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Cytotoxic effects of two edible bivalves *Meretrix meretrix* and *Meretrix* casta

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Liver cancer is the fifth most common cancer in worldwide cancer mortalities. The present study was undertaken to evaluate the anticancer properties of two edible bivalve species *Meretrix meretrix* and *eretrix. casta* on human hepatoma cell line HepG2. The anticancer properties of bivalves have been evaluated by using the Trypan blue exclusion assay, lactate dehydrogenase activity (LDH), caspase 3 activity, glutathione level (GSH) and DNA fragmentation assay. Both mollusc extracts *M. meretrix* extract (MME) and *M. casta* extract (MCE) treated HepG2 cells showed significant inhibition of cell viability at (IC_{50}) 50 µg/ml concentration in the trypan blue exclusion assay. With light microscopic observation the extract treated HepG2 cells showed modified cell morphological features. Lactate dehydrogenase was significantly released from the extracts treated cells. Reduced glutathione levels were observed in MME and MCE treated HepG2 cells. Further DNA ladder assay showed a fragmented laddering pattern of DNA in molluscan extracts treated cells, it further confirms the induction of apoptosis in HepG2 treated cells. As compared to MME, the MCE showed weaker anticancer property. On observation, it can be concluded that extract MME has been a highly selective and effective anticancer drug for human welfare.

Key words: *Meretrix meretrix, Meretrix casta*, trypan blue exclusion assay, lactate dehydrogenase (LDH) assay, caspase 3, DNA ladder assay

INTRODUCTION

Liver cancer or hepatocellular carcinoma (HCC) was one of the leading causes of worldwide cancer mortality (El-Serag and Mason, 1999). The endemic mortalities of HCC were observed in tropical and subtropical countries. The major risk factors involved in HCC were viral particle Hepatitis B and some hepatocarcinogens such as nitrosamines, aflatoxins etc. The therapeutic options are surgical interventions (tumor resection and liver transplantation), radiation therapy, chemotherapy, immune therapies. But these therapeutic methods are producing adverse side effects. Hence it is necessary to evaluate the new active drugs against HCC with the lack of side effects from a cheaper source.

Marine organisms are taxonomically diverse; each and

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every thousand new compounds are derived from marine natural source and they enter into clinical trials for human welfare. Especially in a marine environment, the phylum Mollusca produce a large number of therapeutic drugs including, antibiotic, antiviral, antiphrastic, analgesic and anticancer activities. In early decades *Meretrix meretrix* has been used for traditional medicine in several Asian countries (especially China and India). There are more than 100 new compounds reported from the bivalve molluscs, especially antimicrobial compounds (Sugesh and Mayavu, 2013) and antiviral (Ning et al., 2009). Many bioactive component proteins, peptides and enzyme, enzyme inhibitors were reported from *M. meretrix* (Xie et al., 2012). A peptide extracted from *M. meretrix* was shown to strongly inhibit the growth of human stomach adenocarcinoma BGC-823 cells (Leng et al., 2005). Wu et al. (2006) reported that steroid extract of *M. meretrix* inhibited the growth of human hepatoma cell line, HepG2 and HepG3. Likewise a novel antitumor protein was reported from *M. meretrix* (Ning et al., 2009). Sugesh and Mayavu (2013) also reported antimicrobial properties of two edible bivalve species *M. meretrix* and *Meretrix casta.* On consideration of this fact, the present study was undertaken to evaluate anticancer potential of marine bivalves *M. meretrix* and *M. casta* against human hepatoma cell line (HepG2).

MATERIALS AND METHODS

Cell culture

Human hepatoma cell line (HepG2) was obtained from National Centre for Cell Sciences (NCCS), Department of biotechnology, Pune, India. Cells were routinely grown as monolayer cultures at 37°C in a humidified atmosphere at 5% CO_2 in the air in Dulbecco's modified Eagle medium (DMEM) containing 10% (V/V) fatal calf serum (FCS), penicillin (50 IU/ml) and streptomycin (50 µg/mg). The medium was changed every 3 days.

Preparation of molluscan extracts

The live specimen of marine bivalves (*M. meretrix* and *M. casta*) was collected from Vellar estuary of Parangipettai, south east coast of India (Lat 11° 29' N; 79° 46' E) for the period of study. The collected animals were brought into the laboratory and shells were washed with distilled water and broken by using a hammer. The extraction procedure was followed by Ning et al. (2009). The extracts obtained from *M. meretrix* and *M. casta* were shortly named as MME (*M. meretrix* extract) and MCE (*M. casta* extract).

Cytotoxic assay

The cytotoxicity of the molluscan extracts was assayed by cell viability study using the trypan blue exclusion method (Morita et al., 2003). For the determination of cell viability, monolayer of HepG2 cell was trypsinised and seeded at a density of 1×10^6 cells/well. After 24 h, the medium was replaced with the serum free medium (DMEM medium, supplemented with antibiotics penicillin 100 U/ml, streptomycin U/ml, 1 mm sodium pyruvate) and the cells were cultured for 24 h to arrest the cell growth. The monolayers of HepG2 were treated with various concentrations of bivalve extracts (25, 50, 75, 100, 150 µg/ml) for 48 h and cells were incubated with 1% DMSO as a solvent control. Both attached and floating cells were collected by trypsinization, and aliquot of the cells were mixed with an equal volume of trypan blue dye. The cells excluding dye (viable cells) were counted in duplicate using a haemocytometer and the numbers of these cells were expressed as the percentage of the total number.

$$\% \text{ Inhibition} = \frac{\text{No. of viable cells - No. of viable cells after treatment}}{\text{No. of viable cells without treatment}} \times 100$$

Lactate dehydrogenase (LDH) leakage assay

Lactate dehydrogenase leakage assay was performed by the method of Grivell and Berry (1996). A sample of 100 µl from the growth medium of experimental cultures was added to a 1 ml cuvette containing 0.9 ml of a reaction mixture to yield a final concentration of 1 mM pyruvate, 0.15 mM NADH and 104 mM disodium hydrogen phosphate. After mixing thoroughly, the absorbance of the solution was measured at 340 nm for 45 s. LDH activity was expressed as moles of NADH used per minute per well.

Caspace-3 activity

The caspace-3 activity was assayed using a CASP-3-C calorimetric kit (Sigma St. Louis Mo. USA). Cell lysate of 5 µl was added to 85 µl of assay buffer. The reaction was started by the addition of 10 µl of caspase substrate and incubated at 37°C for 2 h. The concentration of pNA released from the substrate was calculated from the absorbance at 405 nm or from the calibration curve prepared with a standard pNA solution. Positive and negative controls were tested simultaneously according to the manufacturer's instruction. Caspace-3 activity was expressed in µmoles of pNA/min/ml.

DNA fragmentation assay

The DNA fragmentation was followed by the method of Chen et al. (1997). The Hep G2 cells were plated in a 60 mm culture dish at a density of 1×10^6 cells and treated with bivalve extracts of MME and MCE at 37°C for 48 h. The cells attached at the bottom were scraped off and collected together with unattached cells by centrifuging at 1500 g for 5 min at 4°C. The DNA was prepared from pelleted cells. The cells were lysed with lysis buffer and extracted with 2 ml of phenol (neutralized with TE buffer, pH 7.5) followed by extraction with 1 ml of chloroform and isoamyl alcohol in the ratio of 24:1. The aqueous supernatant was precipitated with 2:5 volumes of ice-cold ethanol with 10% volume of sodium acetate in -20°C overnight. After centrifugation at 13,000 rpm for 10 min the pellets were air dried and re-suspended with 50 µl of TE buffer containing 0.5 µl of ethidium bromide. After electrophoresis, the gel was photographed under UV light.

Estimation of glutathione (GSH)

Total reduced glutathione was determined by the method of Moron et al. (1979). TCA (5%) 1 ml was added to the human hepatoma cell line (1×10^6 cells). The precipitate was removed by centrifugation.

To an aliquot of supernatant 2 ml of the DTNB reagent was added to make a final volume of 3 ml. The absorbance was read at 412 nm against a blank containing TCA instead of samples. Aliquots of the standard solution were treated similarly. The amount of glutathione was expressed as nmoles/10^6 cells.

RESULTS

The light microscopic observation showing that extract MME changed the alteration of HepG2 cells. The architecture of untreated HepG2 cells displayed typical baluster shape. The morphological changes were observed in a short time after incubation with MME. The cells detached from the substratum, become rounding and supported each other after exposure for 30 to 60 min. Membrane bulge and detachment from cytoplasmic inclusion were observed at 90 min after treatment. The cells treated with MME became rounded and the surface of the cell membrane was markedly disrupted. But the MCE did not show that much anticancer activity as well as shown by MME. Compared with other anticancer drugs, the column purified extract MME exhibited significant anticancer activities against HCC. Figure 1 and 2 shows the morphological changes of normal and bivalve mollusc extracts of both *M. meretrix* and *M. casta* treated with various concentrations up to 25 to 100 µg/ml for 48 h of exposure. In drug treated HepG2 cells, destruction of monolayer was observed, which was not seen in *M. meretrix* extract (MME) and *M. casta* extract (MCE) treated cells. Both MME and MCE treatment exhibited swelling and rounded morphology of HepG2 cells with condensed chromatin and their membrane also become crooked and vesicle shaped. Progressive structural alterations and reduction of HepG2 cell population were observed in both extracts IC_{50} value.

Cytotoxic assay

The cytotoxic assay of the tryptophan blue exclusion assay was employed to trace active components for cell growth inhibition against human HepG2 cell lines. The column purified extracts of MME significantly inhibited the growth of HepG2 cells and the IC_{50} value was determined to be 50 µg/ml and it was displayed in Figure 1. MCE also displayed the cytotoxic activities to the HepG2 cell lines and its IC_{50} value was determined to be 50 µg/ml and it was displayed in Figure 2. The effect of MME and MCE on hepatoma cell line HepG2 was determined using the tryptophan blue exclusion assay. MME treatment tremendously inhibited the cell growth of HepG2 cells. The population of survival during 48 h in 50 µg/ml concentration of the MME were 81.3% and MCE was 96.18%, respectively. Control DMSO showed 100% survivability (Figure 3). While increasing the concentration of extracts decreased the cell population. The results suggests that the extracts can induce an accumulation during cancer cell developments.

Lactate dehydrogenase (leakage) assay (LDH)

Lactate dehydrogenase enzyme present in the cytoplasm of most cell types. Upon cell membrane damage, this cytoplasmic enzyme is released from damaged cells into extracellular medium, which can be measured colorimetrically. The amount of enzyme activity correlated to the proportion of damaged cells. To investigate the effects of both MME and MCE on cell permeability of HepG2 cells LDH assay was performed, cells were incubated with various concentrations 25, 50, 75, 100 and 150 µg/ml of MME and MCE for 24 to 48 h at 37°C. The activity of LDH leakage was significantly increased in HepG2 cells treated with MME and MCE compared to that untreated cells. The increased LDH activity in a dose dependent manner and more LDH leakage was observed with higher concentration of MME (Figure 4).

Caspase-3

Both extracts MME and MCE were showing the increased amount of caspase-3 activities with increased concentration level (25, 50 and 100 µg/ml). Caspase-3 activation suggested that molluscan extracts caused cell death through apoptosis. The MME extract exhibited high caspase-3 activity as shown in Figure 5.

Glutathione (GSH)

Glutathione (GSH, γ- glutamyl - cysteinyl - glycine) was the most abundant non-protein thiol in eukaryotic cells. GSH is required for the tumor cell proliferation and its metabolism. Cancer cells have higher GSH level than the surrounding normal cells, which is a characteristic of higher cell proliferation rate and resistance to chemotherapy. MME and MCE are exhibited in significant depletion of GSH which was observed in treating HepG2 cells at the concentrations of 25, 50 and 100 µg/ml when compared to control cells (Figure 6).

DNA fragmentation

DNA fragmentation was performed to understand the molecular events of MME and MCE on cancer cells, inducing apoptosis. DNA ladders of the corresponding treated samples are confirmed as the apoptosis and showed that MME and MCE treated HepG2 cells exhibited extensive double strand breaks, thereby yielding ladder appearance, while the DNA of control HepG2 cells

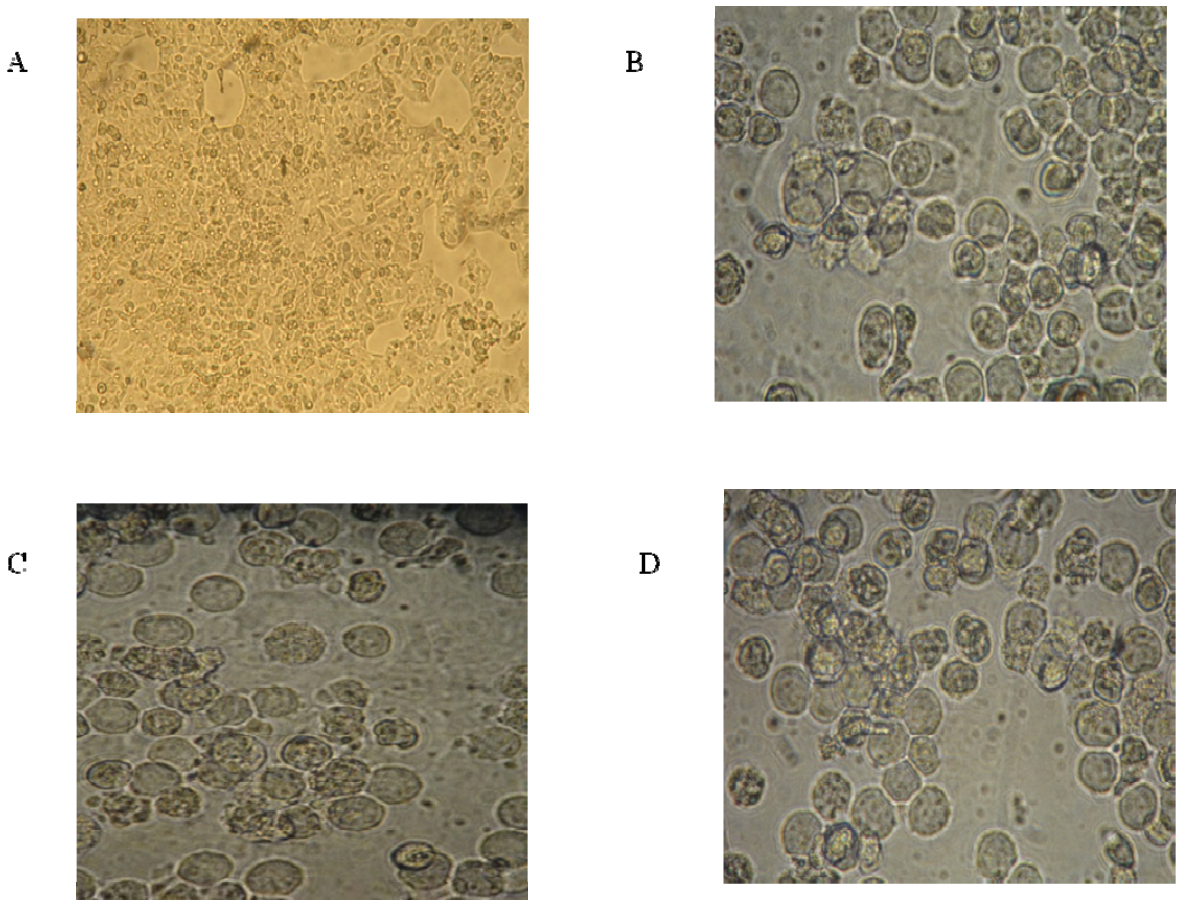

Figure 1. (A) Normal HepG2 cell morphology under light microscope (10×). (B) HepG2 cells showing moderate lysis of cancer cell at treatment of MME extract at 25 µg ml^{-1} concentration (40×). (C) 50 µg ml^{-1} treatment cells showing, well condensed and fragmented nuclei. (D) HepG2 cells treated with 100 µg ml^{-1} concentration, higher lyses of cancer cells.

supplemented with 0.1% DMSO exhibited minimum breakage.

DISCUSSION

In recent years, the researchers paid more and more attention in finding novel anti-cancer drugs from natural resources. Anticancer drugs from marine organisms have attracted recent years, due to its characteristic of multifunction, high sensitivity and stability. There are 'n' number chemical compounds identified in a marine environment, some are in preclinical stages. Example: bryostatin isolated from a bryozoan *Bugula neritina* showed anticancer activities and the compound are in phase II clinical trials. In this present investigation, two bivalve molluscs (*M. meretrix* and *M. casta*) column purified extracts were screened for anticancer activities. The MME showed significant anticancer activities; it was found to strongly inhibit the growth HepG2 cells, it destroyed the cytoskeletal morphology of the cells. While comparing to MME, the MCE showed only acceptable anticancer activities.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. The curative treatment such as tumor resection and liver transplantation are not feasible in advanced stages of HCC (Herold et al., 2002). Therefore, searching a novel anticancer drug for safer and cheaper sources was the treatment of recurrent HCC. Furthermore HCC is well known for its multi drug resistance poor response to current chemotherapeutic agents (Gong et al., 2003). The natural products isolated from marine organisms has been increased rapidly and hundreds of new compounds being discovered every year. Especially, the marine invertebrates (sponges, mollusc, tunicate, etc.) are producing high amounts of bioactive compounds (Burres and Clement, 1989; Corona et al., 2007; Gao et al., 2007; Martinez-Garcia et al., 2007). In the aforementioned objective, the present study was undertaken to evaluate the cytotoxic properties of

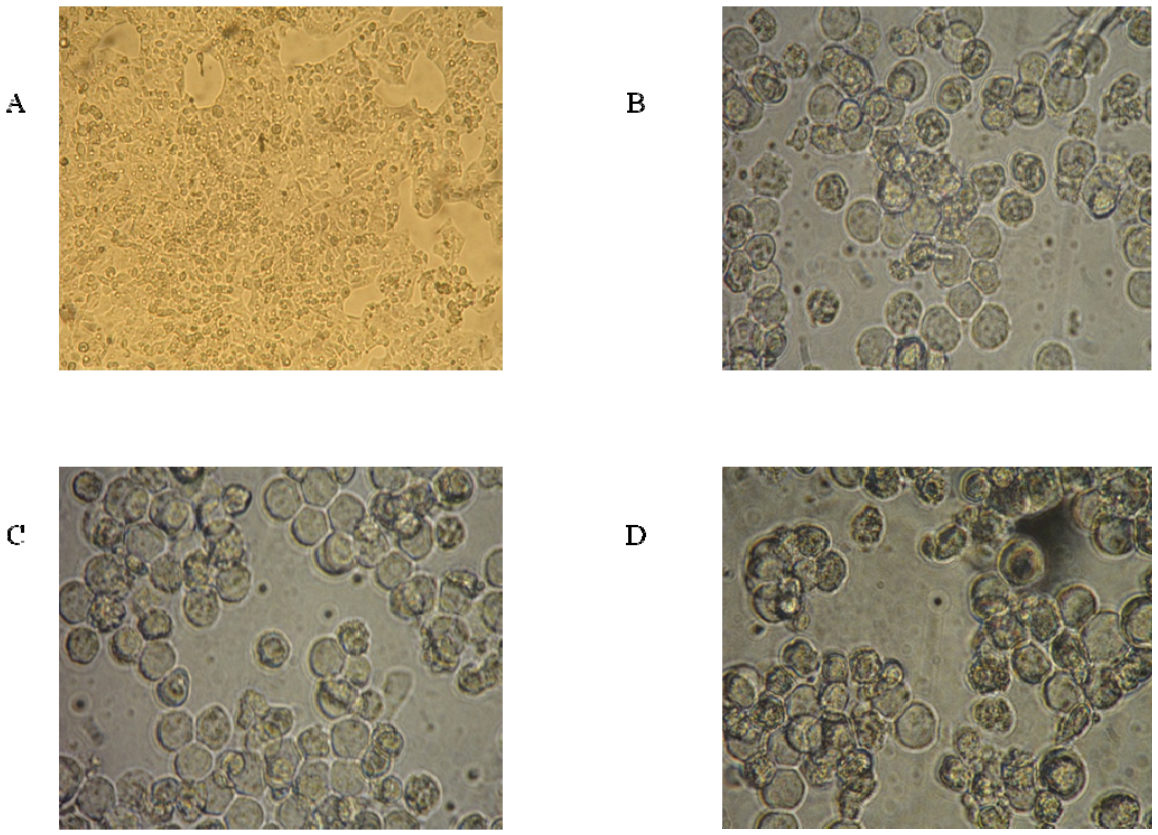

Figure 2. (A) Light microscopic observation of HepG2 cells. (B) Treatment of HepG2 cells with 25µgml^{-1} of MCE. (C) 50 µg ml^{-1} concentration of MCE extracts showing, well reduced and uneven forms of nuclei. (D) HepG2 cells treated with 100 µgml^{-1} concentration.

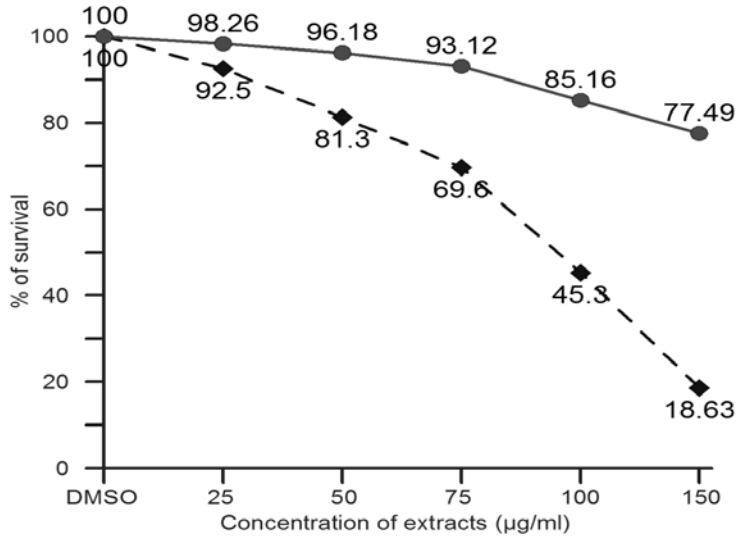

Figure 3. Percentage of HepG2 cell survival on the molluscan extracts determined by tryptophan blue exclusion assay.

two edible marine bivalve mollusc species *M. meretix* and *M. casta* on human hepatoma cell line HepG2. *In vitro* cytotoxic assays are commonly used to screen the chemotherapeutic properties of natural and synthetic

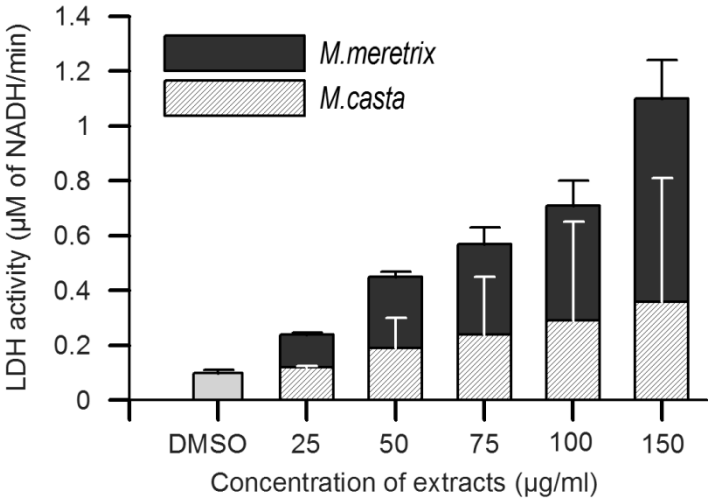

Figure 4. LDH leakage assay was observed in molluscan extracts MME and MCE treated HepG2 cells.

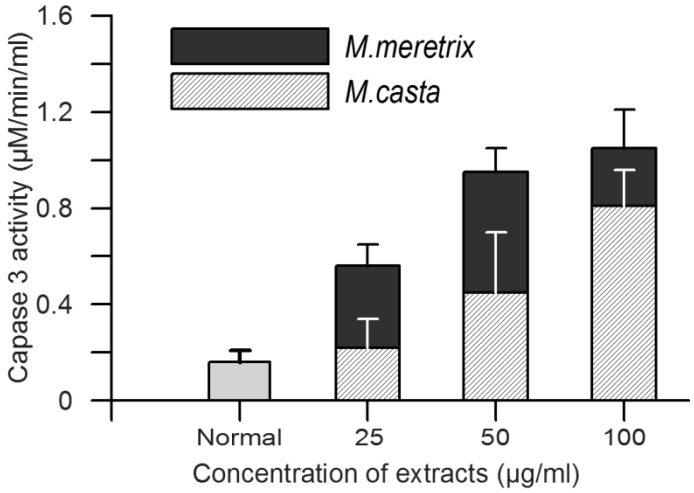

Figure 5. Caspase 3 activities of MME and MCE treated groups, compare to normal extract treated groups showed increased levels of Caspase 3 activities, it confirm the apoptosis.

compounds (Monks et al., 1991). To study the cytotoxic activity of molluscan extracts, MME and MCE was assayed; for cell viability, trypan blue exclusion and lactate dehydrogenase (LDH) leakage assay were performed in human hepatoma cell line HepG2. To destroy the injured cell by a physiological mechanism is called apoptosis. Significant morphological and molecular changes were observed in apoptotic cells (Taraphdar et al., 2001). The rate of apoptosis was calculated from the life span of normal and cancer induced cells. This modulation of apoptosis is important in cancer therapy or prevention of cancer. Apoptosis induction has been a new target for anticancer drug discovery (Workman, 1996). Apoptosis can be characterized by various morphological and molecular changes in the cells. In this connection, light microscopic observation, DNA fragmentation and GSH level were studied in mollusc extract treated HepG2 cells.

Molluscan extracts MME and MCE were shown to markedly reduce the cell viability in a concentration dependent manner. The suppression of cell growth induced by these extracts may be due to induction of cell death rather than the inhibition of cell proliferation. At the concentration of 50 µg/ml, MME showed 81.3% and MCE was 96.18% survivals of HepG2 cells. The inhibitory activities

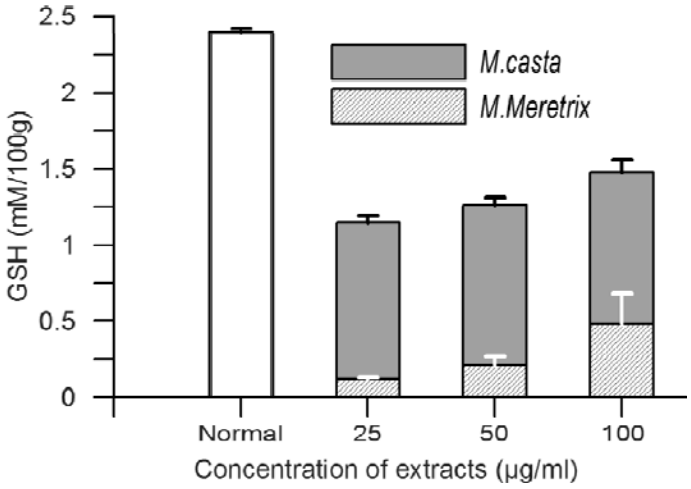

Figure 6. The effect of GSH in HepG2 cells treated with molluscan extracts. The normal group showed increased level of GSH, compared to MME and MCE treated groups.

activities of molluscs extracts were providing evidence for the *in vitro* cytotoxicity. Furthermore, light microscopic observation was proved by anticancer activities in cell line HepG2. The molluscan extracts MME and MCE treated cells showed significant morphological changes. However the control HepG2 cells was seen with high confluence of monolayer without any destruction. Compared to MCE the extract of *M. meretrix* MME showed significant anticancer activities. The column purified extract of *Mercenaria* peptide inhibited the human gastric gland carcinoma cells (BGC-823). At the concentration of 4 µg/ml, the *Merecenaria* peptide strongly inhibited the proliferation of BGC-823 cells and destroyed the skeletal structures of the cells (Leng et al., 2005).

Wu et al. (2006) found out that 7.5 µg/ml concentration of *M. meretrix* showed 60% cell growth inhibition in HepG2 cells and 70% inhibition in HepG3 cells. Likewise, at a concentration of 52.25 µg/ml, column purified novel anticancer protein strongly inhibits the growth of BEL-7402 cells, MCF-7 and human colon cancer cells HCT116 (Ning et al., 2009). Similarly, Lixin et al. (2005) studied antitumor and immune regulation activities of the extracts of some Chinese marine invertebrates and he reported that 95% of ethanol extracts of *Membranipora grandecella, Apostichopus japonicus, M. meretrix* and *Cellana toreuma* have certain antitumor activity in human leukemia cell line HL-60 and human lung cancer cell line A-59.

Recent studies suggested that LDH is more reliable and more accurate marker to study cytotoxicity properties of molluscan extracts MME and MCE. In the present investigation, the LDH leakage was increased significantly in mollusc extracts MME and MCE treated HepG2 cells when compared with control cells. Hence, the LDH in HepG2 cells may be due to the cytotoxicity nature of extracts and it confirms antitumor activity of bivalve molluscan extracts of MME and MCE. The increased LDH activity was in a dose dependent manner and more leakage was observed in high MML concentration. Ning et al. (2009) extracted a novel protein from *M. meretrix* and the protein showed increased cell membrane permeability in human hepatoma cell lines BEL-7402. Compared with untreated cells the LDH leakage was significantly increased in MML treated cells.

Caspases are a class of intracellular cytokine proteases which was considered to be the central components of the apoptotic responses. By breaking down key cellular components that are required for maintaining normal cellular functions caspases are responsible for executing morphological and biochemical consequences directly or indirectly attributed to apoptosis in order to understand the mechanism of antitumor effect of molluscan extracts MME and MCE. Caspases cascade has been demonstrated to be involved in apoptosis signal transduction and execution (Salvesen and Dixit, 1997). Human Caspases 1 to 10 have been described and activation of the caspase cascade involved in chemical-induced apoptosis including degradation of DNA repair enzyme poly ADP ribopolymerase (Lazebnik et al., 1994) and DNA dependent kinase. In the present investigation, molluscan extracts MME and MCE resulted in the activated Caspase-3 activity in HepG2 medium. In molluscan extracts MME and MCE concentration increase, the caspase-3 activity was observed. Usually caspase-3 exists in an inactive form of pro-caspase 3 that becomes proteolytically activated by multiple cleavages of its precursors to generate the active forms in cells undergoing apoptosis. The extract of MME and MCE may induce the

proteolytic cleavage of caspase-3.

In the present study, DNA ladders appeared in molluscan extracts treated HepG2 cells at the concentration of 50 µg/ml for 48 h. There, no fragmentation was observed in control cells. The degradation of DNA into oligonucleosomal fragments is a late event of apoptosis (Compton, 1992). The molluscan extracts MME and MCE induces DNA damage in HepG2 cells and thereby causes apoptosis from this observation, it is inferred that molluscan extracts MME and MCE may exert an anticancer effect through DNA damage in HepG2 cells and promote apoptosis. The reduced tripeptide glutathione (GSH) is a hydroxyl radical and a single oxygen scavenger and participates in a wide range of cellular functions such as protein and DNA synthesis, intermediary metabolism and transport (Deneke and Fanburg, 1989). Glutathione protects the cells from the toxic effects of reactive oxygen species and is an important component of cellular process and depletion of GSH leads to increased accumulation of lipid peroxides and loss of cell viability (Axelsson and Mannervik, 1983). This glutathione is considered to be one of the most important components of the antioxidant defense system in living cells. It plays a critical role in cellular defence against oxidative stress by inactivating free radicals, reactive oxygen species and a variety of cytotoxic electrophiles including alkylating agents.

The GSH level was measured to evaluate the antitumor property of molluscan extracts MME and MCE. In the present investigation, the levels of GSH were significantly decreased in MME and MCE treated HepG2 cells. Studies in a variety of cell types suggested that cancer chemotherapeutic drugs induce tumor cell apoptosis in part by increasing the formation of ROS (Siitonen et al., 1996). The present study indicates that molluscan extracts MME and MCE might rapidly induce intracellular oxidation in HepG2 cells and cause apoptosis.

Conclusion

Hepatoma cell line HepG2 viability was significantly reduced in MME and MCE treated cells at 50 µg ml^{-1} concentrations. With light microscopic observation the extracts treated HepG2 cells showed modified cell morphological features. Lactate dehydrogenase was significantly released from the extracts treated cells. Reduced glutathione levels were observed in MME and MCE treated HepG2 cells. Further DNA ladder assay showed stronger lysis of DNA in MME treated cells, it further confirms the induction of apoptosis in HepG2 treated cells. As compared to MME, the MCE showed weaker anticancer property. On observation, it can be concluded that extract MME has been a highly selective and effective anticancer drug for human welfare.

Conflict of interest

Authors declare that they have no conflicts of interest

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