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Original Article

PRODUCTION AND PURIFICATION OF ANGIOTENSIN-CONVERTING ENZYME INHIBITOR BY SELECTED BACTERIAL STRAIN FOR CANCER THERAPY

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ABSTRACT

Objective: The present study was planned to explore safer, innovative and economic Angiotensin-converting enzyme inhibitors (ACEi) from beef extract by the action of a proteolytic *Micrococcus luteus*. Cytotoxicity of the stable peptide was predicted using MCF-7 cell line *in vitro.*

Methods: ACEi was purified by sequential steps of ethanol precipitation, ion exchange column chromatography (MonoQ) and gel filtration column chromatography (Sephadex G25). The apparent molecular mass was determined by SDS-PAGE. The anticancer property was analyzed by studying the cytotoxicity effects of angiotensin converting enzyme inhibitor using Breast cancer MCF-7 cell lines

Results: The peptide was purified and molecular mass was determined as 4.5 kDa. The IC_{50} value of peptide was found to be 59.5 µg/ml. The DNA fragmentation was not observed in the treated cells. The purified peptide has demonstrated to induce apoptosis of cancer cell. The results proved that the peptide has the ability to be used for cancer therapy.

Conclusion: The presence of ACE inhibition activities in the fermentation of beef extract using *Micrococcus luteus* has been investigated. The Peptide has been determined as an active compound that inhibited the activity of ACE. These properties indicate the possibilities of the use of purified protein as a potent anticancer agent.

Keywords: Angiotensin-converting enzyme inhibitors, *Micrococcus luteus*, Anti-proliferative, Anti-metastatic, MCF-7 cell line, Anticancer activity.

INTRODUCTION

Angiotensin-converting enzyme (ACE) is a dipeptide hydrolase that catalyses both the formation of the potent vasoconstrictor, angiotensin-II (Ang II), and the deactivation of bradykinin, a vasodilator peptide. Given the potential of Angiotensin II in containing the proliferation of the tumour, production of these inhibitors by bacteria could open new doors for anticancer therapy. The protective effect of ACEi cannot be attributed solely to the inhibition of AngII production. Rather, multiple mechanisms that are not yet fully understood could be responsible for the same. ACEi has also been reported to suppress vascular endothelial growth factor (VEGF), which is believed to play a major role in stimulating angiogenesis in human growth [1]. Although genotypic studies on ACE and the risk of cancer in humans have yielded contradictory results, the use of ACE inhibitors in experimental animal models has consistently indicated a protective effect of these drugs against tumor development. Perindopril, a well known ACEi used either alone or in combination with β interferon, was found to inhibit VEGF expression, endothelial cell migration and tubular formation in matrigel, thus proving its role in protection against tumor angiogenesis [2, 3]. Captopril, yet another ACEi, was also observed to attenuate tumor growth and angiogenesis of syngeneic fibrosarcoma when injected in rats [4].

In the recent years, several studies have identified peptides to act as inhibitors against ACE activity. ACE-inhibitory peptides have been discovered, isolated and purified from enzymatic hydrolyzates of different food proteins. Physiological and pharmacological effects of ACE inhibitory peptides derived from food proteins and their prospective applications in preventing hypertension, cancer and for therapeutic purposes have also been reported [5]. Various bioactive peptides including ACE-inhibitory or antihypertensive peptides, immunomodulatory, antioxidative, antimutagenic, anticancer peptides have been released from milk proteins, eggs, meat and fish as well as in different plant protein sources such as soy and wheat through microbial proteolysis [6-8]. The choice of the strain, which influences the release of effective bioactive peptides, is one of the most effective ways of increasing the concentrations of bioactive peptides. Hence, it is imperative that the strain does not exhibit proteolytic properties, failing which the product will be destroyed. It is also important that the strain has the right specificity to produce a higher concentration of bioactive peptides. The concentration of ACE-inhibitory peptides seems to rely on a balance between the formation of bioactive peptides and its consequent breakdown into inactive peptides and amino acids, which in turn depend on storage time and conditions.

With the exception of *Lactobacillus delbrueckii* and *Lactobacillus lactis* which are used for milk fermentation, the uses of microbes as ACE inhibitor source have been less explored. Edible mushrooms *Tricholoma giganteum* have also been proved to have ACE inhibiting peptide. Many research groups have combed for ACE inhibitors in microbial sources such as *Doratomyces putredinis, Nocardia orientalis, Streptomycetes, Actinomycetes, Actinomadura, Spiculospora* and *Actinomadura* [9]. Based on these findings, this research focuses on isolating and identifying ACE inhibitor from the fermentation of beef extract using *Micrococcus luteus*.

MATERIALS AND METHODS

Microorganism and crude enzyme preparation

The isolated strain *Micrococcus luteus* (GenBank accession number Kf303592.1) was inoculated into a protease specific medium broth containing Beef extract (2.0 g/l), $MgSO_4.7H_2O$ (0.1482 g/l), KH_2PO_4 (0.3 g/l), $FeSO_4.7H_2O$ (0.003 g/l), Na_2HPO_4 (1.28 g/l), NaCl (0.05 g/l), NH_4Cl (0.1 g/l), Thiamine (0.03 g/l), $CaCl_2.2H_2O$ (0.0456 g/l). The inoculated broth was incubated at 37 °C for 1 d in an orbital shaker at 150 rpm. After fermentation, the entirely fermented broth was extracted, and the clear supernatant was recovered. The supernatant was filtered through a 0.45 mm cellulose acetate filter paper [10]. The crude enzyme extract was further subjected to the purification process. Before purifying the protein content [11], the ACE inhibitory activity of the crude extract was estimated.

Measurement of ACE inhibitory activity

The ACE inhibitory activity was assayed by the method of Cushman and Cheung [12] with a few modifications. Hip-His-Leu (HHL) was dissolved in 50 mM sodium borate buffer (pH 7.0) containing 1 N NaCl. Following this, 25 µl of 5 mM (HHL) solution was mixed with 10 µl of beef hydrolysate (the pH of which was adjusted to 7.0) and then pre-incubated for 10 min at 37 °C. The reaction was initiated by adding10 µl of ACE and the mixture was incubated for 30 min at 37 ºC. The reaction was stopped by adding 200 µl of 1 N HCl. The hippuric acid liberated by ACE was extracted with 1 ml ethyl acetate, dissolved by adding 1 ml of the buffer after the removal of ethyl acetate by vacuum evaporation, and the optical density was measured at 228 nm. The extent of inhibition was calculated using the formula

$$\text{Extent of Inhibition} = \frac{(B-A)}{(B-C)} \text{ x } 100$$

Result expressed in percentage

Where, A = the optical density in the presence of ACE and ACE inhibitory component; B = the optical density without an ACE inhibitory component; C = the optical density without ACE.

Purification of ACE inhibitory peptide

The crude extract of fermented medium with the selected substrate by test strain was extracted with three volumes of chilled ethanol. The pellet was suspended in Tris-HCl (20 mM; pH 7.0) and further purified by ion exchange column chromatography (Mono Q) and by size exclusion chromatography (Sephadex G25). Each fraction was then tested for ACE inhibition activity and protein content. The protein profile of the active fraction with ACE inhibition was studied using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the molecular weight of the protein was also determined.

SDS-page profile

The apparent molecular weight of the peptide was determined by SDS-PAGE with proteins of known molecular weight along with the protein to be characterized [13] in 15% polyacrylamide gel with a modified protocol [14].

Cytotoxicity of ACE inhibitor on breast cancer cell Line

Cell line and culture

Breast cancer MCF-7 cell lines used in this study were obtained from King Institute of Preventive Medicine and Research, Chennai, India. The cells were maintained in Minimal Essential Media (MEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Preparation of ACE inhibitor

ACE inhibitor was prepared by fermenting the beef extract by *Micrococcus luteus*. The ACE inhibitor was purified by a series of steps namely ethanol precipitation, ion exchange column chromatography and gel filtration column chromatography. The purified fraction in Tris-HCl buffer (pH 7.0, 20 mM) with the protein content of 1 mg/ml was used for cytotoxicity analysis. Various concentrations of the ACE inhibitor were then analyzed for cytotoxicity activity.

***In vitro* anticancer study**

Breast cancer MCF-7 cell lines obtained from King Institute of Preventive Medicine and Research, Chennai, India was used for the present study. The Breast cancer cells were grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). For screening experiments, the cells were seeded into 96-well plate in 100 µl of medium containing 5% FBS, at a plating density of 10,000 cells/well. These cells were then incubated at 37 ºC, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of ACEi. ACEi in 20 mM Tris buffer (pH, 7.0) was diluted in serum-free medium. After 24 h incubation, 100 µl of the medium containing the ACEi at various concentrations (1000 µl, 500 µl, 250 µl, 125 µl, 62.5 µl, 31.2 µl, 15.6 µl and 7.8 µl/ml) were added and incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 48 h. Triplicate was maintained and simultaneously a control was kept without the addition of ACE inhibitor.

Anticancer activity

The effect of ACE inhibitor on MCF-7 cell viability was determined by MTT [3-(4,5-Dimethyl-thiazol-2-yl)-2,5-Diphenyltetrazolium bromide] assay [15]. The yellow tetrazolium salt of MTT was reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals. These crystals were solubilized by the addition of a detergent. Cells (1×10^5 cells/well) were incubated with various concentrations of the compound at 37 ºC for 48 h in an FBS-free medium, before being subject to MTT assay. The absorbance at 570 nm was measured using UV Spectrophotometer. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data have been expressed as the mean percentage of viable cells and compared with respective to control. The half maximal growth inhibitory concentration (IC_{50}) values were calculated.

DNA fragmentation assays by agarose gel electrophoresis

The degradation of DNA into multiple fragments of 39 to 41 base pairs is a distinct biochemical hallmark of apoptosis. DNA fragmentation was analyzed by agarose gel electrophoresis of genomic DNA extracts from the Breast cancer MCF-7 cell lines treated with 20 µl of ACE inhibitor for three hours. The DNA was visualized by placing the gel on a UV transilluminator [16].

RESULTS AND DISCUSSION

In earlier reports, intense proteolysis by endogenous muscle enzymes resulted in an accumulation of peptides [17-19] and free amino acids at the end of the dry-curing process of meat [20, 21]. Similarly, ACE inhibitory peptides were identified in muscle tissues after hydrolysis of pork meat [22, 23] or derived *in vitro* from the digestion of pork meat [24]. In an earlier study, water-soluble fraction of Spanish dry-cured ham was also found to exhibit antihypertensive activity both *in vivo* in spontaneously hypertensive rats (SHR) and *in vitro* ACE inhibitory activity [25]. Based on these findings and observations, in the present study beef extract was used as a substrate for hydrolyzing using *Micrococcus luteus*.

Purification of ACE inhibitory peptides

Crude extract of the fermented beef extract was found to have 78.55±0.42% ACE inhibition and protein content was determined to be 8.66 mg/ml. In the present study, the peptides were concentrated using ethanol precipitation. Upon precipitation the ACE inhibition was 74.96±0.85% and protein content was observed to be 4.00 mg/ml.

Ion exchange chromatography is being widely used at the beginning of a purification scheme and is designed for separating ionic or ionizable compounds in the mobile phase by the counter-ion of the opposite sign in the stationary phase (column packing) [26]. Among the fixed stationary phase, diethyl aminoethyl (DEAE) is being extensively used in the conventional anion exchange chromatography during ACEIP purification [27-30]. Ion Exchange was used to purify hydrolyzate from seaweed pipefish muscle and obtained the reasonable IC_{50} value [29]. Hiprep 16/10 DEAE ion exchange column was used to purify the hydrolyzate of salmon pectoral fin [27]. They found that the purified hydrolyzate provided on an IC_{50} at a concentration of 169 µg/ml after passing through the column and decreased around three times when compared to the native hydrolyzate (IC_{50} = 365 µg/ml). In the present study, the strong anion exchange Mono Q column was used. The soluble crude protein extract was dissolved in a minimal volume of 20 mM Tris-HCl buffer pH 7 and fractionated on Mono Q column. After washing the unbound protein with 20 mM Tris-HCl buffer, the column-bound protein was eluted with 100 ml linear salt gradient (0-100 mM NaCl in 20 mM Tris-HCl, the flow rate was 3 ml/min). The active fraction (Fraction 39 to 41, fig. 1) which displayed ACE inhibitory activity were pooled, dialyzed against 20 mM Tris-HCl and was taken for Sephadex G25 chromatography. The protein content of the pooled

fractions was observed to be 0.92 mg/ml (table 1). The ACE inhibition of the active fraction was observed to be 84.22±0.79%. It is a known fact that the strong anion exchange column tends to bind tightly to the positively charged molecules and release only at a higher concentration of NaCl. Hence, in the present study, the active fractions which were eluted at higher NaCl gradient (~70%) revealed that the peptides may be highly positively charged.

The pooled fractions were further purified on a Sephadex G25 column and eluted with 20 mM Tris-HCl buffer containing 100 mM NaCl. The chromatogram showed three peaks (fig. 2). The fractions (111 to 113) under the highest peak were found to have an ACE inhibitory activity which was pooled and checked for purity. The protein content of the purified fraction was observed to be 0.4 mg/ml with ACE inhibition of 85.43±1.08%. Similar results from earlier work after peptide fractionation through size-exclusion chromatography [25] revealed that the strongest ACE inhibitory activity (85% of ACE inhibition) was obtained from eluted fractions. It may be assumed that the peptides with a lower molecular weight exhibit a higher molecular mobility and diffusivity when compared to these peptides with a higher molecular weight, which appears to improve interactions with cancer cell components and enhances anticancer activity [31]. A study on the mechanism of action revealed that the modulation of hydrophobicity of peptides plays a crucial role against cancer cells [32]. In the purification process during Ion exchange chromatography using Mono Q column, a strong anion exchange column, the peptides were eluted only at higher NaCl concentration could be attributed to its higher positive charges. This positive charge and hydrophobicity might not only impart the ACE inhibitory activity but would also facilitate higher interaction with cancer cells as reported earlier.

Electrophoretic analysis of ACE inhibitory peptide

On SDS-PAGE analysis using 15% gel, several bands were found to appear in the crude extract (lane 1 & 2 of Fig.3), confirming the presence of unwanted impurities. Thus, warranting further purification. The fractions (fractions 39 to 41) of ion exchange column (lane 5, 7 & 8 of Fig.3) showed three prominent bands. The purified fractions of gel filtration column (lane 4 of Fig.3) showed a single band. The apparent molecular weight was found to be around 4.5 kDa. The size of the generated peptides is crucial to the ACE inhibitory effect as previously reported [33, 34]. The peptides with lower MW were easily absorbed compared to peptides with higher MW [35]. In agreement to the earlier report the peptides of smaller molecular weight seem to exhibit ACE inhibitory activity.

Sarcoplasmic and myofibrillar porcine proteins are hydrolyzed by the action of *Lactobacillus sakei* CRL 1862 and *L. curvatus* CRL 705 [36]. They were successful in generating ACE inhibitory peptides by fermentation. It has been reported that muscle foods served as an interesting substrate for producing potential bioactive peptides. Earlier marine proteins from shrimp when fermented with *L. fermentum* SM 605 was found to yield three ACE inhibitor peptides [37]. These reports confirm the ACE inhibitory peptides were produced by microbial hydrolysis which further supports the findings of the present study.

As stated earlier, the mode of action of the majority of ACE inhibitory peptides proves to be competitive substrates for ACE. Most ACE inhibitory peptides found in meat can be classified as true inhibitor type peptides [23, 38, 39]. These peptides may act in one of the two ways: first the peptide may bind to the active site of the ACE enzyme, or it may bind to an inhibitor site located on the ACE, thus modifying the protein conformation and preventing the substrate (Ang I) from binding to the enzyme active site [40].

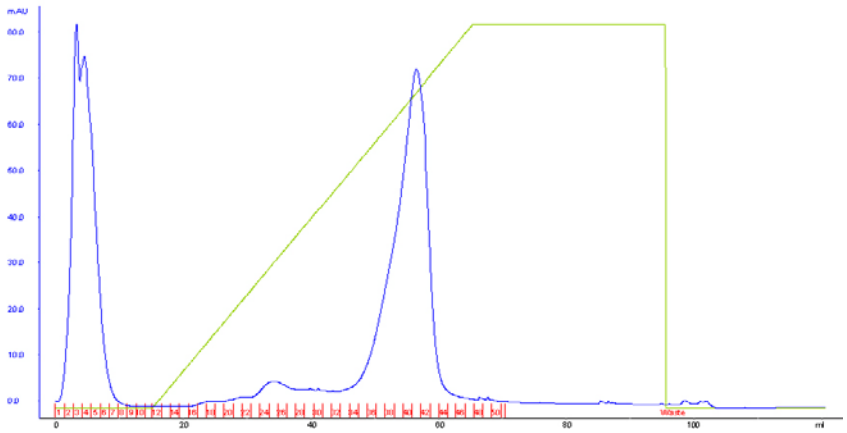

Fig. 1: Ion exchange column chromatogram of ACE inhibitory peptide

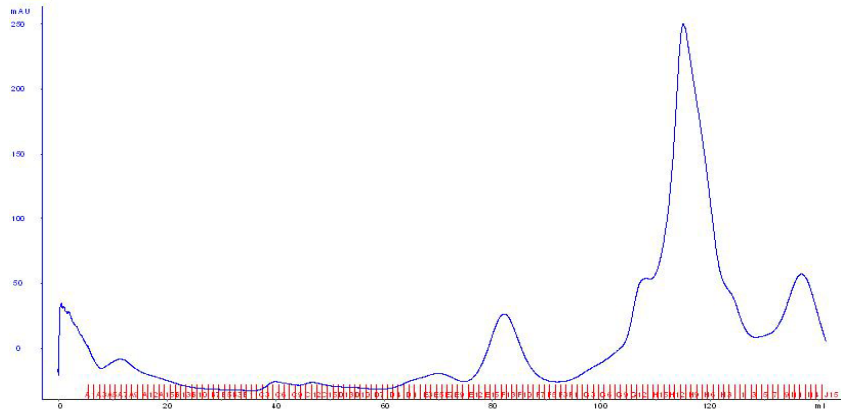

Fig. 2: Size exclusion column chromatogram of ACE inhibitory peptide

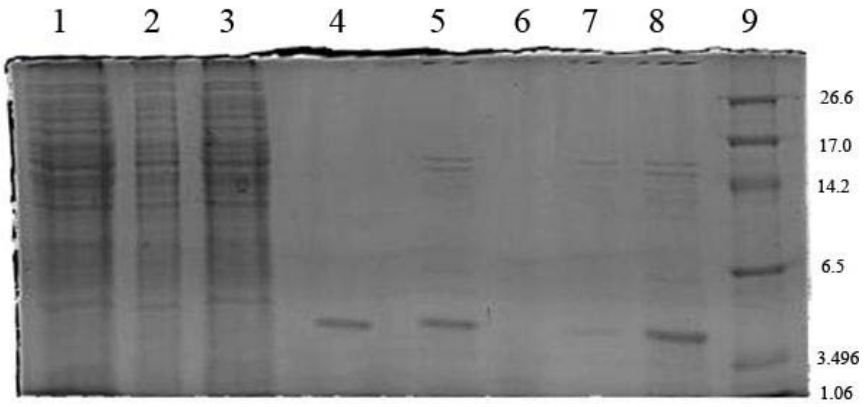

Fig. 3: SDS-PAGE profile of ACE inhibitory peptide purification process

Lane 1,2,3–Crude extract; Lane 4–Protein fraction of sephadex G25 column (size exclusion chromatography); Lane 5,7,8,–Protein fraction of ion exchange column; Lane 9–Molecular Weight Markers (26.6, 17, 14.2, 6.5, 3.496, 1.06 kDa).

Table 1: Purification table of ACE inhibitory peptide

S. No.	Purification step	Volume (ml)	Protein content (mg/ml)	ACE inhibition (%)
1	Crude extract	2000	8.66	78.55±0.42
2	Ethanol precipitation	50	4.00	74.96±0.85
3	Ion exchange column (fraction 39-41)	6	0.92	84.22±0.79
4	Size exclusion column (Fraction 111-113)	6	0.40	85.43±1.08

The experiment was done in triplicates and the values of ACE inhibition (%) expressed as mean±SD.

***In vitro* anticancer analysis**

The peptide isolated in the present study was observed to have a cytotoxic effect on the MCF-7 cell line. The IC_{50} value of the peptide was determined to be 59.5 µg/ml. The cytotoxic effect was found to have a linear relation with the concentration of the peptide, which explains the sequential reduction in viability percentage of cancer cells (MCF-7 cell line) in the graph (fig. 4). The control cells which were not treated with ACE inhibitor were observed to be elongated and proper confluence growth was observed (fig. 5). The cell after treatment with ACE inhibitor were seen as round shaped which confirmed the detachment of the cells from the surface. These rounded cells were indicators of apoptotic cells. The cells treated with 62.5 µg/ml were found to be mostly rounded and as the concentration of ACE inhibitor was increased further the total cell count itself was observed to be reduced (fig. 5).

Oyster hydrolyzates inhibited tumor growth by improving the immune function in S108-bearing mice, which suggested their potential use in tumor therapy [41]. An enzymatic hydrolyzate from jumbo squid skin gelatin showed a cytotoxic effect against MCF-7 and U87 cell lines, with IC_{50} values of 0.13 and 0.10 mg/ml, respectively [42]. Solitary tunicate hydrolyzate was reported to show potent anticancer activity against AGS, DLD-1, and HeLa cancer cells. However, the anticancer activities of these fractions (IC_{50} 577.1–1240.0 µg/ml) were found to be much higher than that of the commercial standards such as Paclitaxel (IC_{50} 2.2–24.6 µg/ml) and 5-Flurouracil (IC_{50} 3.4–34.5 µg/ml) [32].

An appropriate inhibition of Ang II production by ACE inhibition was found to inhibit proliferation via AT1 receptor and calcium channel blockade. ACE inhibition promotes apoptosis in vascular smooth muscle cells [43, 44]. The combined effect of perindropril and IFN proved to induce epithelial cell apoptosis and secondary apoptosis of the tumor cells by attenuating the expression of VEGF [45]. Similarly, the apoptotic activity of the peptides from beef extract fermented by bacteria in the present study could also be attributed to the ACE inhibitory activity of the extract.

Apratoxin A is cyclic depsipeptide extracted from *Lyngbya majuscula* which exhibited cytotoxic effects on human HeLa cervical carcinoma cells by cell cycle inhibition [46]. A similar mechanism has been observed by cyclic depsipeptide Coibamide A which was isolated from *Leptolyngbya* sp. Human lung cancer cell line [47] and Lyngbyabellin B isolated from *Lyngbya majuscula* on human Burkitt lymphoma cells [48]. Linear Pentapeptide Dolastatin 10 and Symplostatin 1 were isolated from *Symploca* sp. which exhibited a cytotoxic effect on human lung cancer cell line and human breast carcinoma cell line by both Bcl-2 phosphorylation and Caspase-3 protein activation [49, 50]. Besides, there were also different types of anticancer peptides isolated from *Lyngbya* sp. and *Nostoc* sp, which exhibited inhibiting activity against cancer on different cell lines through microfilament disruption, secretory pathway inhibition, etc [51, 52].

The peptide transporters *viz.* PEPT1 and PEPT2, in mammals, are being investigated for peptide-based delivery systems. These transporters are integral plasma membrane proteins that mediate the cellular uptake of di and tri peptides and peptide-like drugs. They are present predominantly in epithelial cells of the small intestine, bile duct, mammary glands, lung, choroid plexus and kidney, but are also localized in other tissues (Pancreas, liver, Gastrointestinal tract). Intriguingly, they seem to be overexpressed in some types of tumors but not in their corresponding healthy tissues [53, 54]. These peptide transporters act as Trojan horses facilitating the delivery of pharmacologically active compounds due to their wide substrate binding capacity (bind molecules with different size, hydrophobicity and charge [55]. This peptide transporter delivery system is an added advantage for the peptides to be delivered to the cancer cells without imparting any negative effects on the healthy cells.

In the present study, the DNA laddering assay revealed that the peptides induced DNA fragmentation was not clearly seen in the agarose gel pattern of DNA from treated cells (fig. 6). Even though an earlier report [48] stated that the ACE inhibitor produce DNA fragmentation, there are several reports which suggest cell death by an alternate mechanism as well Didemnin depsipeptides were reported to have a cytotoxic effect on cancer cell lines by inhibiting protein synthesis *in vitro* [56]. The peptide was supposed to inhibit protein synthesis by ribosome-EF-1α complex, as a correlation

between inhibiting protein synthesis in cell lysates and in human adenocarcinoma MCF-7 cells was observed [57]. Studies with Jaspamide in HL-60 human leukemia cell line revealed that the nanomolar concentrations of this depsipeptide inhibited cell proliferation and also increased polynuclear cells [58]. In yet another study, Cryptophycin-52, a member of the family of the marine depsipeptides-Cryptophycins, produced by total chemical synthesis, showed antitumor activity at picomolar concentrations. This compound was found to exhibit anti-proliferative activity by stabilizing spindle microtubules, and by binding tightly and non-covalently to a single high-affinity site on tubulin while also inducing a conformational change in the tubulin molecule [57, 59]. ACE inhibitors were also reported to have antioxidant properties [60]. In view of these earlier reports, the peptide isolated in the present study might have induced cytotoxicity by any one of the mechanisms without involving DNA fragmentation.

Studies on peptides obtained from protein hydrolyzates have shown that these biomolecules contain antioxidant, antiproliferative, and antimutagenic activities which could confer on them anticancer properties. In agreement with the earlier reports, the peptide isolated in the present study from beef extract could exhibit anticancer activity through not only the ACE inhibition pathway but also through other pathways already reported. However, the effects of the antiproliferative peptides on cell cycle of normal and transformed cells and, on the structure of the bioactive peptides, and *in vivo* studies of these activities need to be investigated further. Nevertheless, there is a need for scaled-up production of these compounds, which could be achieved by the utilization of microbial by-products.

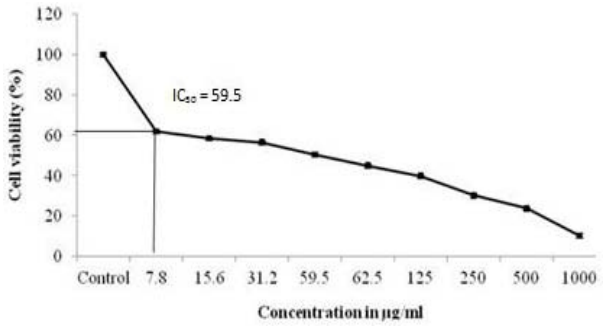

Fig. 4: Cytotoxicity of ACE inhibitor showed the viability percentage of cancer cells (MCF cell line)

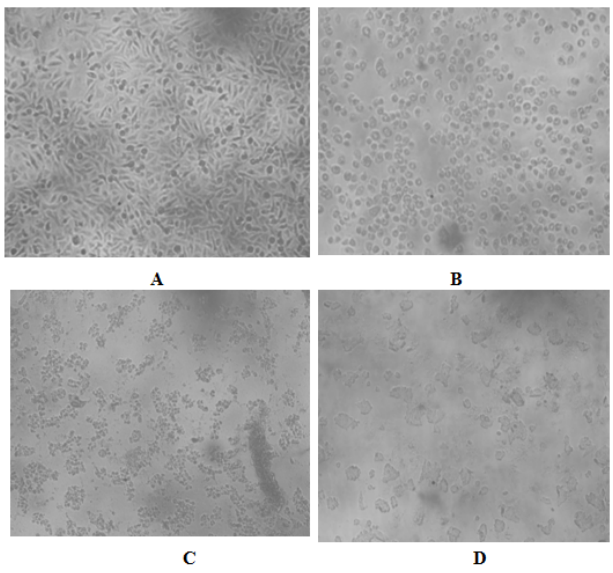

Fig. 5: Cytotoxicity effect of ACE inhibitor on breast cancer cell line MCF-7

A: MCF-7 control cells untreated with an ACE inhibitor; B: MCF-7 cells treated with 62.5 µg/ml of ACE inhibitor; C: MCF-7 cells treated with 125 µg/ml of ACE inhibitor; D: MCF-7 cells treated with 250 µg/ml of ACE inhibitor

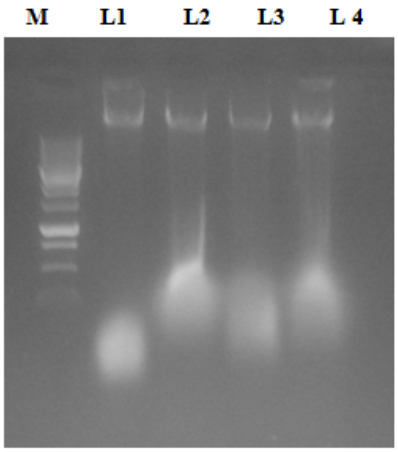

Fig. 6: DNA fragmentation of MCF-7 cells treated with an ACE inhibitor M-Marker1000bp (base pairs) DNA ladder; L1-l; L2-250µg/ml; L3-125µg/ml; L4-62.5µg/ml

CONCLUSION

The presence of ACE inhibitory peptide in beef extract fermented by *Micrococcus luteus* was investigated. The present study succeeded in isolating and purifying a potent ACE inhibitor by ethanol precipitation followed by ion exchange chromatography and size exclusion chromatography. The molecular weight of the ACEi was determined to be around 4.5 kDa. The anticancer property was analyzed by studying the cytotoxic effect of the purified ACEi on MCF-7 cell lines and the IC_{50} value of the peptide was found to be 59.5 µg/ml. This property enthuses to explore further the possibilities of the purified ACEi as a potent anticancer agent.

CONFLICT OF INTERESTS

The authors report no conflicts of interest.

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