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Research Article

NOVEL ANGIOTENSIN CONVERTING ENZYME INHIBITOR FROM *ASPERGILLUS* SP. BY SOLID STATE FERMENTATION

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ABSTRACT

Aspergillus sp., was isolated from soil and cultivated in Solid state fermentation for ACE (Angiotensin converting enzyme) inhibitor extraction. ACE Enzyme was extracted from rabbit lung and ACE inhibitory assay was conducted. Further ACE Inhibitor extract was analysed for apoptotic effect. DNA laddering, MTT assay and cell morphological change assay were performed which confirmed the apoptotic effect of the extract. Antioxidant assay of both cell line and *invitro* assays for ACE inhibitor extract was conducted. Radical scavenging activity of ACE Inhibitor was very low and all other antioxidant activities like reducing power, lipid peroxidation, GST, reduced glutathione activity were high. GC-MS analysis was conducted for crude ACE Inhibitor. Among the 12 compounds, 2 compounds (4-Hydroxy-2-methyl acetophenone and 2, 5-Dihydroxy propio phenone) were reported to have both anticancer and vasodilator activity in the database. HPLC analysis was conducted for both crude and purified fraction. The chromatogram of crude extract showed a single major peak with other minor peaks. Chromatogram of pooled active fraction showed a single major peak with two minor peaks. Fungal extract was subjected to ultrafilteration. Sephadex G-25 gel filtration chromatography was used for purification of ACE inhibitor and 14 fractions were collected. ACE inhibition activities of all fractions were conducted of which fraction 4 showed 75.01% inhibition. This fraction was subjected to SDS-PAGE which showed single band with the molecular weight of 31KDa.

Keywords: Angiotensin converting enzyme inhibitors, *Aspergillus* sp., ACE inhibition assay anticancer activity, Antioxidant activity.

INTRODUCTION

Synthetic chemicals are used as a drug for many diseases with a lot of side effects. Fortunately, this situation is changing and we are now entering in to a new era in which microbial metabolites are being applied to cure diseases. In pharmaceutical industry, secondary metabolites have been screened almost exclusively for hypertensive, anti-inflammatory and anticancer agents[1]. More than thousands of secondary metabolites from microbial source have been discovered. Among them fungi were exploited as the rich source of secondary metabolites. A complex metabolic pathway in fungal cells favors the production of different types of secondary metabolites and these metabolites have high pharmacological values.

Besides supplying amino acids and energy that are essential for growth and maintenance, some proteins can act as an important source of biologically active peptides with antihypertensive, opioid, immunomodulating, antioxidative, antimicrobial, antithrombotic, antiamnestic, hypocholesterolemic, and other activities[2]. These peptides are inactive within the sequence of parent proteins, but they can be released by enzymatic proteolysis *in vivo* or *in vitro*, for example during gastrointestinal digestion or during food processing. Once bioactive peptides are liberated, they may act as physiological modulators with hormone-like activity. Thus, these peptides represent potential health-enhancing nutraceuticals for food and pharmaceutical applications. Among the bioactive peptides, Angiotensin I–converting enzyme (ACE) inhibitory peptides derived from food proteins have attracted particular attention and have been studied comprehensively for their ability to prevent hypertension. These peptides could be used as a potent functional food additive and represent a healthier and natural alternative to ACE inhibitor drugs.

Angiotensin (renin substrate), a glylcoprotein mainly synthesized by the liver and secreted in the circulating blood, is cleaved by the enzyme renin (EC 3.4.99.19) releasing the decapeptide Ang I. Angiotesin I is then cleaved by ACE, forming the octapeptide Ang II. Ang II is the principal biologically active peptide that causes arteriolar vasoconstriction and stimulates aldosterone secretion and thus plays an important role in hydro mineral balance.

Inhibition of ACE is considered to be an useful therapeutic approach in the treatment of hypertension. Therefore, in the development of drug to control high pressure, ACE inhibition has become an important target. A large number of highly potent and specific ACE inhibitors have been developed as orally active drugs that are used in the treatment of hypertension and congestive heart failure[3]. There are reports on ACE inhibitory peptides derived from food proteins, their physiological and pharmacological effects and their prospects for application in preventing hypertension and for therapeutic purposes.

The unique character of tumor vasculature has not been fully utilized for cancer chemotherapy and little attention has been paid to this subject in drug design or tumor targeting. Ang II has a potential role in various aspects of tumor progression and targeting Ang II production or action could prove useful in anticancer therapy[4]. Researchers hope a better understanding of the angiogenesis process will help them in cancer treatment.

MATERIALS AND METHODS

Isolation of fungus and spore staining

Soil samples were collected from Bharathiar University Campus, Coimbatore, India. Using serial dilution method, the fungal species were isolated on saboroud dextrose agar (SDA) medium. The pure cultures were maintained on SDA slants. The spores of pure culture isolates were stained for morphological characterization using Lacto phenol cotton blue method[5]. Selected six *Aspergillus species* isolates were taken and used for further studies of ACE inhibitor production.

Solid state fermentation

Solid substrate (wheat bran) was dried in an oven at 60° C for 2h. Dried wheat bran was accurately weighed to 10g in Petri dishes, and appropriately moistened with distilled water to make a slurry and autoclaved at 121° C for 40min. After cooling, the media was inoculated with 1ml (10% v/w) of spore suspension of each *Aspergillus* sp., in different plates. It was followed by incubation at room temperature for 6 days[6].

ACE extraction from animal tissue

ACE enzyme source rabbit was decapitated and lung tissue sample was harvested. Lung tissue samples were rinsed, blotted and homogenized in 0.4M BB (borate buffer pH 7.2), containing 0.34M sucrose and 0.9M NaCl (1 g tissue =10 ml buffer). Homogenates were centrifuged at 3000 rpm for 10 min and the supernatant was frozen at –70°C. Protein content[7] and ACE enzyme content in the extract were analysed[8].

Angiotensin converting enzyme assay

200µl of Hip-His-Leu (HHL) and 10µl of lung ACE enzyme were added. Immediately mixed by swirling and incubated for 15 minutes at 37°C and then added 250µl of HCl and 2ml of ethyl acetate. After shaking vigorously for1 minute, centrifugation was done at 7000rpm for 2 min. Pipetted out 1ml of clear upper layer of each vial and placed the vials in a boiling water bath for 15 min in a hood. After the ethyl acetate has evaporated, added 3ml of deionized water and mixed by inverting, without shaking until, the residue is dissolved and transferred to suitable quartz cuvettes. For determining hippuric acid (HA) content recorded the absorbance at 228 nm for both test and blank using spectrophotometer. The same procedure was carried out for blank, except HCl was added previously to stop the enzyme reaction. The amount of HA released was determined by plotting standard with HA. The enzyme activity is expressed as µg of HA released per mL per min.

Extraction of ACE inhibitory active principles from *Aspergillus* species

Fermented material of different *Aspergillus* sp. were dried at 40° C for 24h, and crushed to powder. One gram of the powdered material was extracted with 50ml of Methanol: Water: Ethanol (1:1:1 v/v) in 250ml Erlenmeyer flask keeping the flask at 30° C in a rotary shaker at 200rpm for 2h. After 2h, the mixture was centrifuged at 10,000 rpm for 10min and the supernatant was filtered through 0.45 µm cellulose acetate filter paper[6]. Protein content of the supernatant was estimated by Lowry *et al*.,[7] method.

ACE inhibition studies of fungal extract

The optimum substrate concentration for maximum enzyme activity was determined in terms of maximum reaction velocity (Vmax) and Michaelis constant (Km at which reaction velocity is half maximum). For this, various concentrations of HHL (6M) in 50mM HEPES HCl buffer with 300mM NaCl (pH 8.3) were incubated with lung ACE enzyme preparation. The accurate values of Vmax and Km were obtained from double reciprocal Line Weaver-Burk plot[9].

GC-MS and HPLC analysis

The crude fungal extract was then subjected to GCMS analysis. Gas chromatography –Mass spectral data were recored on a instruments GC Clarus 500 Perkin Elmer, Mass detector-Turbo mss gold – perkin Elmer, Turbo mass 5.1 software. The biological activity of the identified compounds was also observed from the data base. Pooled active fraction was subjected to HPLC analysis. High performance liquid chromatography on a reversed phase column (RP – HPLC) is widely utilized to generate a peptide map. The peptide mixture was separated by RP – HPLC on a symmetry C18 column. The mobile phase was delivered at a flow rate of 0.8 ml/min. Separation was made under linear gradient elution conditions used acetonitrile as the organic modifier and trifluroacetic acid (TFA) as the volatile buffer. Eluent consisted of 0.1% TFA in milli-Q water (v/v); eluent B of 0.07% TFA in acetonitrile. The chromatographic column was conditioned with 100% of eluent A, after which 20 µl of the peptide solution was applied on the C18 column and eluted by the remaining eluent A for 10 min followed by increasing eluent B concentrations: 0-10 min, 0%, 10-30 min, 0.65% and 10 min, 100%. The UV absorbance of the eluent was monitored at 214 nm[10].

Anticancer property of fungal extract on cell line

Lung carcinoma cell line A549 was used for anticancer assays. MTT assay was performed to analyse antiproliferation activity. Morphological changes and DNA fragmentation induction by fungal extract were observed to analyse apoptic activity[11].

Analysis of antioxidant assay

Animal cell line antioxidant assay

Antioxidant property of the extract was analysed both in cell line and in vitro condition[12]. In cell line, three analysis viz; glutathione s transferase, reduced glutathion and lipid peroxidation were carried out.

***Invitro* Antioxidant assay**

Four assays were performed to analyze the antioxidant properties of ACE inhibitor. They are reducing power assay, DPPH free radical scavenging activity, hydroxyl radical scavenging activity and *in vitro* lipid peroxidation assay

Purification of ACE inhibitor from *Aspergillus* sp

The fungal extract was ultra filtered with a 500MW cut off filter, and the filtrate was checked for ACE inhibition and protein content. The active extract solution was lyophilized, and 2ml was applied to Sephadex G-25 column (10X1.6cm), equilibrated with distilled water and eluted with the sodium borate buffer at a flow rate of 2mL/h. Protein content and ACE inhibition activity was determined in all the fractions. Active fractions were then pooled together and used for further analysis[13].

Molecular weight determination

The purity of the ACE inhibitor was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE][14] and stained with silver nitrate[15].

RESULTS

Isolation of fungus from soil

Nine fungal species were isolated by serial dilution technique by plating on saboroud dextrose agar (SDA). Six out of nine pure cultures were found to be *Aspergillus* species by morphological characterization using Lacto phenol cotton blue stain (Table 1).

Table 1: Fungal isolates

S. No.	Fungal species
1	*Aspergillus 1*
2	*Aspergillus 2*
3	*Aspergillus 3*
4	*Aspergillus 4*
5	*Aspergillus 5*
6	*Aspergillus 6*
7	*Mold 1*
8	*Mold 2*
9	*Mold 3*

ACE enzyme extraction

Lung tissue was used for extraction since the ACE enzyme is present mainly in lungs[16]. For the present study, rabbit lung tissue was used for ACE enzyme extraction. The enzyme extract was observed to contain 2.806U/mL of ACE enzyme (Table 2). This crude enzyme extract was used as ACE enzyme source for further studies.

Table 2: ACE enzyme activities in rabbit lung tissue extracts

S. No.	ACE enzyme activity (U/mL)	Protein (µg/mL)	Specific activity (U/ml)
1	2.806	3	0.935

Screening of Aspergillus species for ACE inhibition

Extracts from the selected six *Aspergillus species* were then screened for ACE inhibition. Among the six samples, the extract from *Aspergillus* strain 6 only showed an inhibition of 98.6% (Table 3). Based on the observation, the extract of *Aspergillus 6* strain alone was used for further studies as ACE inhibitor source.

Inhibition analysis of *Aspergillus* 6 extract

To analyse the inhibitory pattern of the fungal extract, Line Weaver Burk plot was plotted for the ACE enzyme with varied concentrations of fungal extracts and captopril a known ACE inhibitor was used as reference. The fungal extract was observed to have non competitive inhibition. The Vmax and Km values were reduced from 848 to 255 and 66 to 45.5 respectively with HHL as substrate. In case of captopril, the inhibition was 848 Vmax was reduced to 252 and Km 66 was reduced to 42.

Table 3: Screening of *Aspergillus* species extract for ACE inhibition

S. No.	Organism	Enzyme activity with inhibitor (U/mL)	Inhibition (%)
	Control	2.806	
1	*Aspergillus* 1	2.806	0
2	*Aspergillus* 2	2.806	0
3	*Aspergillus* 3	2.806	0
4	*Aspergillus* 4	2.806	0
5	*Aspergillus* 5	2.806	0
6	*Aspergillus* 6	0.03897	98.611
7	Mold 1	2.806	0
8	Mold 2	2.806	0
9	Mold 3	2.806	0

Compound analysis of crude ACE inhibitor by GCMS and HPLC

The crude extract was then analysed for identifying the compounds present in the extract by GC-MS (Figure 1). The mass spectrum obtained when compared with database showed the presence of 12 compounds (Table 4). On HPLC, the chromatogram showed a major peak. The fourth fraction was identified as the most active fraction by gel chromatography, was subjected to RP-HPLC and than eluted with acetonitrile under linear gradient elution conditions : 0-10 min, 0%, 10-30 min., 0-65% and 30-40 min 100% in order to improve the separation of the peptide (Figure 2). Acetonitrile and TFA were used as the organic modifier and the volatile buffer, respectively.

The major peak at 3.7 min retention time showed a peak area of 1.29% which was the maximum.

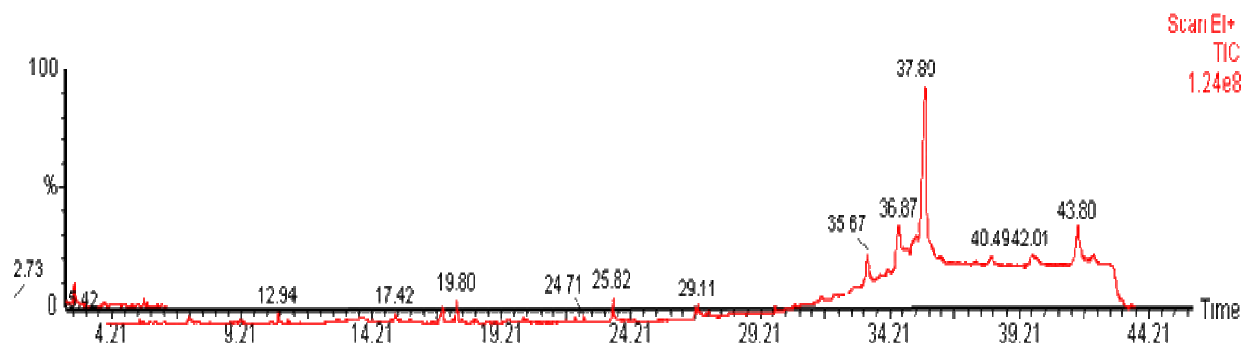


Fig. 1: GC-MS analysis of crude extract of *Aspergillus* 6

Table 4: List of compounds in crude extract of *Aspergillus* 6

S. No.	RT	Name of the compound	Molecular Formula	MW	Peak Area (%)	Compound Nature	Activity
1	5.42	Acetic acid, phenyl-, isopentyl ester	$C_{13}H_{18}O_2$	206	5.63	Ester	Antimicrobial
2	7.59	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	144	1.78	Flavonoid fraction	Antimicrobial
3	8.22	2(3H)-Furanone, 5-ethoxydihydro-	$C_6H_{10}O_3$	130	1.27	Lactone compound	Antimicrobial
4	9.50	Acetic acid, (trimethylsilyl)-	$C_5H_{12}O_2Si$	132	8.30	Silica compound	Antimicrobial
5	11.49	4-Hydroxy-2-methylacetophenone	$C_9H_{10}O_2$	150	2.25	Phenolic compound	Analgesic, Anesthetic, Antioxidant, Antiseptic, Antibacterial, AntiviralCancer preventive,Fungicide, RodenticideEmetic, Vasodilator.
6	12.94	Ethyl á-d-riboside	$C_7H_{14}O_5$	178	9.54	Sugar moiety	No activity reported
7	13.32	Undecanoic acid, ethyl ester	$C_{13}H_{26}O_2$	214	1.27	Fatty acid Ester	No activity reported
8	17.42	Undecanoic acid	$C_{11}H_{22}O_2$	186	6.88	Fatty acid	No activity reported
9	19.25	2,5-Dihydroxypropiophenone	$C_9H_{10}O_3$	166	23.21	Phenolic compound	Analgesic, Anesthetic, Antioxidant, Antiseptic, Antibacterial, Antiviral Cancer preventive, Fungicide, Rodenticide Emetic, Vasodilator.
10	20.00	3-Pyrrolidin-2-yl-propionic acid	$C_7H_{13}NO_2$	143	2.52	Alkaloid	Antimicrobial
11	24.38	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O$	210	5.75	Alkaloid	Antimicrobial
12	25.82	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	31.59	Palmitic acid	Antioxidant Hypocholesterolemic Nematicide Pesticide Lubricant Antiandrogenic Flavor Hemolytic

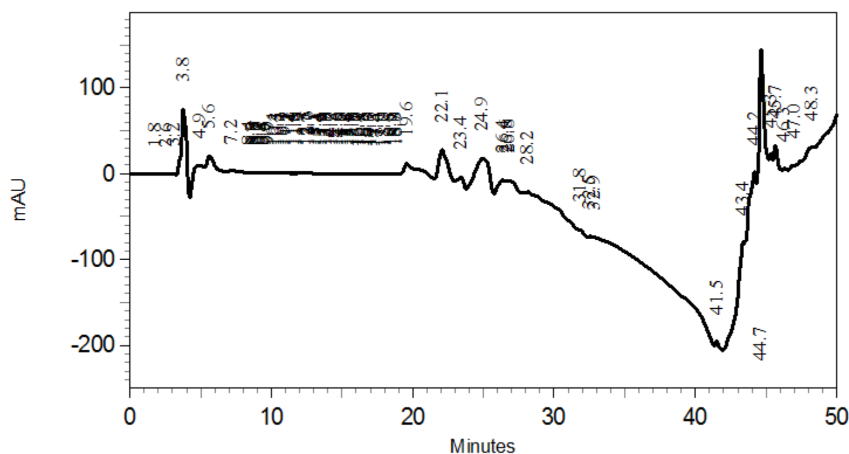


Fig. 2: HPLC analysis of purified fraction of *Aspergillus* 6

Anticancer effect of *Aspergillus 6* extract on cell line

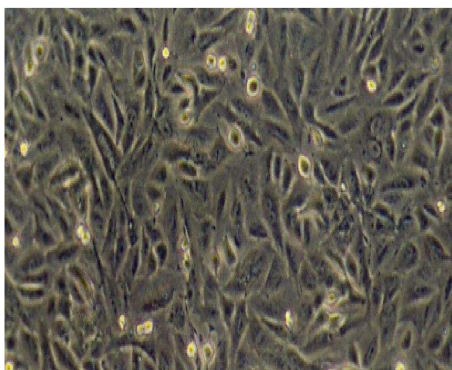
A549 lung carcinoma cell line was treated with the *Aspergillus 6* extract (Figure 3). MTT assay was done to determine its antiproliferation effect. DNA fragmentation study was performed to analysis the apoptic effect. From the results observed, it was confirmed that the fungal extract has a compound with anticancer property.

ACE inhibitor induced DNA fragmentation

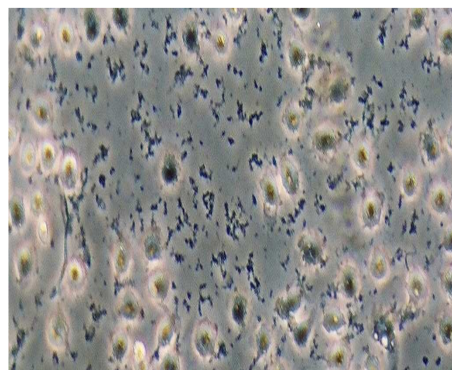
The degradation of DNA into multiple fragments of 180-200 base pairs is a distinct biochemical hallmark of apoptosis. DNA fragmentation was confirmed by electrophoresis of genomic DNA extracts from A549 cells treated with 200µl of ACE inhibitor for three hours. The ladder formation was observed in ACE inhibitor treated cells, where as the untreated cells did not show a typical ladder (Figure 4). These results indicated that ACE inhibitor led to apoptotic cell death in A549 cells.

MTT cell viability assay

In order to show the concentration dependent action of ACE inhibitor in A549 cells were treated with different (5-100µg) concentration of A549 and viability assessed after 4h. 50% of cell viability was attained in 50µg concentration of ACE inhibitor. It was observed that the cell viability decreased with increased concentration of ACE inhibitor (Figure 5).



Control cells



ACE treated cells

Fig. 3: Effect of ACE inhibitors on A549 cells

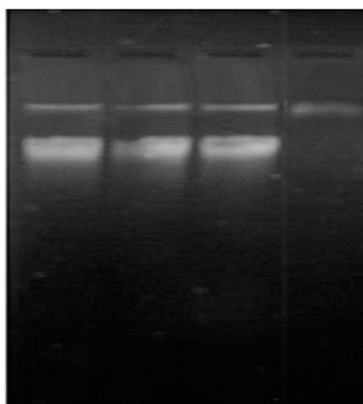


Fig. 4: ACE inhibitor induced DNA fragmentation

Lane 1,2,3 – cells treated with 5µl of inhibitor; Lane 4 – control cell

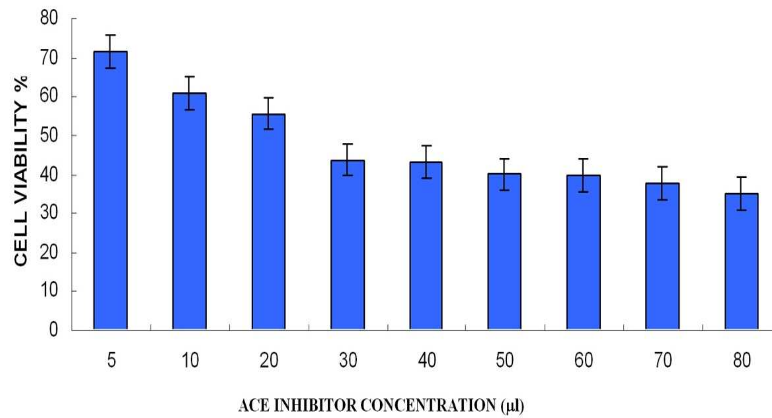


Fig. 5: MTT assay

Table 5: Purification steps

S. No.	Purification Steps	Volume (ml)	Protein (mg/mL)	Inhibition activity (%)
1.	Solvent extraction	5	3.6	86.11
2.	Sephadex G-25	2	1.4	75.00

Fig. 6: SDS gel electrophoresis

Lane 1- protein markers; Lane 2, 7- crude extract; Lane 3, 4, 5, 6- purified extract

Analysis of Antioxidant property

To analyze the antioxidant property, both *invitro* cell line studies were performed. Reducing power, DPPH radical scavenging activity Hydroxyl radical scavenging activity, *invitro* and lipid peroxidation assay were carried out *invitro* analysis in cell line studies GST reduced to glutathione and lipid peroxidation assay were performed.

Purification and molecular weight determination of ACE inhibitor

The crude extract from the selected fungi was filtered through 0.45µm filter. The filtrate was lyophilized and applied on to Sephadex G-25 column and eluted with sodium borate buffer pH. The entire fraction were collected and checked for protein content and ACE inhibition assay. Among these fractions only 4, 5, 6 fractions showed high ACE inhibitory action. These fractions were run on SDS PAGE. On PAGE a single band was observed at approximately 31kDa. The purified enzyme fraction had an inhibition activity of 75%.

DISCUSSION

Angiotensin II the biologically active peptide of the Renin Angiotensin system is a major regulator of blood pressure and cardiovascular homeostasis and is also recognized as a potent mitogen. AngII has a potential role in various aspects of tumor progression[4]. ACE enzyme which catalyses the AngII formation from Ang I could prove useful in anticancer therapy. Based on this view, the present study is focussed to identify the ACE inhibitor from soil fungus. In the recent report Solid State Fermentation was observed to result in increased secondary metabolite production. Hence in the present study, Solid-State Fermentation with wheat bran[17] was used for ACE inhibitor production. Lung tissue was used for extraction since the ACE enzyme is present mainly in lungs[16] but also in plasma and other tissues. At these locations, ACE can influence the production of AngII and contribute to cardiovascular homeostasis maintenance[8]. One of the most common anti-hypertensive therapies that prevent the production of Ang II is ACE inhibition. Owing to its multifarious impact, ACE inhibition nowadays is being focused for cancer therapy. ACE inhibitors are either natural peptides or synthetic element derivatives. Earlier studies for ACE inhibition have been mostly on bioactive peptides from dairy products[18]. ACE inhibitory peptides have also been studied from animal extracts such as beef hydrolysates[1] and plant extract from soybean[2]. Microbes as ACE inhibitor source have been less explored with the exception of *Lactobacillus delbrueckii* and *Lactobacillus lactis* which are used for milk fermentation[18]. Edible mushrooms *Tricholoma giganteum* has also been proved to have ACE inhibiting peptide[21]. Many research groups have combed for ACE inhibitors in microbial sources such as *Doratomyces putredinis, Nocardia orientalis, Streptomycetes, Actinomycetes, Actinomadura, Spiculospora* and

Actinomadura[22]. In the present study, the *Aspergillus sp* was selected for the isolation of ACE inhibitors. ACE inhibitors so far analyzed have been found to be either competitive or non competitive inhibitors. Peptides from crude fraction of *Lactobacillus lactis* sub sp *cremoris* showed competitive inhibition when Dixon plots was drawn[18]. Ono *et al*.,[23] have studied the inhibition of dipeptides Phe-Leu and Leu-Phe on ACE which showed non-competitive inhibition and competitive inhibition respectively. This result indicates that the sequence of ACE inhibitory peptides can alter both inhibitory potency and inhibition mechanisms. Pepstatin, a pentapeptide isolated from the culture filtrate of *Actinomycetes* strains inhibited ACE in a noncompetitive way[24, 25]. Corvol *et al*.,[26] have proposed a competitive type of inhibition of pepstatin.This contrast inhibitory pattern was then later explained as the change in pH which cause change in degree of dissociation leading to a different inhibition pattern. Apart from pH the concentration of inhibitory peptide also had been proved to alter inhibitory pattern[27]. The observed results in the present study, higher non competitive inhibition pattern at low concentration (10µg) of extract and low inhibition at higher concentrations (30µg and 50µg) could also be due to change in pH or concentration. Ang II stimulated pathways are responsible for proliferation of vascular smooth muscle cells. Appropriate inhibition of Ang II production by ACE inhibition could inhibit proliferation via AT1 receptor and Calcium channel blockage[28]. ACE inhibition promotes apoptosis in vascular smooth muscle cells[29,30]. The combined effect of perindropril and IFN proved to induce epithelial cell apoptosis and secondary apoptosis of the tumour cells by attenuating expression of VEGF. ACE inhibition resulted in a DNA laddering pattern characteristic of apoptosis[31]. Similarly the apoptotic activity of the fungal extract in the present study could also be attributed to the ACE inhibition activity of the extract. ACE inhibitors were also reported to have antioxidant property[32]. Extracts of ACE inhibitor obtained by solid substrate fermentation were tested for antioxidant activity. To analyze the antioxidant property, both *invitro* cell line studies were performed. Reducing power, DPPH radical scavenging activity Hydroxyl radical scavenging activity, *invitro* and lipid peroxidation assay were carried out *invitro* analysis in cell line studies GST reduced to glutathione and lipid peroxidation assay were performed. The ACE inhibitor of protein nature in the crude extract in the present study could also be the reason for antioxidant and free radical scavenging properties. To prove that the ACE inhibitor is of protein origin, the protein purification was carried out. The purified protein also exhibited ACE inhibition.

CONCLUSION

The present study revealed that the peptides obtained from *Aspergillus* species have the ability to inhibit ACE activity and also antioxidant activity *in vitro*. The use of ACE inhibitors as anticancer agents will be a challenge but in view of the necessity for anticancer drugs, such an approach is worth of further investigation. The compounds identified in the present study after further structural analysis and explanation on mode of action might serve as a potent drug against ACE.

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