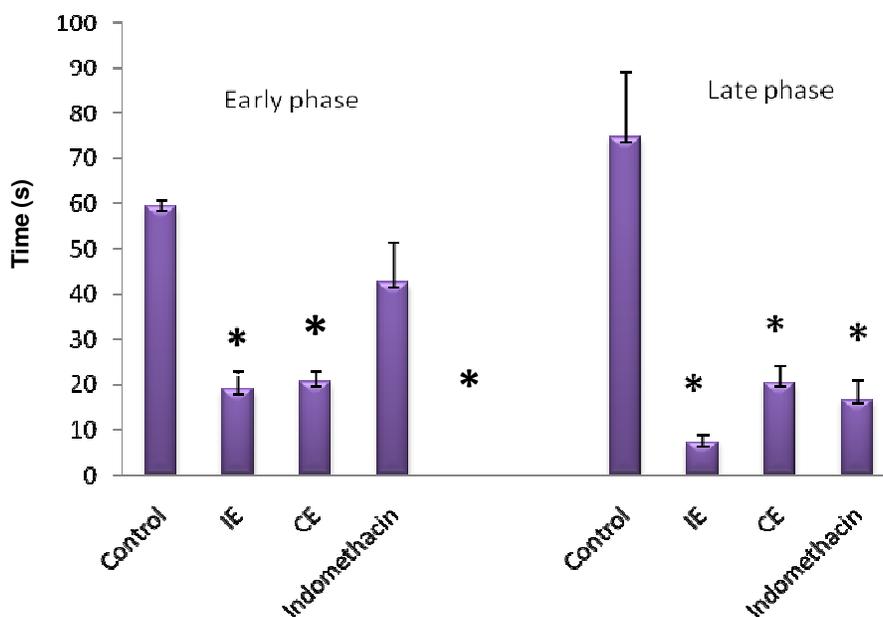


Figure 4. Effect of both cuttlefish ink extract (IE) and freshwater clam extract (CE) on formalin-induced paw licking in mice. Each vertical column represents the mean \pm SEM of change of 6 mice. Both extracts and indomethacin were effective compared to control.

efficient free radical scavengers and antioxidants (Prota, 1992). Katritzky et al. (2002) have proposed that sepi melanin is a copolymer of eumelanin constituted of

approximately 20% of units of 5, 6-dihydroxyindole (DHI) and 75% of units of 5,6-dihydroxyindole-2-acid carboxylic (DHICA). Zhang et al. (2003) reported that sepi ink

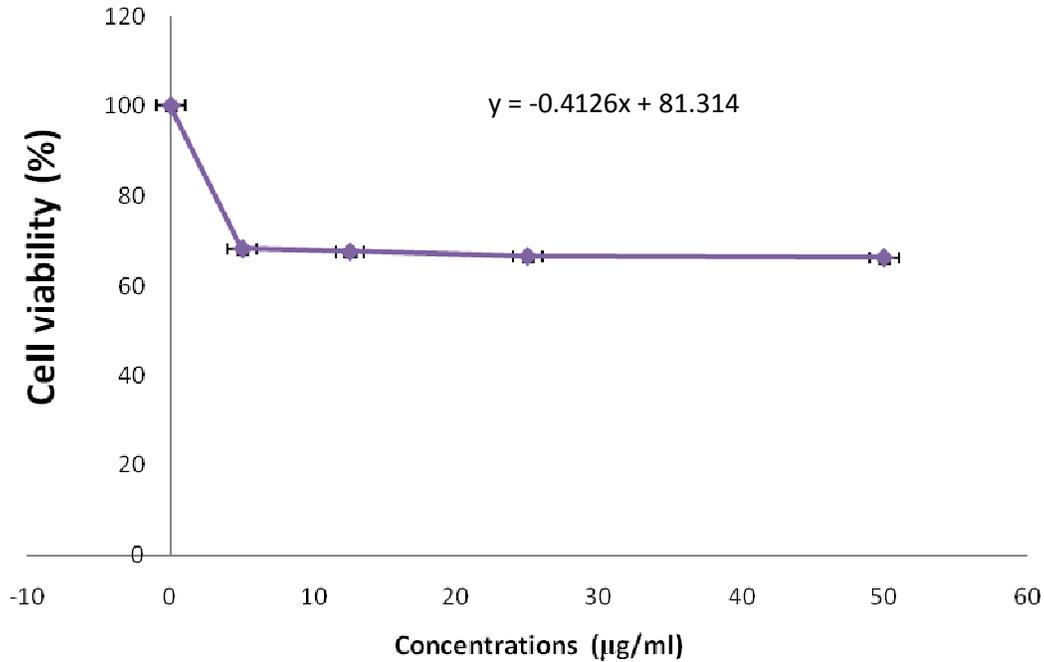


Figure 5. *In vitro* cytotoxic activity of cuttlefish ink extract (IE) on HepG2 liver hepatocellular carcinoma.

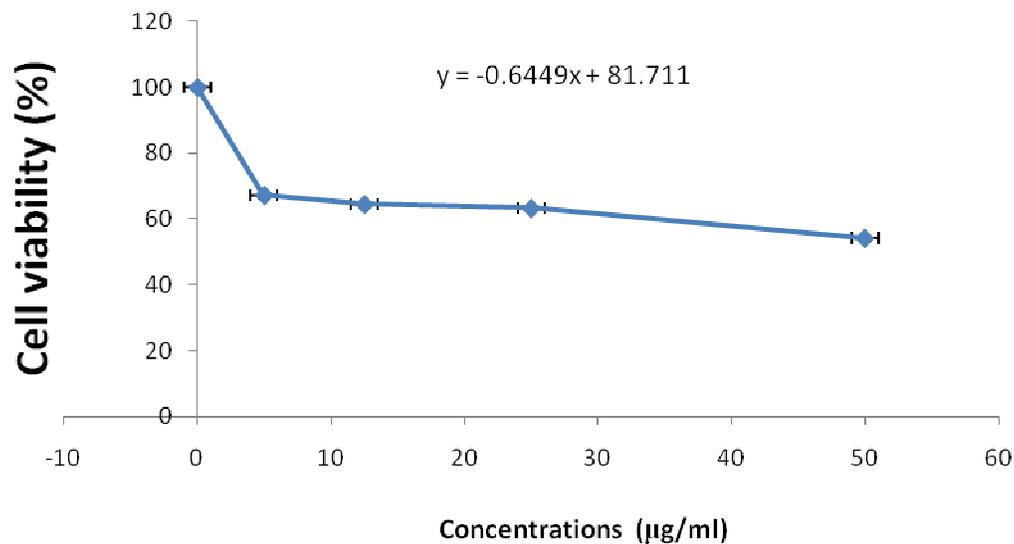


Figure 6. *In vitro* cytotoxic activity of freshwater clam extract (CE) on HepG2 liver HCC.

elevated SOD activity in the liver and kidney of mice in a dose dependent manner. Background researches showed that melanin of sepia ink, like SOD, can catalyze O_2^- to H_2O_2 , and thus avoid the free radical chain reaction triggered by O_2^- (Chen et al., 2007). Melanin of squid ink may act as SOD due to the presence of DHI which catalyzes the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Meyskens et al., 2001). Moreover, melanins also absorb cationic metal ions such as iron and copper *in*

vivo that can dramatically affect the redox state of the polymer by promoting the production of the highly reactive HO^\bullet in a Fenton type reaction (Fisher, 2003). Also, two different metabolites in the melanin, L- Dopa and dopamine effector molecules in concentrations sufficient to produce physiological effects have been identified (Lucero et al., 1994). In consonance with the study of Lucero et al. (1994) and Fiore et al. (2004) demonstrated that HPLC analysis of crude ink gland extract

in *S. officinalis* indicated the presence of L-dopa (2.18 ± 0.8 nmol/mg of protein) and dopamine (0.06 ± 0.02 nmol/mg of protein). The pro-oxidant and antioxidant actions of L-DOPA and dopamine in vitro were evaluated (Spencer et al., 1996).

Taurine is a sulfur containing amino acid which has been previously found to exhibit antioxidant properties (Das et al., 2009; Li et al., 2009). Viewed in conjunction with the report of Derby et al. (2007) and Soliman (2011), both squid ink extract and freshwater mussel extract of *C. aegyptiaca*, contain considerable amounts of taurine. Moreover, Soliman (2011) have shown the presence of high levels of precursor amino acids of GSH (glycine, glutamine and cysteine) in the freshwater mussel extract of *C. aegyptiaca*. It can be concluded from the aforementioned investigation that IE and CE extracts may be two potent antioxidants which is based on their chemical structures.

All the currently available analgesic drugs such as NSAIDs have more or less few adverse effects (Raquibul et al., 2009). As a result, more and more people are turning to natural products as alternative treatment of pain. Clark (2002) reported that pain continues to exert significant burdens on patients and the healthcare system. Pain represents the sum of reactions which include specialized and non-specialized tissues, as well as psychological and cognitive reactions to painful stimuli (Vaz et al., 1997). A useful way of categorizing pain for purposes of studying analgesics is to distinguish between visceral and somatic types (Pasternak, 1993). Visceral pain is perceived as a diffuse and burning sensation, while somatic is localized and sharp. As no single analgesic drug is ideal for all pains (Pirmohamed et al., 2004), additional therapies are always being sought. According to Raffa and Pergolizzi (2011), natural products might contain ingredients that offer such alternatives by acting through novel mechanisms of action or by interacting additively or synergistically through known mechanisms of action. The results of the present study revealed that, IE and CE extracts showed analgesic action in mice, by inhibiting the acetic acid-induced writhing as compared to the positive drug indomethacin. Acetic acid-induced writhing is a highly sensitive and useful test for analgesic screening. Acetic acid causes inflammatory pain by inducing capillary permeability and in part through local peritoneal receptors from peritoneal fluid concentration of prostaglandin E2 (PGE2) and prostaglandin F2 α (PGF2 α) (Bentley et al., 1983). So, IE and CE extracts might have some chemical constituents that are responsible to inhibit prostaglandin synthesis or to block pain sensation or might exert other specific mechanism to counteract the pain induced by acetic acid. But, this test alone can not specify the involvement of either central or peripheral activity (Chan et al., 1995). Thus, formalin test and hot-plate test are usually carried out in addition to the earlier mentioned test to distinguish between peripheral and central pain.

The hot plate test is considered to be selective for centrally acting opioid-like analgesics (Woolfe and MacDonald, 1994). Insight to hot plate test, it can be concluded that IE exhibited strong anti-nociceptive actions in mice by increasing the latency period in the hot-plate test as compared to the indomethacin, while CE did not. In these models, the increase in stress tolerance capacity of the animals as in the case of IE, indicates the possible involvement of a higher center (Vogel and Vogel, 1997). It is therefore thought that the analgesic effect of IE in the present study may involve central activity, while that of CE may involve peripheral activity.

In regard to formalin induced paw licking test in mice, the results of the present study showed that both extracts significantly decreased the number of paw lickings in both early and late phases. The formalin test in mice is a useful test for evaluating mild analgesics. The test employs an adequate painful stimulus, the animals show a spontaneous response and the test is sensitive to the commonly used analgesics. The test has two different phases, reflecting different types of pain. The first phase reflects a direct effect of formalin on nociceptors (non-inflammatory or neurogenic pain) whereas the second phase reflects inflammatory pain (Elizabetsky et al., 1995). In this study, IE and CE extracts are capable of attenuating both the neurogenic and the inflammatory phases of pain. This probably shows that the anti-nociceptive action of the extracts was mediated by both neurogenic and inflammatory mechanisms. It is well known that, centrally acting drugs such as opioids inhibit both phases equally (Shibata et al., 1989), but peripherally acting drugs such as aspirin and indomethacin only inhibit the late phase. Therefore, IE and CE extracts in the present study indicated to exert their action centrally not peripherally.

HCC represents the fifth most common malignancy and regarded as the main cause of mortality in patients with chronic liver diseases. HCC is a tumor characterized by high local invasiveness and high metastatic efficiency. Because of the high demand for anti-cancer drugs, attention has recently been focused on investigation and screening of pharmaceutical anticancer compounds in the natural products. The results of this study revealed that IE and CE showed potent and cytotoxic activities against HepG2 cell lines with IC50 67 and 49.24 μ g/ml, respectively.

Results of the present study showed the antinociceptive and the antioxidant effects of both IE and CE extracts. It is well known that there are links between the inflammatory and nociceptive, oxidative and cancer processes and the ability to inhibit any of the processes will definitely lead to the inhibition of the others (Zakaria et al., 2011). Takaya et al. (1994) confirmed the antitumor activity of a peptidoglycan fraction from the squid (*Illex argentinus*) ink against Meth A fibrosarcoma in mice. Our results are in conformity with the results obtained by them.

Lu et al. (1994), working with cuttlefish ink in mice found increased humoral immunity in ink treated mice. The antitumor fraction of the *I. argentinus* ink was separated by Phenyl Sepharose CL-4B chromatography into three fractions: illexin peptidoglycan, tyrosinase, and the complex of them (Naraoka et al., 2000). The third fraction containing the illexin peptidoglycan and tyrosinase showed the highest activity against Meth A tumour in BALB/c mice, suggesting the role of both components in antitumour activity of squid ink. The melanin free ink of the cuttlefish, *S. officinalis* is shown to have toxic effect on a variety of cell lines and the active factor was identified as tyrosinase (Russo et al., 2003).

Chemotherapeutic drugs can induce cell death through two ways: necrosis and apoptosis. Apoptosis is an energy-dependent form of programmed cell death that differs from necrosis (Koh et al., 2005). Necrosis is an acute form of cell death and causes cell lysis that is typically followed by an inflammatory response, which then can produce side effects. Therefore, new types of antitumor drugs should induce apoptosis, not necrosis, in tumor cells. Russo et al. (2003) showed that, purified sepia tyrosinase was found to induce a significant increase in caspase 3 activity in PC 12 cells, leading eventually to an irreversible apoptotic process.

Conclusions

The HCC remains a malignant disease leading to death. Significant progress must be made in the management of the disease. Because of its complexity, a multidisciplinary approach must be implemented to support the different aspects in HCC. It can be concluded from the results of this study that, IE might exert its antitumor effect through apoptotic effect rather than necrotic effect. Further study will be needed to deduce the pathway by which CE exert its antitumor effect. A better understanding of the molecular and histological changes may be responsible for the occurrence of the disease and should allow the development of new diagnostics and effective treatments for HCC.

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