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Full Length Research Paper

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

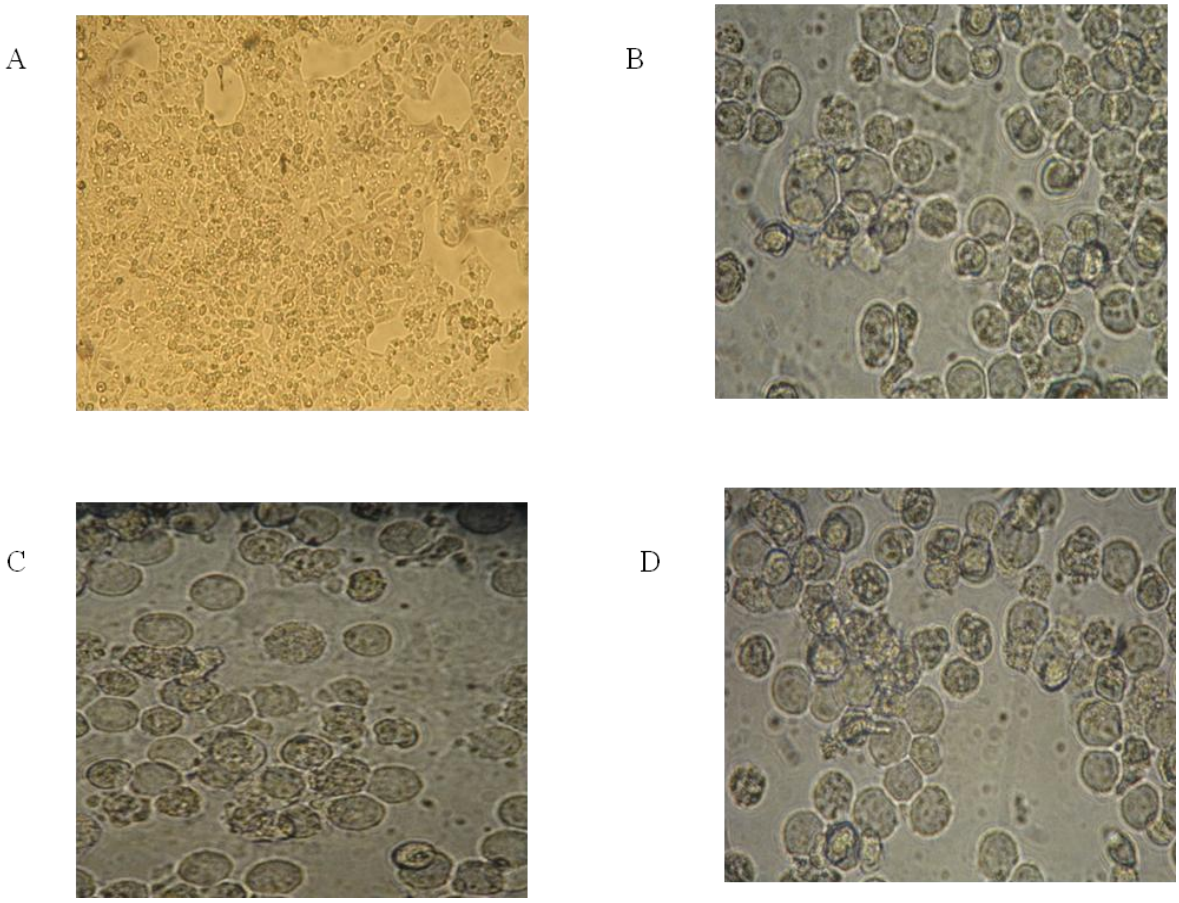

Figure 1. (A) Normal HepG2 cell morphology under light microscope (10×). (B) HepG2 cells showing moderate lysis of cancer cell at treatment of MME extract at 25 µg ml^{-1} concentration (40×). (C) 50 µg ml^{-1} treatment cells showing, well condensed and fragmented nuclei. (D) HepG2 cells treated with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

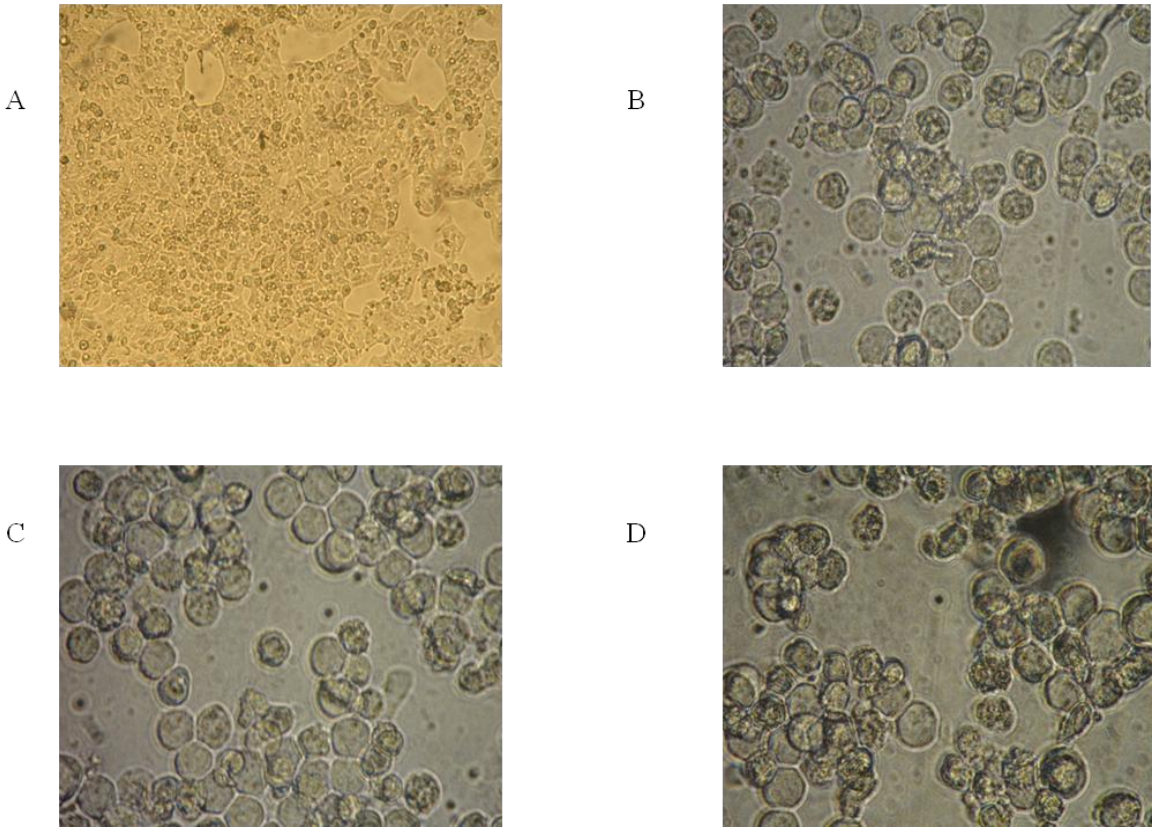

Figure 2. (A) Light microscopic observation of HepG2 cells. (B) Treatment of HepG2 cells with 25µgml^{-1} of MCE. (C) 50 µg ml^{-1} concentration of MCE extracts showing, well reduced and uneven forms of nuclei. (D) HepG2 cells treated with 100 µgml^{-1} concentration.

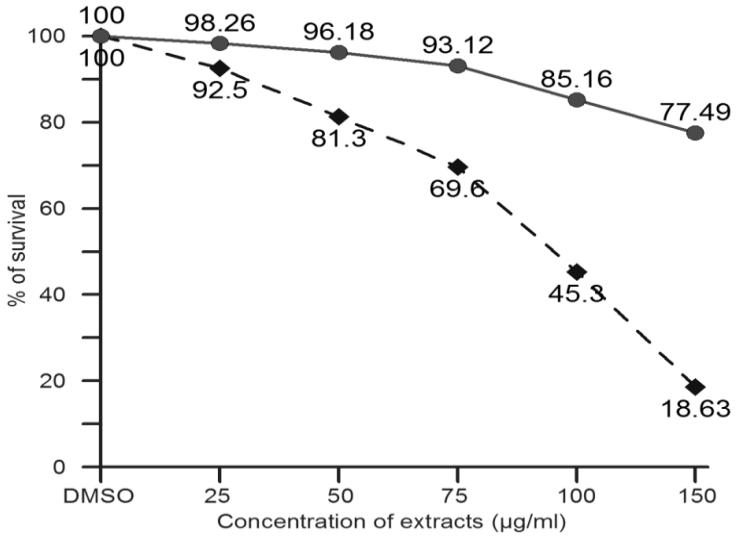

Figure 3. Percentage of HepG2 cell survival on the molluscan extracts determined by tryptophan blue exclusion assay.

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

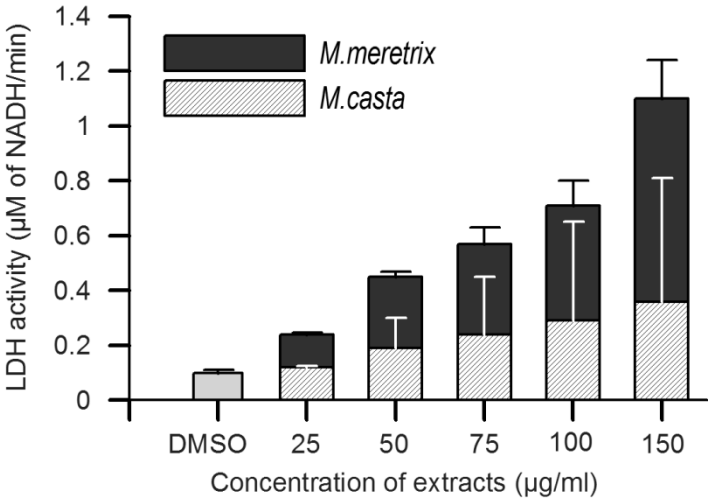

Figure 4. LDH leakage assay was observed in molluscan extracts MME and MCE treated HepG2 cells.

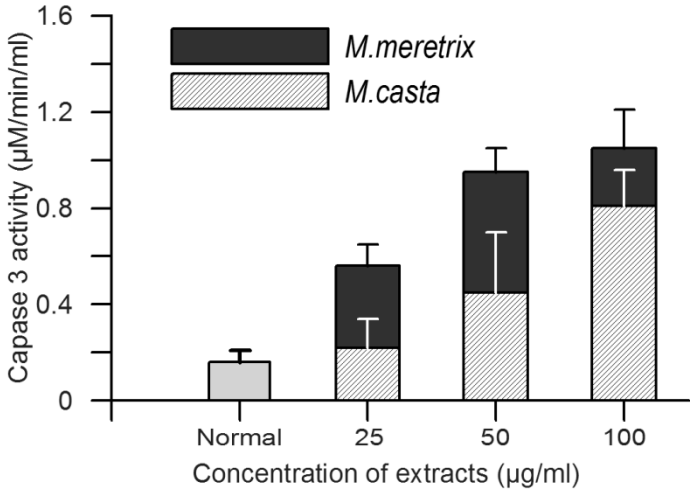

Figure 5. Caspase 3 activities of MME and MCE treated groups, compare to normal extract treated groups showed increased levels of Caspase 3 activities, it confirm the apoptosis.

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

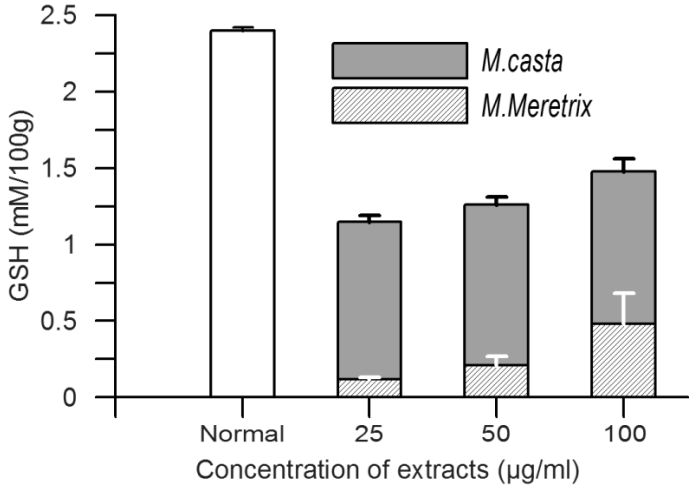

Figure 6. The effect of GSH in HepG2 cells treated with molluscan extracts. The normal group showed increased level of GSH, compared to MME and MCE treated groups.

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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Optimization of enzyme-assisted extraction of anthocyanins from blackberry (*Rubus fruticosus* L.) juice using response surface methodology

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Preparations of pectic enzymes are used for more efficient extraction of desirable blackberry pigments, facilitating faster release. In this study, we validated the use of response surface methodology (RSM) for the optimization of enzymatic treatment for extraction of anthocyanins from blackberry juice. Tristimulus colorimetry was used to quantitatively and qualitatively evaluate the process. Our results showed that the optimal yield (639 g/L) of anthocyanins extracted from blackberries by this study's enzymatic mixture was obtained under the following conditions: enzyme loading 0.2% and 52°C for 1.1 h. The yield of anthocyanins showed high correlations with lightness (L*) (r = -0.833), chroma (C*) (r = 0.796) and hue angle (h) (r = 0.752), and was significantly affected by the extraction temperature (p = 0.0011).

Key words: Anthocyanins, blackberry, response surface methodology, optimization, pectic enzyme.

INTRODUCTION

Anthocyanins are found widely in higher plants in their roots, caudexes and leaves as well as in their flowers and fruits. They have been in high demand by the food industry as replacements for synthetic dyes due to legislative action against and consumer concerns about synthetic food additives (Francis, 1989; Fabre et al., 1993). Anthocyanins also possess pharmacological properties and are used by humans for therapeutic purposes. Increasing numbers of studies have shown that anthocyanins have beneficial effects in the context of myriad human diseases, including liver dysfunction, hypertension, vision disorders, microbial infections and diarrhea (Bors et al., 1998; Smith et al., 2000; Wang et al., 2000). Mechanical crushing of berries results in a highly viscous fruit puree from which it is difficult to directly extract juice by pressing. For this reason, the addition of pectinolytic enzyme preparations to the fruit pulp prior to pressing is a prerequisite to obtaining satisfactory juice yield and efficient use of the press in the industrial production of black currant juice and concentrates. Pectic enzymes that are used in the fruit juice industry and increasingly in wine manufacturing, originate largely from fungal sources, notably Aspergillus niger spp. (Grassin and Fauquembergue, 1996). These enzyme preparations are generally multicomponent since they contain various pectinolytic and other plant cell wall

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degrading enzymes. During the pressing of liquefied berry mash, the juice is separated from the skin and seeds. The resulting juice contains relatively high levels of phenolics with an intense dark-purple color from anthocyanins, but the press residue is still rich in anthocyanins and other phenolics (Landbo and Meyer, 2004).

Preparations of pectic enzymes are used for more efficient extraction of desirable fruit pigments and other phenol compounds bound in plant cells, accelerating their release. These preparations also shorten the time required for maceration, settling and filtration, resulting in quicker release of red grape pigments and aroma compounds (Meyer et al., 1998; Schieber et al., 2001; Capounova and Drdak, 2002; Muñoz et al., 2004).

Blackberries are a good source of anthocyanins, with a reported anthocyanin content ranging from 67 to 230 mg/100 g fresh weight (Sellappan et al., 2002; Benvenuti et al., 2004). Blackberry juice (like all fruit and vegetable juices) is considered an ingredient when used in foods as a colorant. Current studies on anthocyanins from blackberries are focused on their stability and antioxidant properties (Wang and Lin, 2000; Elisia et al., 2007; Wang and Xu, 2007). However, few details are available on extraction process parameters for anthocyanins from blackberries using pectic enzymes in the literature, suggesting that an optimization method is needed for the extraction process of anthoycanins from blackberries using pectic enzymes.

Response surface methodology (RSM) is an effective statistical technique for optimizing complex processes. It is widely used in optimizing process variables. The basic theoretical and fundamental aspects of RSM have been previously reviewed (Farooq et al., 1997; Chandrika and Fereidoon, 2005). Color is one of the most important attributes of natural colorants and can be mostly attributed to anthocyanin pigments. The application of colorimetric systems based on uniform (CIELUV and CIELAB) and non- (CIEXYZ) uniform color spaces is useful in the quantification and characterization of the color properties of pigments and foods. The correlation between some color parameters and pigment content in food has been evaluated in previous studies (Kammerer et al., 2004; Montes et al., 2005), but the relationship between the total anthocyanin content and the color parameters in blackberries have not been studied during the extraction process.

In this study, we investigated some of the potential main factors (enzyme loading, extraction temperature and time) that may be related to the extraction of anthocyanins from blackberries; the color properties of the anthocyanins extracts were established by tritimulus colorimetry. The aim of this study was to validate the use of RSM to optimize the process conditions for quantitative and qualitative (relative to color properties) extraction of anthocyanins from blackberries using a commercial pectic enzyme preparation.

MATERIALS AND METHODS

Blackberries were purchased from a local farm in Nanjing City and stored at -20°C until use. A commercial pectic enzyme preparation (Klerzyme-150) was purchased from Shanghai Chemical Reagent Co., Shanghai, China. This preparation is 1 to 10% liquid pectinase derived from *Aspergillus niger.*

Enzymatic treatment

Frozen blackberries were thawed at 25°C for 6 h, and then crushed for 30 s using a triturator (Model DS-1, Shanghai Specimen and Model Factory, Shanghai, China). Crushed berries were transferred to a 50 ml conical flask and pectic enzyme was added to the crushed berries with different enzyme loadings (0.0 to 0.5%, m/m). Mixtures were then put in a thermostatic water bath at specific temperatures (20 to 70°C) for varying periods of time (0 to 2.5 h). Afterwards, the mixtures were centrifuged at 4000 rpm for 15 min. The supernatant was collected and transferred into a 50 ml volumetric flask for the determination of anthocyanin yield. Twenty grams of each sample was used for each treatment condition.

Experimental design

First, the single factor experiment for extraction was performed analyzing the effect of three factors (enzyme loading, temperature and time) on the extraction of anthocyanins from blackberries. Then the optimization of extraction process parameters (temperature, enzyme loading and time) was performed using Box-Behnken design (Table 1) and a model of extraction efficiency incorporating the 3 process parameters was developed and validated. Finally, the composition of anthocyanins was detected in the blackberries by high performance liquid chromatography (HPLC).

RSM was used to determine the optimal conditions for anthocyanin extraction from blackberries using Klerzyme-150. Experimental design and statistical analyses were performed using Stat-Ease software (Design-Expert 6.0.10 Trial, Delaware, USA Echip, 1993). A three-level, three-factor Box-Behnken design was chosen to evaluate the combined effect of the three independent variables of enzyme loading, temperature and time, which were coded as A, B and C, respectively. The minimum and maximum values were set for extraction temperature at 40 and 60°C, extraction times of 0.5 and 1 h and enzyme loadings of 1 and 3% (m/m) (Table 1). The measured response values were anthocyanin pigment yield, L*, *C** and *h*. The complete design consisted of 17 combinations including five replicates of the center point (Table 3) (Myers and Montgomery, 2002). The response function (Y) was partitioned into linear, quadratic and interactive components:

$$Y = \beta_0 + \sum_{i=1}^{k} B_i X_1 + \sum_{i=1}^{k} B_{ii} X^2 + \sum_{i>j}^{k} B_{ij} X_i X_j ,$$

Where β_0 is defined as the constant, B_i the linear coefficient, B_{ii} the quadratic coefficient and B_{ij} the cross product coefficient. X_i and X_j were defined as the levels of the independent variables, while k equaled the number of tested factors (k = 3). Analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were

Table 1. Independent variables and their coded and actual values used for optimization.

Independent variable	Units	Symbol	Code levels		
			-1	0	1
Enzyme loading	%	*A*	0.1	0.2	0.3
Temperature	°C	*B*	40	50	60
Time	h	*C*	0.5	1	1.5

Table 2. Effect of different conditions for extraction on anthocyanins yield.

Index	Effect of enzyme loading on blackberry anthocyanin yield (1 h, 40°C) [Enzyme loading (%, m/m)]					
	0	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
	376±18^{c}	413±11^{b}	452±12^{a}	461±11^{a}	462±15^{a}	428±10^{b}
	Effect of temperature on blackberry anthocyanin yield (2%, 1 h) [Temperature (°C)]					
	20	**30**	**40**	**50**	**60**	**70**
Anthocyanin yield (g/L)	442±16^{d}	496±11^{c}	535±17^{b}	568±12^{a}	541±15^{b}	542±18^{b}
	Effect of time on blackberry anthocyanin yield (2%, 50°C) [Time (h)]					
	0	**0.5**	**1**	**1.5**	**2**	**2.5**
	466±16^{c}	536±18^{b}	572±10^{a}	564±17^{a}	588±12^{a}	536±19^{b}

determined. The significances of all terms in the polynomial were statistically evaluated by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models.

Determination of anthocyanin yield

The quantification of total juice anthocyanin content was determined by pH-differential methods as previously described by Giusti and Wrolstad (2001). Total anthocyanins were calculated as cyanidin-3-glucoside according to the following equation:

Total anthocyanins (mg/L) = A × MW × DF × 1000 / (ε × 1)

Where A = (A_{510} – A_{700}) pH 1.0 – (A_{510} – A_{700}) pH 4.5, molecular weight (MW) = 449.2 g/mol for caynidin-3-glucoside, DF = dilution factor, 1 = pathlength in cm, ε = 26,900 (molar extinction coefficient) in L/mol/cm for caynidin-3-glucoside and 1000 = conversion from g to mg. All analyses were done in triplicate (n = 3).

Other anathlytes

Brix was measured at 25°C using an Abbe refractometor (Atago, Tokyo, Japan). The turbidity of juice was measured by a STZ-A24 turbidimeter (Guangming Turbidimeter Plant, Wuxi, China) and expressed in nephelometric turbidity units (NTU). Proteins concentrations were determined by the micro-Kjeldahl nitrogen method × 6.25 (AOAC, 1990). Titratable acidity and total sugar were determined according to the standard method (AOAC, 1990).

Color coordinates

The weighted-ordinated method (constant intervals, Δλ = 2 nm) was applied to obtain tristimulus values, using as references the CIE Standard Illuminant D_{65}, the CIE 1964 Standard Observer, and water as the reference blank. Following the most recent recommendations made by the CIE, CIELAB System (the variables related with psychometric color attributes: L*, *C** and *h* for color specification was applied (Cevallos-Casals and Cisneros-Zwvallos, 2004).

Statistics

All trials were carried out in triplicate and all data were reported as means ± standard deviation (SD). Statistical significance was evaluated using Student's t-test and *P* values < 0.05, 0.01 or 0.001 were considered significant.

RESULTS AND DISCUSSION

Effect of enzyme loading, temperature and extraction time on anthocyanin yield

The effect of enzyme loading on extraction of anthocyanins was shown in Table 2. Anthocyanin yield increased when enzyme loading was increased from 0 to 0.2% (m/m), but did not significantly increase when the ratio was higher than 0.2%. These data suggested that the solvent-solid ratio of 0.2% was the optimal ratio for

Table 3. Observed and predicted values of L*, C*, *h* and anthocyanin yield obtained by the Box-Behnken experiment.

No.	Independent variables			Dependent variables			
	Enzyme loading (%, m/m)	Temperature (°C)	Time (h)	Anthocyanin yield (g/L)	L*	C*	*h*
1	0.1(-1)	40(-1)	1.0(0)	542±11	31.00±0.18	31.24±0.05	10.93±0.12
2	0.1(-1)	60(1)	1.0(0)	589±10	30.47±0.10	32.34±0.07	12.98±0.07
3	0.3(1)	40(-1)	1.0(0)	511±11	31.34±0.10	30.88±0.07	9.53±0.13
4	0.3(1)	60(1)	1.0(0)	579±10	30.72±0.09	31.02±0.03	12.02±0.03
5	0.2(0)	40(-1)	0.5(-1)	529±18	31.28±0.07	31.14±0.11	10.60±0.09
6	0.2(0)	60(1)	0.5(-1)	585±17	31.21±0.14	31.33±0.09	10.27±0.01
7	0.2(-1)	40(0)	1.5(1)	575±16	31.00±0.04	31.37±0.07	8.87±0.18
8	0.2(1)	60(1)	1.5(1)	589±12	30.54±0.09	32.37±0.05	12.72±0.07
9	0.1(-1)	50(0)	0.5(-1)	585±10	30.37±0.11	32.54±0.06	13.03±0.06
10	0.3(0)	50(0)	0.5(-1)	595±11	31.31±0.06	32.35±0.18	13.92±0.05
11	0.1(0)	50(0)	1.5(1)	589±14	30.20±0.04	32.37±0.07	13.99±0.09
12	0.3(1)	50(1)	1.5(1)	595±14	30.36±0.07	32.31±0.01	13.49±0.14
13	0.2(0)	50(0)	1(0)	619±18	29.32±0.09	32.35±0.09	13.92±0.07
14	0.2(0)	50(0)	1(0)	623±15	29.49±0.07	32.88±0.15	13.85±0.08
15	0.2(0)	50(0)	1(0)	624±10	29.31±0.06	32.28±0.03	13.24±0.11
16	0.2(0)	50(0)	1(0)	627±17	29.35±0.15	32.99±0.04	14.03±0.15
17	0.2(0)	50(0)	1(0)	631±14	29.31±0.07	32.35±0.02	13.30±0.05

anthocyanin extraction for this case. Anthocyanin yield also increased with increasing temperatures from 20 to 50°C, but declined when the extraction temperature was above 50°C. These results indicated that 50°C was the optimal temperature for anthocyanin extraction from blackberries using Klerzyme-150 (Table 2). Table 2 showed the effect of different extraction times on anthocyanin yield: yield increased when the extraction time was extended from 0 to 1 h, but remained approximately the same when the time was extended from 1 to 2 h. Anthocyanin yield significantly declined when the extraction time was extended from 2 to 2.5 h, indicating that extraction times between 1 to 2 h were the optimal duration for anthocyanin extraction in this study.

Analysis of the Box-Behnken experiment

The results for each dependent variable and their coefficients of determination (R^2) were summarized in Tables 3 and 4. Statistical analyses indicated that the proposed model was adequate, possessing no significant lack of fit and with very satisfactory of the R^2 for all responses. The R^2 values for anthocyanin yield, L*, *C** and *h* were 0.955, 0.989, 0.883 and 0.986, respectively. Coefficient of variances (Table 4) for anthocyanin yield, L*, *C** and *h* were within the acceptable range. In general, a high coefficient of variance indicates that variation in the mean value is high and does not result in an adequate response model (Chandrika and Shahidi, 2005). The probability (*p*) values of all regression models were less than 0.05. The effects of enzyme loading, temperature and time on anthocyanin yield, L*, *C** and *h* were reported (Table 4) by the coefficient of the second-order polynomials. Response surface and contour plots were used to illustrate the effect of extraction temperature, extraction time and solid-liquid ratio on the responses. Response surfaces for anthocyanins yield were shown in Figures 1 to 3.

The effect of temperature and enzyme loading on anthocyanin yield was shown in Figure 1. When enzyme loading increased to approximately 0.2 %, temperature became a critical factor in improving anthocyanin yield. The fluctuation in temperature could lead to great differences in anthocyanin yield. It could be observed that the optimal temperature and enzyme loading for anthocyanins extraction were about 50°C and 0.2%. We consider that the dissolving of anthocyanins was inhibited when the temperature was lower than 50°C; and when the temperature was exorbitantly higher than 50°C, anthocyanins could be degraded and its structure could be rearranged (Chigurupati et al., 2002). When the optimal temperature was at about 50°C, the highest yield of anthocyanins could be achieved when the enzyme loading was about 0.2% and the yield slight decreased when the enzyme loading was higher than 0.2%. Considering the cost of enzyme loading, it was advisable that the enzyme loading should be set at 0.2%.

The effect of temperature and extraction time on anthocyanin yield was illustrated in Figure 2. When the

Table 4. Regression coefficients (R^2) and CV values for four dependent variables for anthocyanin extraction from blackberries.

Coefficient	Anthocyanin yield	L*	C*	*h*
β_0 (intercept)	636.20	23.96	32.57	13.67
Liner				
B_1	-3.12	0.21**	-0.24	-0.13
B_2	23.13**	-0.21**	0.15***	1.01**
B_3	6.75	-0.26***	-0.02	0.04
Quadratic				
B_{11}	-29.73**	0.54	-0.03	-0.27
B_{22}	51.23***	0.99***	-1.17	-2.04***
B_{33}	-15.47*	0.66***	-0.15	-1.02*
Cross product				
B_{12}	5.25	-0.02	-0.24	0.11
B_{13}	-1.00	-0.19*	0.03	-0.12
B_{23}	-10.50*	-0.10	-0.10	1.05**
R^2	0.955	0.989	0.883	0.936
CV	2.07	0.41	1.13	4.93
Probability (*p*)	0.0006***	<0.0001***	0.0145*	0.0021**

*Significant at 0.05; **significant at 0.01; ***significant at 0.001

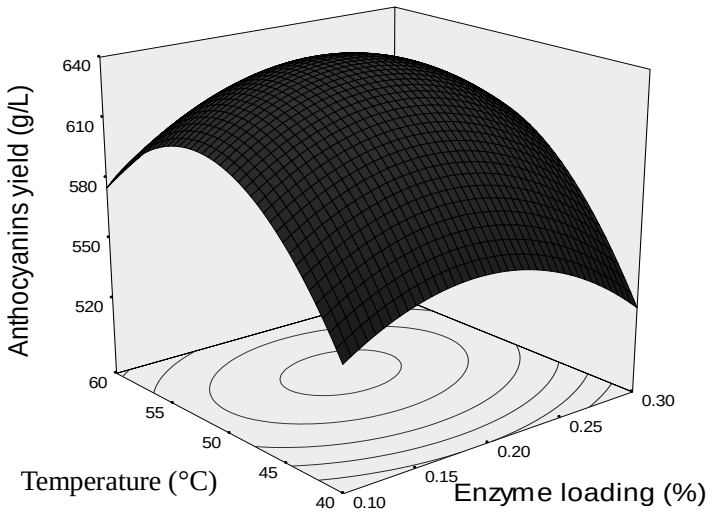

Figure 1. The effect of enzyme loading and temperature for enzyme extraction on anthocyanin yield.

extraction time reached approximately 1 h, temperature became the critical factor for improving anthocyanin yield. Fluctuation in temperature could lead to large differences in anthocyanin yield. It can be seen from Figure 2 that the optimal temperature and time for anthocyanin extraction was approximately 50°C and 1 h, respectively. If the extraction time was shorter than 1 h, the dissolution of anthocyanins did not reach equilibrium with the anthocyanins remaining in the blackberry. When the time was longer than 1 h, the anthocyanin yield slightly decreased. This may be due to the long time of exposure for dissolved anthocyanins to oxygen, light and microorganisms

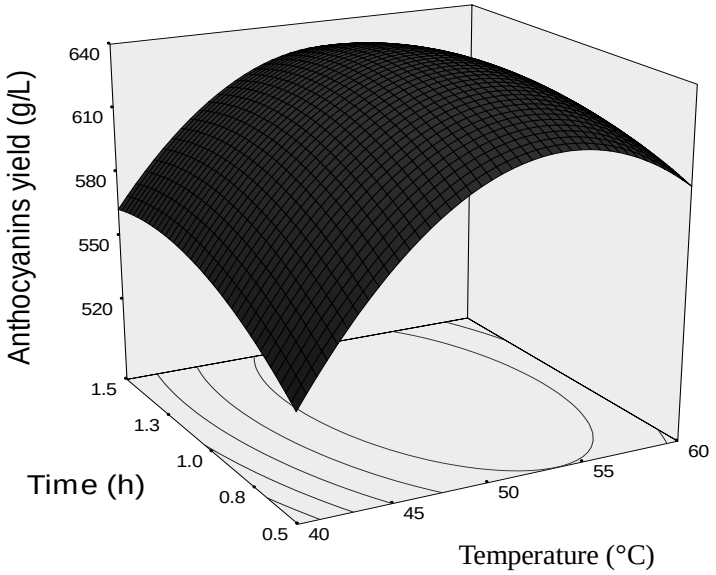

Figure 2. The effect of temperature and extraction time on anthocyanin yield.

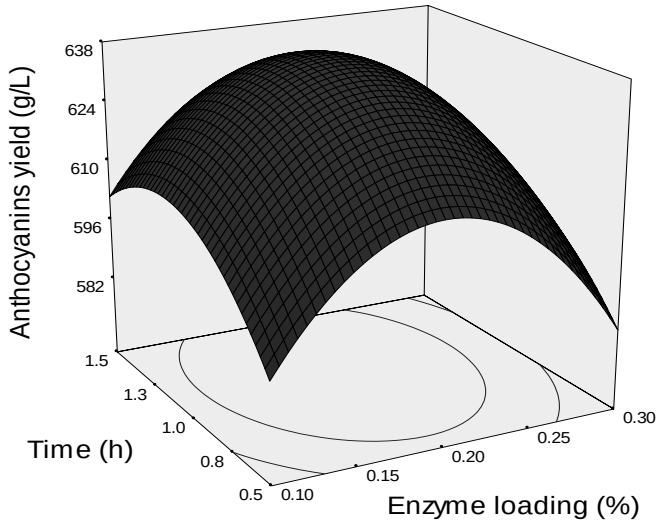

Figure 3. The effect of enzyme loading and extraction time on anthocyanin yield.

in the environment leading to increased chances of oxidation or degradation of dissolved anthocyanins (Chigurupati et al., 2002).

The effect of enzyme loading and time on anthocyanin yield was shown in Figure 3. Both enzyme loading and time had a significant, quadratic effect ($p < 0.05$) on anthocyanin yield. Yield increased when the enzyme loading was increased from 0.1 to 0.2%, but did not continue increasing when the enzyme loading was higher than 0.2%. The response surface shows the optimal conditions for the extraction process relative to the anthocyanin yield. It was observed that the optimal conditions for anthocyanin yield were slightly different. There were multiple combinations of variables that could give maximum levels of anthocyanin yield. Since the optimal response for each dependent variable did not fall in exactly the same region, all the response surfaces were superimposed. During anthocyanin extraction, the

Table 5. Design and results of confirmatory trials.

Trial	Enzyme loading (%, m/m)	Temperature (°C)	Time (h)	Anthocyanin yield (g/L)	
				Observed value	Predicted value
Optimal condition	0.2	52	1.1	620±10	639
Random condition 1	0.2	40	1.5	550±15	564
Random condition 2	0.2	60	1	590±12	608

parameters of enzyme loading, temperature and time are important. Therefore, the best combination of process variables for the response functions was found. The process variables resulting in the best combination of response functions were enzyme loading of 0.2% at a temperature of 52°C and a time of 1.1 h. The response functions were calculated from the final polynomial, resulting in a response of 639 g/L for anthocyanin yield, 29.32 for L*, 32.54 for *C** and 13.80 for *h*.

Correlations between anthocyanin yield and color parameters

Correlations between the yield of anthocyanins and color parameters were also explored in this study. A negative correlation (r = -0.833) was found between yield and L*, indicating that higher L* values correlated with lower yield of anthocyanins. Positive correlations were identified between the anthocyanin yield and chroma (*C**) (r = 0.796). This positive correlation indicated that high *C** values correlated with high anthocyanin yield. These results are in agreement with Montes et al. (2005) who evaluated the correlations between anthocyanin yield and *C** and L* in Jaboticaba fruit. Similarly, the correlation between yield and *h* was positive (r = 0.752).

Verification of the model

Within the scopes of the variables investigated in the Box-Behnken design, additional experiments were performed under different conditions for anthocyanins extraction to assess the validity of the model (Equation 1). The design and results of the confirmatory trials were shown in Table 5. It was demonstrated that there was a high degree of fit between the values observed in the experiment and the values predicted by Equation 1.

Conclusion

The use of pectolytic enzymes can give high yields of anthocyanins from blackberries. Testing different process conditions (enzyme loading, temperature and time) for the extraction of anthocyanins revealed that the extraction temperature significantly affected the yield of anthocyanins from blackberries processed with Klerzyme-150. The relationship of yield to extraction conditions can be modeled by second-order polynomials. The optimization of the extraction process using Klerzyme-150 was performed by RSM, and the optimal conditions for anthocyanin yield from blackberries were found to be an enzyme loading of 0.2% (m/m) at a temperature of 50C for a time of 1.1 h under these study conditions. The correlations between anthocyanin yield and the color parameters (L*) were relatively high (r = –0.833). The results of this study show the utility of RSM as a process optimization approach for extraction. Future studies are necessary to make broader conclusions about the optimization of different process parameters on anthocyanin extraction by testing and controlling for blackberries of different origins, and for different pectolytic enzyme mixtures with well-controlled purity, activity and composition.

Conflict of interest

Authors declare that they have no conflicts of interest.

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Evaluation of treatment of suspected meningitis according to guidelines in a hospital in the United Kingdom

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Acute meningitis is a potentially life-threatening neurological emergency which requires rapid diagnosis and early administration of appropriate empirical therapy. The compliance regarding empirical treatment of acute meningitis to the Barts and the London Trust (BLT) guidelines was assessed along with the use of corticosteroids. A retrospective audit for the time period of 1st January, 2008 to 21st May, 2009 was carried out in order to determine the level of compliance to the Trust guidelines in empirical treatment and the use of corticosteroids in acute meningitis. Patients were identified from cerebrospinal fluid specimens sent to the Microbiology Department from five wards in the BLT. This project's primary outcome is the extent to which prescribers follow current Trust guidelines regarding empirical antimicrobial therapy of community acquired meningitis/encephalitis and the extent to which corticosteroids are prescribed as adjuvant treatment. Twenty nine patients with suspected meningitis were identified. Eighty-nine percent of patients were initiated on appropriate antibiotics in accordance with Trust guidelines and 79% of patients on antiviral agents accordingly. When all elements of guideline compliance was assessed (antimicrobial choice, dose, time of administration etc), compliance fell to 38%. Empirical treatment was delayed for more than 6 h from admission in 30% of patients receiving antibiotics and 47% of patients receiving antivirals. Corticosteroids were not used. After identifying fairly low level of compliance, a suitable strategy for the improvement has to be developed. The place of corticosteroid therapy for the treatment of meningitis will be more specific.

Key words: Meningitis, antimicrobial agents, hospital, corticosteroids.

INTRODUCTION

Despite the availability of effective antimicrobial treatment, meningitis remains an important cause of

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morbidity and mortality globally (Van de Beek et al. 2002; Lepur and Baršić 2007; Van de Beek et al. 2004a). Around 1.2 million cases of acute meningitis occur every year in the world, resulting in 135,000 deaths (Cullen 2005; Van de Beek et al. 2006). Hence, early recognition, assessment of disease severity and administration of appropriate antibiotic therapy are considered to be crucial in achieving a beneficial clinical outcome in patients with meningitis (Fitch and van de Beek 2007). Possible beneficial effects of corticosteroids on morbidity and mortality in meningitis is seen in their ability to attenuate subarachnoid space inflammation caused by inflammatory response, but there is a concern that corticosteroids might decrease cerebrospinal fluid (CSF) penetration of antimicrobials (Heyderman et al. 2003; Van de Beek et al. 2004b; Begg et al. 1999; O'Donnell et al. 2009; Korshin et al. 2007; Peterković et al. 2012).

Empirical antimicrobial therapy of suspected meningitis is needed in the case of delayed lumbar puncture (LP), presence of purulent meningitis or negative CSF Gram stain results and it should be initiated as soon as possible (preferably inside of one hour after admission) (Chadwick and Lever 2002; Gjini et al. 2006). Empirical treatment should depend mainly on the most common meningeal pathogen(s) causing the disease, the patient's age and underlying conditions, and it should last at least 48 to 72 h or until the diagnosis of meningitis/encephalitis can be ruled out (Sáez-Llorens and O'Ryan 2001; Mitropoulos et al. 2008). Meningitis guidelines for the management of adult patients with suspected bacterial meningitis and meningococcal sepsis have been available on the Bart's and the London intranet since 2001. This guideline represents a complete clinical care pathway for the patient presenting with signs and symptoms of meningitis to Barts and the London Trust (BLT) (Appendix 1) (Begg et al. 1999; Chaudhuri et al. 2008; Solomon et al. 2007; Tunkel et al. 2004).

Aforementioned stated facts along with the Trust guidelines all advocate the importance of early and proper management of patients with suspected or proven meningitis. In that sense it is reasonable to conduct an audit at Barts and The London Trust to assess the quality of patients' care, level of compliance to the Trust guidelines and to identify a strategy for improvement of clinical practice if needed. The aim of this retrospective audit is to review the pharmacological management of patients admitted to the Barts and The London NHS Trust with suspected community acquired meningitis and to assess compliance with the Trust guidelines.

MATERIALS AND METHODS

Hospital numbers and specimen details were obtained from the Microbiology Department, for patients whose CSF specimens were taken by LP and sent for analysis from five wards at the Royal London Hospital (RLH) from 1st January, 2008 until 21st May, 2009. One-hundred and sixty-seven patients' hospital numbers were obtained and their electronic patient records (EPRs) were reviewed in order to determine if they were admitted with suspected community acquired meningitis/encephalitis on admission to the RLH. After looking into EPRs, 94 patients were selected for further review of their medical notes. The selection decision was based on data presented in discharge notes of patients: patients had suspicion of community acquired meningitis/encephalitis and given empirical intravenous antimicrobials initially without clear diagnosis or patients where there was insufficient data to exclude by EPR alone. Seventy-three patients were excluded from the study initially on account of differential/confirmed diagnosis that did not include meningitis/encephalitis. The inclusion criteria for the patients in this audit were:

1. Admission to five wards at the BLT (Ward 1, Ward 2, Ward 3, Ward 4 and Ward 5) with the suspicion of community acquired meningitis/viral encephalitis between 1st January, 2008 and 21st May, 2009.
2. Lumbar puncture performed and CSF specimen sent to the Microbiology for analysis.
3. > 1 month of age.

The exclusion criteria were:

1. Nosocomial meningitis.
2. Immunosuppressive disease (HIV) or long term treatment with immunosuppressive drugs.
3. Presence of ventricular shunt.
4. Head trauma or surgical procedures in the two weeks prior to presenting with signs and symptoms of meningitis.
5. Tuberculous meningitis.

The collected data were analysed using statistical package for social sciences (SPSS) software Student Version 17.0 for Windows. Categorical variables were analysed and described using descriptive statistics such as numbers and percentages. Continuous variables were described using maximum, minimum, mean and standard deviation.

RESULTS AND DISCUSSION

Demographic data

Out of 94 patients' case notes requested from the BLT Health Records department, only 62 were available for review. After reviewing these notes, 29 patients with suspected meningitis or viral encephalitis fulfilled the inclusion criteria were selected for the data analysis. Their characteristics are presented in Table 1.

Choice of antimicrobials

Ceftriaxone was used in all adult patients and in 57.1% (4/7) paediatric patients for the empirical treatment of suspected meningitis. Children younger than 3 months (3/7) were commenced on a combination of cefotaxime and amoxicillin. Cefotaxime is the equivalent cephalosporin to ceftriaxone for the empirical treatment of meningitis in children < 3 months and the addition of amoxicillin is recommended. Aciclovir was the only antiviral drug used for empirical treatment of suspected encephalitis. Compliance to the Trust guidelines in use of

Table 1. Demographic data of patients with suspected meningitis/encephalitis.

Parameter	All (%)	Suspected meningitis (%)	Suspected encephalitis (%)	Undifferentiated (%)	Difference (χ^2 test)
Age					
Adults* (mean ± SD: 36.41±12.14)	22 (75.9)	2 (50)	11 (91.7)[§§]	9 (69.2)	p = 0.182
Children* (mean ± SD: 36.29±57.62)	7 (24.1)	2 (50)[§]	1 (8.3)	4 (30.8)	
Gender					
Female	17 (58.6)	2 (50)[§]	8 (66.7)	7 (53.8)	p = 0.754
Male	12 (41.4)	2 (50)	4 (33.3)[§§]	6 (46.2)	
Ward					
Ward 1	16 (55.2)	3 (75)[§]	6 (50)	7 (53.8)	
Ward 2	12 (41.4)	1 (25)	6 (50)[§§]	5 (38.5)	p = 0.721
Ward 3	1 (3.4)	0	0	1 (7.7)	

*****Age of adults in years; age of children in months. § - meningitis confirmed in one patient (*S. pneumoniae*). §§- encephalitis confirmed in one patient (HSV)

Table 2. Compliance to the BLT guidelines in choice of antimicrobials.

Parameter	Compliance-antibiotics (%)	Compliance-antivirals (%)
All	24/27 (88.9)	19/25 (76)
Age		
1 month-18 years	7/7 (100)	3/5 (60)
18-50 years	17/17 (100)	13/17 (76.5)
>50 years	0/3 (0)	3/3 (100)
Aetiology		
Meningitis	4/4 (100)	-
Encephalitis	9/10 (90)	10/12 (83.3)
Undifferentiated	11/13 (84.6)	9/13 (69.2)
Ward		
Ward 1	14/15 (93.3)	11/13 (84.6)
Ward 2	9/11 (81.8)	7/11 (63.6)
Ward 3	1/1 (100)	1/1 (100)

antibiotic/antiviral in the empirical treatment of acute meningitis/encephalitis in relation to differential diagnosis was assessed and results are shown in Table 2. Compliance to the Trust guidelines in use of antibiotics was 100% in children and adults between 18 and 50 years, in contrast to older patients where amoxicillin was not introduced to any of 3 patients to cover possible *Listeria* infection. Compliance to the Trust guidelines in introducing aciclovir in empirical treatment of patients with suspected encephalitis was 76% and it varied among age groups, aetiology groups and different wards. 100% compliance was noted in patients above 50 years of age and in 1 patient treated on a ward 3, while the lowest was seen on the ward 2 (63.6%).

Dose of antimicrobials

The dose of antimicrobial/antiviral drugs used for the empirical treatment of acute meningitis/encephalitis was studied and compared to those recommended by the Trust guidelines. The mean value for daily dose of ceftriaxone in adults was 2.85 g ± 1.09 (min: 1 g, max: 4 g) and in children 1.31 g ± 0.71 (min: 0.54 g, max: 2.50 g). The mean value for the daily dose of aciclovir in adults was 1851.56 mg ± 666.72 (min: 855 mg, max: 3000 mg),

Table 3. Compliance to the Trust guidelines in dose of antimicrobials.

Parameter	Compliance antibiotic dose (%)	Compliance antiviral dose (%)
All	15/27 (55)	13/20 (65)
Age		
1 month-18 years	6/7 (85.7)	3/4 (75)
18-50 years	9/17 (52.9)	9/13 (69.2)
>50 years	0/3 (0)	1/3 (33)
Aetiology		
Meningitis	3/4 (75)	-
Encephalitis	4/10 (40)	5/10 (50)
Undifferentiated	8/13 (61.5)	8/10 (80)
Ward		
Ward 1	11/15 (73.3)	8/12 (66.7)
Ward 2	4/11 (36.4)	4/7 (57.1)
Ward 3	0/1 (0)	1/1 (100)

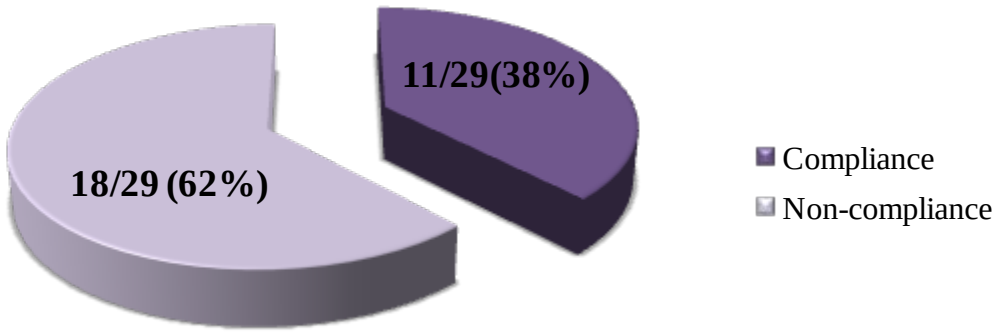

Figure 1. Overall compliance to the Trust guidelines in the use of antimicrobials.

while in children it was 813 mg ± 543.10 (min: 264, max: 1350). Compliance to the Trust guidelines in dosing antimicrobials for the empirical treatment of acute meningitis/encephalitis is presented in Table 3. Where doses varied against the recommended regimen, intentional noncompliance to guidelines were investigated in the medical notes. The dose was deemed non-complaint if there was no intentional or measured deviation documented in the medical notes. Compliance to the Trust guidelines was higher with dosing of antivirals compared to antibiotics (65% vs. 55%) and it differed among age groups, aetiology groups and different wards, but a significant difference was seen only in compliance among different age groups in antibiotic dosing (χ^2 = 2, N = 29) = 6.376; p = 0.041) with significantly lower compliance in patients above 50 years of age compared to other two groups. Overall compliance to the Trust guidelines in the use of antimicrobials (antibiotics and antivirals) is calculated and presented in Figure 1.

Use of corticosteroids in the treatment of suspected meningitis/encephalitis

Corticosteroids were not used in any of 29 patients admitted to the BLT with suspicion of acute meningitis/encephalitis who were included in this study; hence indication for their usage (type of meningitis), dose, timing and duration could not have been assessed.

Time to first dose of antimicrobials from presentation/arrival

Time to first dose of antibiotic or antiviral was calculated as a difference from date and hour of the patient's admission to the hospital written in medical notes to the

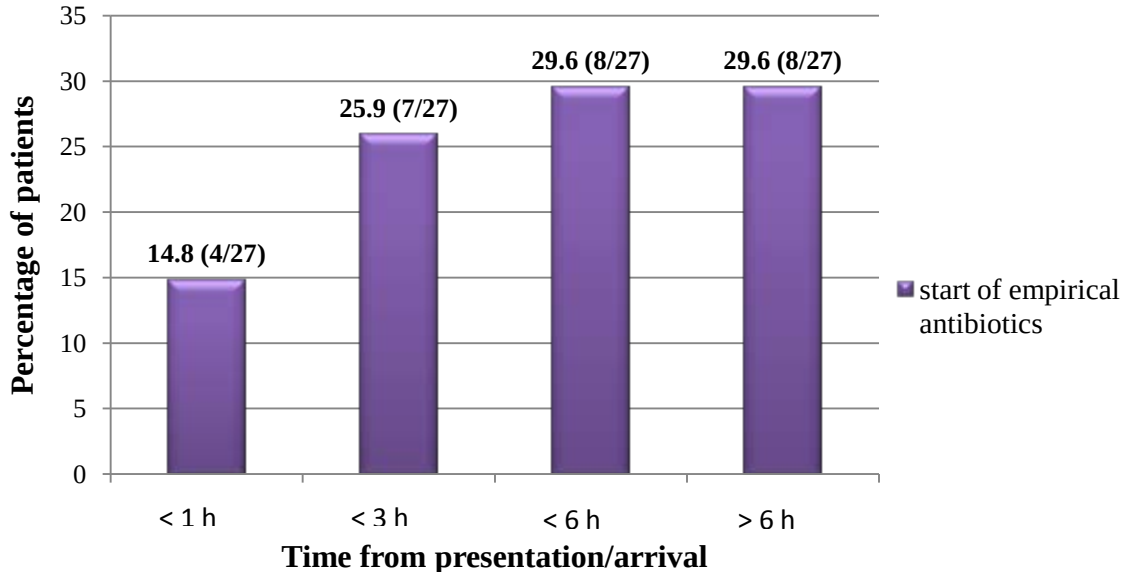

Figure 2. Start of empirical antibiotic therapy from presentation/arrival at hospital.

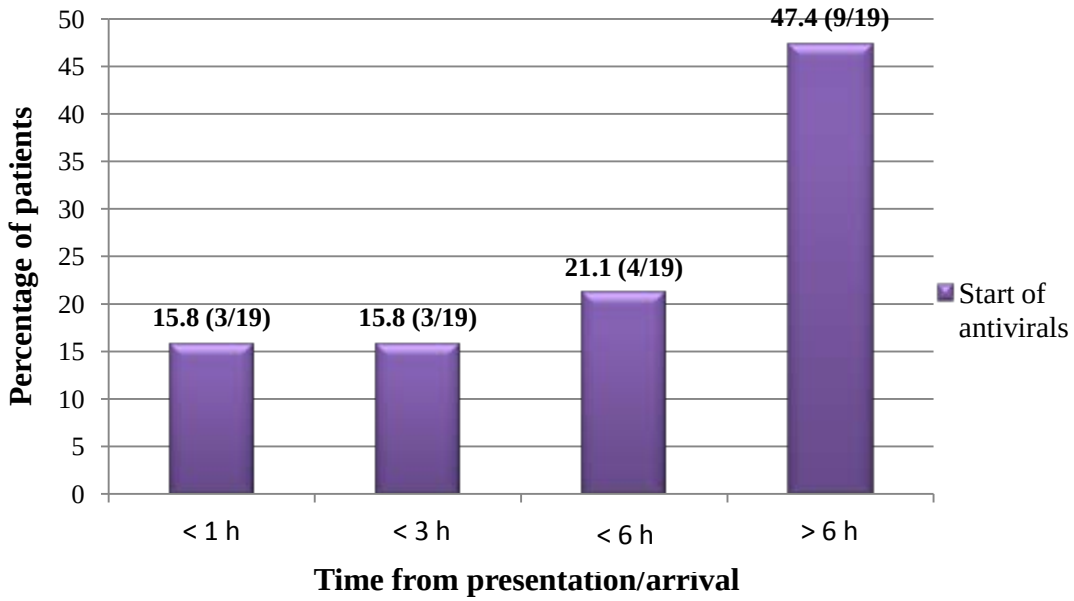

Figure 3. Start of empirical antiviral therapy from presentation/arrival at hospital.

time of administering of antibiotic or antiviral noted on the patient's drug chart. Figures 2 and 3 represent the percentage of patients to whom antibiotics/antivirals were given in the first hour, in the period less than 3 h (1 to 3 h), in the period less than 6 h (3 to 6 h) or after 6 h from the patient's presentation/arrival at the hospital.

Mean time to first dose of antibiotic from presentation was 6.18 ± 8.39 h (min: 0, max: 32.16), while mean time to first dose of antiviral from presentation was 8.19 ± 9.41 h (min: 0, max: 35.16). The ANOVA test was initially chosen for finding any difference between three wards, but it was not possible to perform post-hoc analysis since one of the wards had only 1 patient (ward 3). An independent sample t-test was performed instead which revealed a significant difference in time to first dose of antibiotic between ward 1 and ward 3 {$t(14) = -11.51$, $p < 0.001$} and between ward 2 and ward 3 {$t(10) = -2.69$, $p = 0.023$}, while the difference was not significant between ward 1 and ward 2. A significant difference was seen between the same wards in time to first dose of antivirals

as well: ward 1 and ward 3 {t (10) = -8.203, p < 0.001}; ward 2 and ward 3 {t (6) = -2.526, p = 0.045). Time to first dose of antibiotics and antivirals at ward 3 was much longer compared to both ward 1 and ward 2.

The primary outcome of this study was the extent to which prescribers comply with the Trust guidelines regarding empirical antimicrobial therapy of suspected acute meningitis/encephalitis and the use of corticosteroids. Firstly, the choice of antibiotics and antivirals was studied and it was found to be appropriate in most of the patients. Ceftriaxone was used as antibiotic of choice in adults and children older than 3 months while a combination of cefotaxim and ampicillin was introduced in children younger than 3 months which was all in accordance with the Trust guidelines. However, all 3 patients above 50 years were treated outside of the Trust guidelines, since amoxicillin was not added to ceftriaxone to cover possible *Listeria monocytogenes* infection. The reason for this might be the unawareness of the probable causative microorganisms or the choice of antibiotic in this age group by the prescribers. Although acyclovir was the only antiviral drug used for empirical treatment in patients included in this study, it was sometimes omitted without any clear reason (contraindication such as renal insufficiency) in the management of patients with suspicion on both meningitis and encephalitis (undifferentiated group). Generally, compliance to the Trust guidelines was lower with use of antivirals and the ward 2 was identified as the one with the lowest compliance regarding choice of antimicrobials in empirical treatment.

Fairly low compliance to the Trust guidelines in antimicrobial dosing was noticed in this retrospective audit when all aspects are considered. A trend of lower compliance with increase in patients' age was observed. The reason for noncompliance in antibiotic (ceftriaxone) dosing was mostly a smaller dose given with appropriate frequency (1 g b.d. instead of 2 g b.d.) or the dose was appropriate but the drug was not given frequently enough (o.d instead of b.d.). This lower dose was mainly seen in patients over the age of 50 implying prescribing may feel the need to dose reduce in older patients, however no calculated renal impairment was found and no intentional deviation was documented. Regarding aciclovir, lower doses were given instead of the recommended dose stated in the Trust guidelines (that is, 5 mg/kg t.d.s. instead of 10 mg/kg t.d.s.). It is important to note that the ward pharmacist intervened for the change of antimicrobial dose in two patients, which was accepted after 1 and 2 days of the treatment start.

Overall, compliance in the use of antimicrobials for empirical treatment of suspected meningitis/encephalitis showed that only one-third of prescribers were complying fully with the Trust guidelines. This result is similar to a Dutch study conducted in 2002, just one year after implementation of the national guidelines (Van de Beek et al. 2002). Higher compliance to Bispectral index (BIS) guidelines was found in a University teaching hospital where in 85% of patients choice of antibiotics was appropriate (Zimmerli 2003). A 10-year retrospective study revealed 65% compliance with Infectious Diseases Society of America (IDSA) guidelines in the management of Intensive Care Units (ICU) patients, which decreased mortality (Brouwer et al. 2010). Low overall compliance in this audit is mostly attributed to using unsuitable dose of generally appropriately chosen antimicrobials. It is possible that prescribers are not aware of the fact that higher doses of antimicrobials are used in meningitis compared to other infections or not familiar with the Trust guidelines for acute meningitis. There was also a tendency to dose lower where there was a lower suspicion of meningitis in the differential diagnosis.

Regarding the use of corticosteroid, these were not used in empirical treatment of any patients involved in this study including the patient with proven *Streptococcus pneumoniae* meningitis. Reasons for this might be various: recommendation in the Trust guidelines is not precise enough regarding indication, dosing and possible benefits of corticosteroids in empirical treatment of suspected meningitis/encephalitis; prescribers' suspicion of its efficacy due to lack of large amounts of evidence from randomised controlled trials or the fact that none of the patients had suspicion of *S. pneumoniae* meningitis accompanied with presence of focal neurologic signs and Glasgow Coma Scale (GCS) score of 8 to 11 (1 patient had GCS = 11 and 1 had GCS = 10), since these patients benefit mostly from corticosteroid treatment (Auburtin et al. 2006; Begg et al. 1999; Korshin et al. 2007; Køster-Rasmussen et al. 2008).

There is no precise recommendation in National BIS, British National Formulary (BNF) and hence not in the BLT guidelines about the timing of antimicrobials in empirical treatment of suspected meningitis/encephalitis. However early treatment is advocated (without any delay) administration of therapy. Unicentre retrospective 14-year study conducted on 286 patients and multicentre 2-year study on 187 patients both showed that in the group of patients with unfavourable outcome antibiotic treatment was significantly delayed and that early adequate antibiotic treatment related to the onset of overt signs of meningitis was independently associated with favourable outcome (OR = 11.19; 95% CI 4.37 to 32.57; p < 0.001 and OR = 1.09/h, CI: 1.01 to 1.19, respectively) (Lepur and Baršić 2007; Stockdale et al. 2011). Study conducted in Addenbrooke's hospital (Cambridge) showed that 70% of empirical antibiotics were given in a time period of less than 1 h from arrival of 116 adult patients with suspected meningitis to the hospital (Chadwick and Lever 2002). Median time between arrival and first dose of antibiotic was 90 min, while in the study presented here it was 3.53 h (211.8 min) which is almost 2.5 times longer. Empirical antimicrobial treatment was started after 3 h of presentation in more than half of the patients and after 6 h in one third of patients. There were further delays in the

introduction of antiviral therapy since almost half of the patients received treatment after 6 h of presenting to the BLT. Reasons for these delays have not been clear, and some future prospective audit could help to identify this.

One study identified computed tomography (CT) head scan as one of the reasons for the delay of antibiotics, which when greater than 6 h, significantly increased risk of death for 8.4 times (95% CI, 1.7 to 40.9) (Georges et al. 2009). Another study showed that delay in antibiotic introduction longer than 3 h was one of the predictors of 3-months mortality (OR, 14.12; 95% CI, 3.93 to 50.9; $p < 0.0001$) (Proulx et al. 2005). It was found that time to first dose of antimicrobials was significantly further delayed in ward 3 compared to ward 1 and ward 2 which is consistent with the logistics of patients admission pathway. The patient on the ward 3 received their antimicrobial 32 h after admission. Results obtained from this retrospective audit can be added to other studies with similar research objectives. The advantage of this study can be found in the fact that, while others were mostly concentrating on one aspect of management of patients with suspected meningitis/encephalitis, this retrospective audit looked at antimicrobial choice, dose as well as the timing of pharmacological management of these patients in all age and aetiology groups. In this way a complete picture of patients' treatment was observed.

A few limitations were identified in the audit. A relatively small number of patients was included in the study out of which only two patients had confirmed diagnosis of meningitis/encephalitis. Only patients with lumbar puncture performed were involved in this audit, since it was a method of recruiting patients. The interpretation of data was limited by the quality of medical note keeping and availability of medical record.

A retrospective review conducted at the Barts and the London NHS Trust of 29 patients who presented with suspected acute meningitis/encephalitis over a period of 18 months showed a relatively low level of compliance to the full application of Trust meningitis guidelines. Although, the choice of antimicrobials was mostly appropriate (except for older patients where amoxicillin was not added), the doses of drugs were often smaller than recommended. The start of empirical therapy was significantly delayed in relation to the hospital admission. Corticosteroids were not introduced in the empirical treatment of patients enrolled in this study, including the one patient later diagnosed of *S. pneumoniae* meningitis.

Relevance of the findings to practice

1. The Trust guidelines need to be reviewed in the light of some recent evidence about the use of corticosteroids in acute meningitis.
2. Causative microorganism in certain age and therapy indicated (for example addition of amoxicillin to cover *Listeria monocytogenes* infection) are not on the same place in the guidelines (it could be missed by junior physician who is using the guideline for the first time). Hence, it would be useful to place all information, such as age group, causative microorganism, recommended therapy (dose, frequency and duration) in one table or algorithm.
3. The recommendation for the timing of empirical therapy could also be more specific in the Trust guideline, by changing it from 'give empirical therapy without delay' to 'give empirical therapy within the first hour of patient's arrival to the hospital'.
4. After review of the Trust guidelines for the management of acute meningitis/encephalitis, education in form of lectures and presentations (including the results of this audit) should be provided for junior physicians and pharmacists where the importance of liaison with the Microbiology department and Microbiology pharmacist would be outlined.

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