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# Effect of Mycelial Extract of *Clavicorona pyxidata* on the Production of Amyloid β-Peptide and the Inhibition of Endogenous β-Secretase Activity *in vitro*

**Tae-Hee Lee[1], Young-Il Park[2], and Yeong-Hwan Han[1,3,*]**

[1]*Department of Life Science, College of Natural Science, Dongguk University, Gyeongju 780-714, Republic of Korea*
[2]*Department of Biology, Graduate School, Dongguk University, Seoul 100-715, Republic of Korea*
[3]*Myco Co., Gyeongju, Gyeongbuk 780-921, Republic of Korea*

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**Amyloid β-peptide (Aβ), which is a product of the proteolytic effect of β-secretase (BACE) on an amyloid precursor protein, is closely associated with Alzheimer's disease (AD) pathogenesis. There is sufficient evidence to suggest that a BACE inhibitor may reduce Aβ levels, thus decreasing the risk of AD. In a previous study, an extract of *Clavicorona pyxidata* DGUM 29005 mycelia was found to inhibit the production of a soluble β-amyloid precursor protein (sβAPP), Aβ, and BACE in neuronal cell lines. We sought to determine whether this mycelial extract exerts the same effect in human rhabdomyosarcoma A-204 and rat pheochromocytoma PC-12 cells. We found that the production of Aβ decreased in a dose-dependent manner in the presence of the mycelial extract and that the concentration of Aβ never exceeded 50 μg/ml. The presence of sAPP was detected in every culture medium to which the mycelial extract had been added and its concentration remained the same, regardless of the concentration of the extract used. Endogenous β-secretase activity in A-204 and PC-12 cellular homogenates also decreased in the presence of this extract. These cells, in culture, were not susceptible to the cytotoxic activity of the mycelial extract.**

***Keywords***: Alzheimer's disease, amyloid β-peptide, amyloid precursor protein, β-secretase, *Clavicorona pyxidata*

Alzheimer's disease (AD) is the most common neurodegenerative disorder and it is characterized by progressive dementia accompanied by the occurrence of extracellular senile plaques and intracellular neurofibrillary tangles (Selkoe, 1989). The major component of these plaque deposits is amyloid β-peptide (Aβ), a 40- to 43-amino acid peptide cleaved from an amyloid precursor protein (APP) by β-secretase (BACE) and by a putative γ-secretase (Estus, 1997; Vassar, 1999). The proteolytic cleavage of Aβ from APP is a key step in the AD pathogenesis.

BACE is a type-1 transmembrane protein characterized by N-linked glycosylation and two active-site motifs consisting of aspartic proteases. An immature BACE of approximately 60 kDa is produced in the endoplasmic reticulum then undergoes propeptide cleavage and further glycosylation to produce a mature BACE in the Golgi apparatus (Capell *et al.*, 2000; Haniu *et al.*, 2000; Huse *et al.*, 2000).

The importance of BACE in AD pathogenesis was demonstrated when BACE expression and activity were found to be elevated in the brain tissue of patients with sporadic AD. BACE expression and activity were also increased in the presence of a mutant APP, which contained additional cleavage sites, revealed by an in increase in β-site cleavage products and by the observation of a lack of Aβ production in BACE knockout mice (Cai *et al.*, 2001; Luo *et al.*, 2001). These findings underscore the importance of targeting β-secretase enzyme activity inhibition as the basis for developing anti-AD drugs. Numerous studies on BACE activity in human cells have used cells transfected with constructs harboring a Swedish mutation proximal to the β-secretase cleavage site in APP. A several-fold increase in Aβ secretion was observed in these cells (Citron *et al.*,

* To whom correspondence should be addressed.
(Tel) 82-54-770-2213; (Fax) 82-54-770-2515
(E-mail) yhhan@dongguk.ac.kr

1992; Felsenstein *et al.*, 1994), showing that Aβ is minimally expressed in the majority of cell types. Reverse transcription-polymerase chain reaction (RT-PCR) analysis using a BACE cDNA-specific primer demonstrated that BACE is found in various types of cells and tissues and that it is particularly abundant in the pancreas and brain (Hiroshi and Takeshi, 2001). Studies of Aβ expression have shown that rhabdomyosarcoma A-204 expresses a greater amount of Aβ than any other type of cell (Potempska *et al.*, 1999). Therefore, we used A-204 cells in this study. We also used PC-12 cells because they have neuronal properties and because BACE expression is readily detectable in neuronal cells (Acquati *et al.*, 2000; Bennett *et al.*, 2000; Hussain *et al.*, 2000).

The pharmacotherapeutic agents currently used to inhibit BACE include substances that can be found in natural products. The mycelial and culture broth extracts of *Clavicorona pyxidata* have been found to inhibit the effects of BACE on acetylcholinesterase and proryl endopeptidase activity (Lee *et al.*, 1999; Lee *et al.*, 2006b).

In this study, we investigated the ability of the *C. pyxidata* mycelial extract to inhibit endogenous β -secretase activity, as well as Aβ and soluble APP (sAPP) activity in A-204 and PC-12 cells. We also investigated the cytotoxicity of the mycelial extract in A-204 and PC-12 cells.

## Materials and Methods

### *Materials*

The *C. pyxidata* used in this study was isolated from a fruiting body, identified, and designated as *C. pyxidata* DGUM 29005. The RNA sequences of ITS1, 5.8S and ITS2 of *C. pyxidata* DGUM 29005 (Lee *et al.*, 2006a) were deposited in the Genbank database (Accession No. AY 588248). All of the chemicals used in this study were of the highest possible purity. Protein G-agarose was obtained from Santa Cruz Biotechnology (USA). The horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig) secondary antibody and the anti-rabbit Ig secondary antibody were purchased from Zymed Laboratories, Inc. (USA). The ECL kit used to detect the HRP-labeled antibody was obtained from Amersham Biosciences (USA). The mouse anti-Aβ-protein monoclonal antibody 4G8, which was used to induce immunoprecipitation of Aβ and sAPP, was purchased from Chemicon International, Inc. (USA). The monoclonal antibody 6E10, which was used for Aβ detection, was obtained from Abcam Ltd. (UK). The mouse anti-APP A4 monoclonal antibody 22C11, which binds the N-terminus to APP, was obtained from Chemicon International, Inc. (USA).

### *Cell culture*

Human rhabdomyosarcoma A-204 and rat pheochromocytoma PC-12 cells were obtained from the American Type Culture Collection (ATCC, USA). The A-204 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and the PC-12 cells were maintained in a 1:1 mixture of DMEM/Ham's F-12 medium supplemented with 5% heat-inactivated FBS and 10% heat-inactivated horse serum at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. The cell culture reagents were obtained from GIBCO-BRL (USA). The human rhabdomyosarcoma A-204 and rat pheochromocytoma PC-12 cells were incubated for 48 h with various concentrations of the mycelial extract in order to induce the expression of Aβ and APP, and to determine their rates of expression.

### *Measurement of cell viability*

Human rhabdomyosarcoma A-204 and rat pheochromocytoma PC-12 cells were plated at an appropriate density ($0.5 \times 10^4$ cells/well) on 96-well plates. The cell viability was determined using a sodium XTT reduction assay. The extract cytotoxicity was determined by exposing the cells to various concentrations of the mycelial extract (range: 1-100 μg/ml) for 48 h. The results were expressed as the percentage of XTT reduction, based on the assumption that the absorbance of the control cells was 100%.

### *Immunoprecipitation*

Immunoprecipitation was induced in the media or in the cells in each dish to which mycelial extract had been added. After harvesting, the media samples were centrifuged at 15,000xg for 10 min to remove any non-adherent cells. The cell samples were harvested in RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 100 μg/ml sodium orthovanadate. The lysed cells were then transferred to a tube on ice and clarified by centrifugation at 15,000xg for 10 min at 4°C to remove the cellular debris. Immunoprecipitation was performed on the supernatant using mAb 4G8 [anti-Aβ -(18-22)] at a dilution of 1:100. After the supernatant and cell lysates were treated with mAb 4G8, the mixture of the protein and antibody was incubated for 1 h at 4°C. Protein G-agarose beads were added to this mixture, which was then incubated overnight at 4°C. The immunoprecipitation complexes were washed for 10 min in RIPA buffer containing 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 100 μg/ml sodium orthovanadate, and were then washed 3 times in RIPA

buffer. The immunoprecipitation complexes were re-suspended in sample buffer containing 10% SDS and β-mercaptoethanol, and were then boiled for 5 min.

***Western blot analysis***

The Aβ and sAPP expression levels were determined by loading 10 μl of each sample onto 10% and 15% Laemmli SDS-polyacrylamide gel electrophoresis (PAGE) columns. The proteins that separated in the gels were transferred to polyvinylidene difluoride membranes by electrophoresis (Millipore, USA), and were exposed to a 15 V current for 25 min. The membranes were blotted and then heated in boiling Tris-buffered saline (TBS) for 5 min to enhance the signal. They were then blocked for 1 h with 5% skim milk in TBS containing 0.1% Tween 20 (TBS-T buffer). The membranes were washed with TBS-T, followed by the addition of mAb 6E10 (1 μg/ml) or mAb 22C11 (1 μg/ml) to the mixture, which was then incubated overnight at 4°C. The bound antibodies were detected using the HRP-conjugated anti-mouse Ig secondary antibody in order to detect Aβ and sAPP. The membrane treated with the secondary antibody was then incubated for 1 h at room temperature, washed 3 times in TBS-T, and analyzed using an ECL detection system according to the instructions supplied by the manufacturer. Synthetic Aβ (0.5 ng/μl) was loaded onto the same gel and measured in parallel to identify the cellular Aβ.

***Assay of BACE inhibitory activity***

The BACE inhibition assay was conducted using a β-secretase assay kit from ANASPEC (USA) in accordance with the protocol supplied by the manufacturer. When the A-204 and PC-12 cells growing on the 100-dishes were 90% confluent, they were added to various concentrations of the mycelial extract and incubated for 48 h at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. The A-204 and PC-12 cells were homogenized using a Glass-Col homogenizer in 150 μl of β-secretase buffer (BioVison, USA) in order to obtain an endogenous enzyme source. The lysed cells were then incubated on ice for 10 min and clarified by centrifugation at 15,000xg for 10 min at 4°C to remove the cellular debris. The resultant supernatants were used as endogenous sources of BACE. The BACE purified from the A-204 and PC-12 cells was then detected at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

***Statistical analysis***

The data were expressed as the mean ± SD (standard deviation). The mean values were compared using Duncan's Multiple Range Test and calculated using SPSS software (SPSS Inc., USA). $P$ values of $< 0.05$ were considered significant.

## Results and Discussion

***Effect of an extract of C. pyxidata mycelia on sAPP, and Aβ expression in A-204 and PC-12 cells***

Alternative APP cleavage results in the production and secretion of sAPP and Aβ. The primary foci of AD research is currently aimed at developing approaches to reduce Aβ levels based on the hypothesis that a reduction in Aβ, a primary component of the extensive extracellular amyloid plaques and intracellular neurofibrillary tangles that characterize AD (Selkoe, 1991), will reduce the pathophysiological effect of the disease, Such approaches include the inhibition of Aβ production, reduction of soluble Aβ

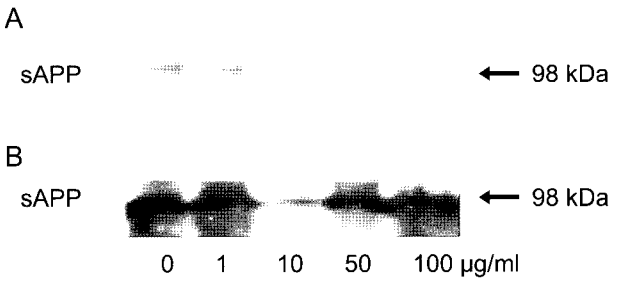


**Fig. 1.** ffect of *C. pyxidata* mycelial extract on sAPP production in A-204 (A) and PC-12 (B) cells.
The sAPP levels in the cultured medium were detected by western blot assay after both types of cells were incubated with various concentrations of the mycelial extract for 48 h at 37°C in a humidified atmosphere of 5% $CO_2$/ 95% air.

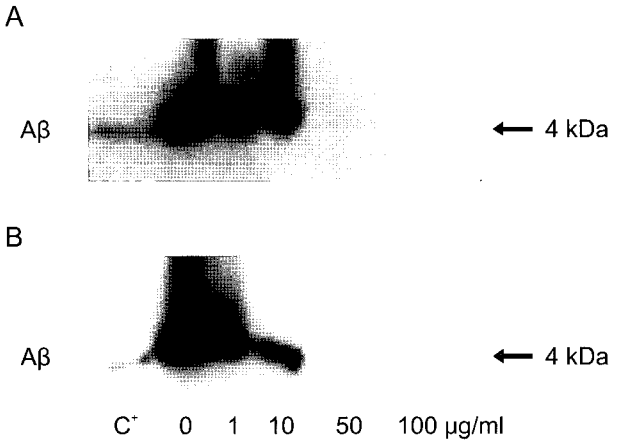


**Fig. 2.** Effect of *C. pyxidata* mycelial extract on Aβ production in A-204 (A) and PC-12 (B) cell lines. Aβ expression was detected using a western blot assay after each type of cell was incubated with various concentrations of the mycelial extract for 48 h at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. *Synthetic Aβ (0.5 ng/μl) was used to identify cellular Aβ production.

levels, and enhanced clearance of Aβ from the brain (DeMattos *et al.*, 2001; Koldamova *et al.*, 2005; Walsh *et al.*, 2005). In this study, we investigated Aβ levels in A-204 and PC-12 cells.

We incubated the A-204 and PC-12 cells with various concentrations of the *C. pyxidata* mycelia extract for 48 h in order to explore the effect of an extract of *C. pyxidata* mycelia on sAPP and Aβ expression. The cell lysates were examined for evidence of Aβ expression, and the culture media were evaluated for levels of sAPP expression. The presence of sAPP was detected in the media that had been treated with various concentrations of the *C. pyxidata* mycelial extract, and there were no significant differences in expression levels detected among the various extract concentrations used (Fig. 1).

The Aβ expression in these cells decreased in a dose-dependent manner in 0 μg/ml to 10 μg/ml concentrations of the mycelial extract; the higher concentrations of extract inhibited Aβ expression (Fig. 2). Based on these results, we concluded that the extract of *C. pyxidata* mycelia inhibits Aβ expression, but not sAPP expression. These results also indicate that the *C. pyxidata* mycelial extract either inhibits or completely prevents the release of Aβ from APP without inhibiting the expression of APP.

### *Inhibitory effect of BACE by mycelial extracts of C. pyxidata*

BACE mediates a critical step in the production of A β, which is responsible for neuronal death and the appearance of insoluble amyloid plaques in AD patients. Because BACE has been identified as a secretase enzyme that converts APP into Aβ, a BACE blockade can be expected to slow the production of Aβ (Sinha *et al.*, 1999; Vassar, 1999; Yan *et al.*, 1999). β -secretase enzyme activity inhibition has been the targeted mode of action for drugs currently being developed to prevent AD. The importance of this approach is underscored by the finding that human mutations at the P1 and P2 β-secretase cleavage subsites result in early-onset familial AD (Hass *et al.*, 1995; Cai *et al.*, 2001). We sought to determine whether an extract of *C. pyxidata* mycelia could inhibit intracellular BACE activity after the extract was incubated with intact A-204 and PC-12 cells.

BACE activity inhibition was apparent at various concentrations of the mycelial extract (Fig. 3, 4). A 100 μg/ml volume of the extract resulted in a 58.7% and 43.5% inhibition of BACE activity in A-204 and PC-12 cells, respectively. Thus, the *C. pyxidata* mycelial extract exhibited dose-dependent inhibition of endogenous BACE activity. These results are consistent with the patterns of Aβ expression identified though Western blot analysis.

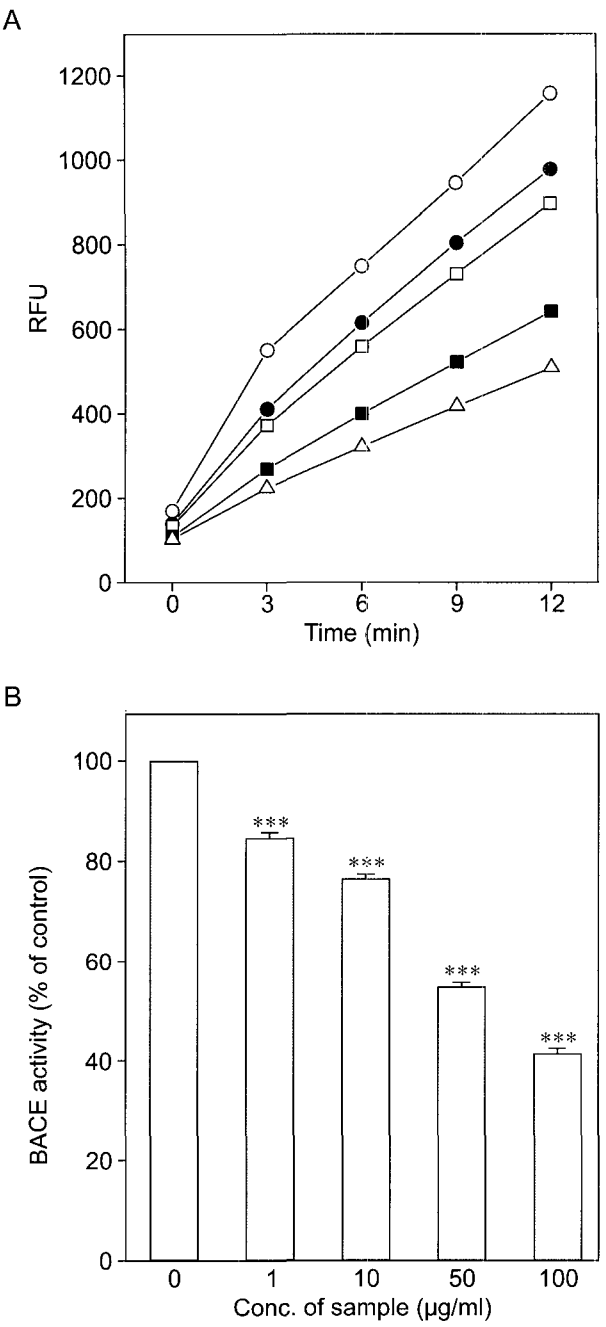


**Fig. 3.** Inhibitory effect of *C. pyxidata* mycelial extracts on endogenous BACE activity in A-204 cells. (A) Kinetics activity of BACE activity in A-204 cells that had been incubated with various concentrations of the mycelial extract for 2 days at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. Symbols used: 0 μg/ml (○), 1 μg/ml (●), 10 μg/ml (□), 50 μg/ml (■), 100 μg/ml (△). The increase in fluorescence intensity was monitored at Ex: 488 nm and Em: 520 nm. (B) Histograms were created to compare the inhibitory activity of BACE in various concentrations of the mycelial extract. Values are expressed as the means ± SD of three separate experiments, each performed in triplicate. *** $P$ <0.001.

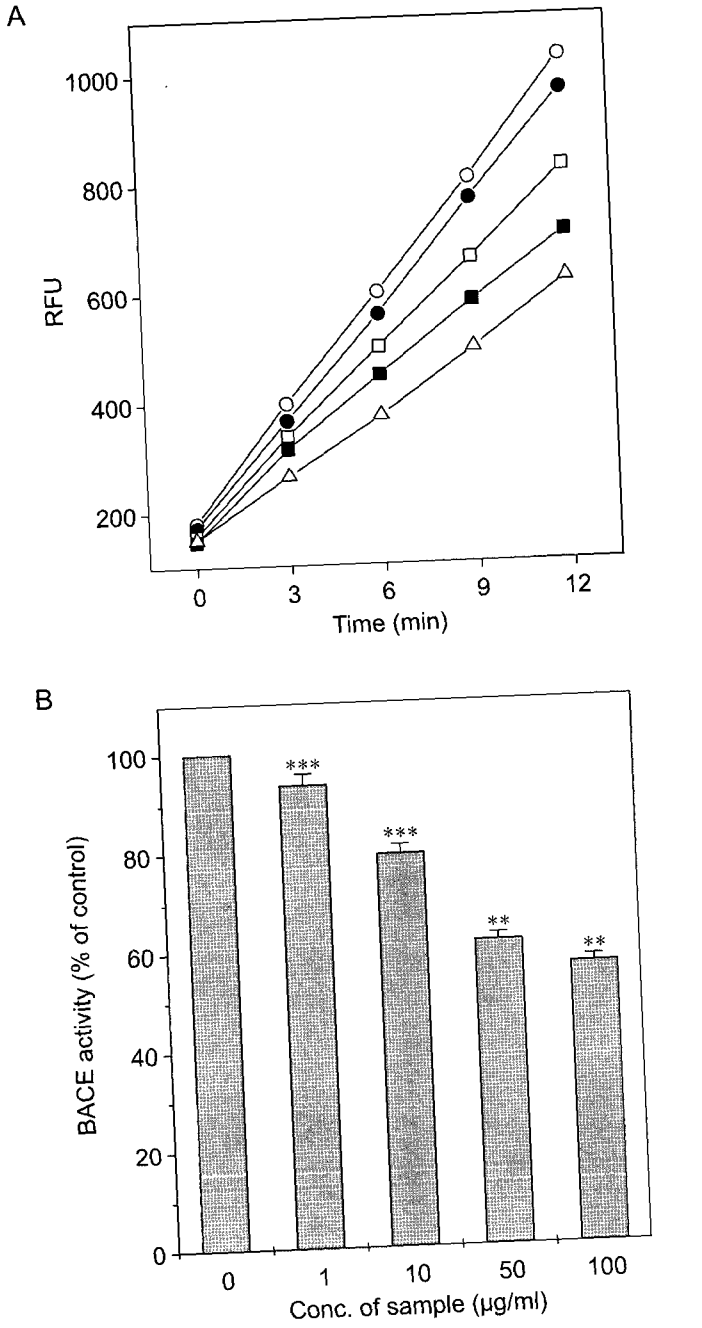


**Fig. 4.** Inhibitory effect of *C. pyxidata* mycelial extracts on endogenous BACE activity in PC-12 cells.
(A) Kinetics activity of BACE activity in PC-12 cells that had been incubated with various concentrations of the mycelial extract for 2 days at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. Symbols used: 0 μg/ml (○), 1 μg/ml (●), 10 μg/ml (□), 50 μg/ml (■), 100 μg/ml (△). The increase in fluorescence intensity was monitored at an Excitation wavelength of 488 nm and an emission wavelength of 520 nm. (B) Histograms were created to compare the inhibitory activity of BACE in various concentrations of the mycelial extract. Values are expressed as the means ± SD of three separate experiments, each performed in triplicate. ** $P$ <0.01; *** $P$ <0.001.

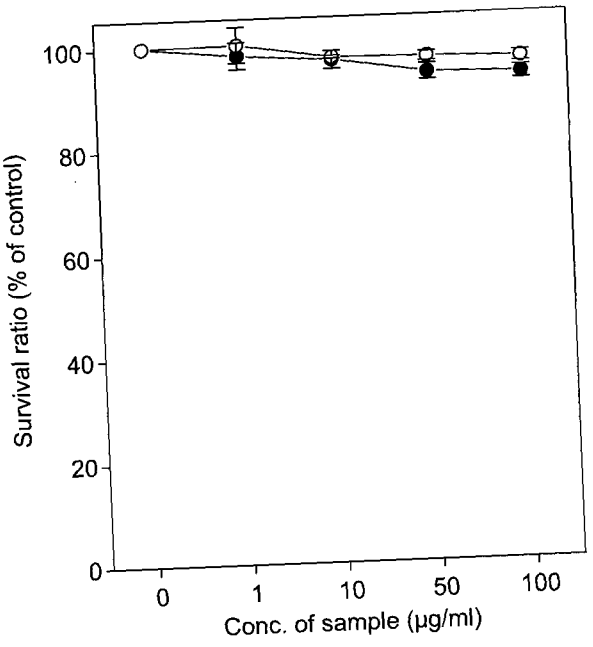


**Fig. 5.** Cytotoxic effect of *C. pyxidata* mycelial extracts on A-204 (○) and PC-12 (●) cells.
Each type of cell was incubated with various concentrations of the mycelial extract for 48 h at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. The survival ratio, expressed as the percentage of the survival of the controls, was measured using the XTT assay. Values represent the means ± SD of three separate experiments that were each performed in triplicate.

### *Cytotoxic effect of mycelial extract of C. pyxidata*

This *in vitro* study was conducted in order to determine whether an extract of *C. pyxidata* mycelia has any cytotoxic effects. An XTT-based cytotoxic assay was used in human A-204 and PC-12 cells. The results indicated that the extract had only a minimal effect on the survival of either cell type (Fig. 5). After being exposed to 100 μg/ml of the extracts, the cell viabilities for the A-204 and PC-12 cell lines were 96.6% and 93.6%, respectively. These results suggest that the *C. pyxidata* mycelial extract did not affect cell survival.

Further study will be required to determine whether the mycelial extract inhibits γ-secretase activity and to identify the method by which β-secretase activity might be blocked. It also remains to be seen whether the extract can alter Aβ production in animals that overproduce Aβ. These topics are currently under investigation in our laboratory.

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