CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

ISBN 978-88-95608- 56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864050

Filamentous Fungi as Source of Biotechnologically Useful Metabolites and Natural Supplements for Neurodegenerative Diseases Treatment

Laura Ciribelli Borges Dominguete*[a], Jacqueline Aparecida Takahashi[ab]

[a] Post-Graduation Course on Food Science, Pharmacy Faculty, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[b] Department of Chemistry, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
ciribellilaura@gmail.com

Filamentous fungi are microorganisms that have been increasingly used for obtaining biologically active substances, due to their extraordinary biosynthetic capacity. These fungal metabolites have extensive industrial applications and their production is cheap compared to plants metabolites. The most prominent applications of fungal metabolites are as pigments, antimicrobials, immunosuppressants or immune stimulants, antioxidants, cytotoxic and enzyme inhibitors, among other compounds used in the pharmaceutical or food industry. In recent years, a significant number of natural compounds with antioxidant activity has been reported. Since oxidative stress is related to various diseases, including the neurodegenerative ones, some of these natural metabolites have been screened and presented significant inhibitory activity of acetylcholinesterase (AChE), an enzyme involved in Alzheimer's disease (AD). This enzyme degrades the neurotransmitter acetylcholine, which level is much reduced in AD patients. That disease affects mainly elderly individuals, and with the increase in life expectancy, AD is considered a serious and costly public health problem since treatment is only symptomatic and still limited by price and adverse effects. Therefore, the search for drugs of natural origin, in particular, from fungi to the treatment or prevention of AD has become a huge and urgent need. The vast majority of scientific works that investigate fungal secondary metabolites with regard to possible biological activities, use extracts obtained from the use of solvents such as methanol, ethanol, ethyl acetate, or other, demanding great spent of time, labour, and reagents, generating high volumes of chemical waste, and, at the end, often only a few extracts presents satisfactory results. In view of the urgency of obtaining effective treatment for AD, this study, in an innovative way, targeted, in the biological assays, fermented broths containing secondary metabolites, which provided time optimization and elimination of solvents use, avoiding, therefore, negative impacts to the environment. The aim of this study was to determine the antioxidant potential and AChE inhibitory capacity of fermented broths from different fungi. For this, about 50 filamentous fungi isolated from soil with different macroscopic characteristics were selected. They were grown in culture medium constituted by peptone, sucrose, potassium phosphate dibasic (K_2HPO_4); sodium nitrate ($NaNO_3$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$); ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), potassium chloride (KCl) and cupric sulfate pentahydrate ($CuSO4.5H_2O$), by 28 days at 25 ºC. After this period, the broths containing secondary metabolites were screened for assessing their total antioxidant activity by colorimetric phosphomolybdate method and their AChE inhibitory activity. The broth of *Hypocrea lixii* stood out among the other fungal species, presenting promising results. Even with bioactive compounds very diluted in the medium, this broth presented significant total antioxidant activity (18.22 µg AAE/mL broth) and efficient inhibition of AChE (65.4%) in relation to eserine (positive standard).

1. Introduction

Filamentous fungi are microorganisms that have been increasingly used for obtaining biologically active substances, due to their extraordinary biosynthetic capacity and relatively fast growth. Fungal metabolites have potencial for use mainly in the food industry and pharmaceuticals, such as pigments which was reported by Dufossé et al. (2014), antimicrobial agents (Nisa et al., 2015) and anticancer substances (Zhao et al.,

Please cite this article as: Dominguete L., Takahashi J.A., 2018, Filamentous fungi as source of biotechnologically useful metabolites and natural supplements for neurodegenerative diseases treatment, Chemical Engineering Transactions, 64, 295-300 DOI: 10.3303/CET1864050

2013). In recent years, a significant number of natural compounds with antioxidant activity has been investigated, since oxidative stress is related to various diseases, including the neurodegerenative ones (Li et al., 2015). Fungal metabolites have been screened and demonstrated strong antioxidant activity (Suresha and Srinivasan, 2013). These metabolites have also been investigated for inhibition of AChE, an enzyme involved in the pathological process of AD (Abraham et al., 2015). Mendiola-Precoma et al. (2016) reinforce that, with the increase in life expectancy and the aging of population, AD is already considered a serious and costly public health problem whose current treatment is only symptomatic, consisting mainly in the use of AChE inhibitors (Hajlaoui et al., 2016). Since the cause of AD is a complex and multifactorial pathology, treatment effectiveness improves when more than a single target is attacked (Zhang et al., 2008). Due to the high cost, low efficiency and adverse effects of the drugs available in the market, search for natural compounds derived from fungi and vegetables that have potential to be used in prevention or treatment of Alzheimer has been growing (Abirami et al., 2014; Aly et al., 2011).

Filamentous fungi have been increasingly used for different biotechnological applications due to their biosynthetic versatility, varied chemical profile, large-scale cultivation facility and big arsenal of strains information already produced (Dufossé et al., 2014).

The vast majority of scientific studies that investigate biological activities of fungal metabolites uses extracts obtained from the extraction with solvents such as ethyl acetate or others (Saravanakumar et al., 2015). Extracts preparation is a process that requires time, labor, reagents, and often only a small number of the extracts screened presents satisfactory results. Current works have increasingly addressed the issue of sustainable development and the reduction of environmental impact in today's society (Lakioti et al., 2017). Therefore, in this study, in an innovative way, the bioassays target were the fermented broths containing secondary metabolites, which provided time optimization and elimination of solvents use, avoiding, thus, negative impacts to the environment. Thus, the aim of this work was to analyze the antioxidant potential and AChE inhibitory capacity of broths produced by fermentation of different fungi. For this, about 50 filamentous fungi with different macroscopic characteristics were isolated from soil. They were grown in complex culture medium constituted by peptone, sucrose, potassium phosphate dibasic (K_2HPO_4); sodium nitrate ($NaNO_3$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$); ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), potassium chloride (KCl) and cupric sulfate pentahydrate ($CuSO_4.5H_2O$) for 28 days at 25 ºC. After that, the fermented broths were evaluated *in vitro* for the total antioxidant activity (first screening) and later some of the most active ones were tested for the ability to inhibit AChE. Among the samples analyzed, *Hypocrea lixii* broth was one of the most promising ones. Therefore, through the use of this methodology of work, it was possible to screen a large number of fungi and find, in a short time, and without expense of organic solvents, a fungal species with potential biotechnological application, whose metabolites had not been investigated for AChE inhibition and total antioxidant activity so far.

2. Materials and methods

2.1 Materials

The reagents used for analysis of total antioxidant activity were: sulphuric acid (Química Moderna, São Paulo, Brasil), ammonium molybdate (Labsynth, Diadema, Brazil), sodium phosphate dibasic (Labsynth, Diadema, Brazil) L-Ascorbic acid (Neon, São Paulo, Brazil), and for analysis of AChE inhibition were used albumin bovin serum (Sigma, St. Louis, USA); 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB) (Sigma, St. Louis, USA); TRIS/HCl acetylthiocholine iodide (ATCI) (Sigma, St. Louis, USA); acetylcholinesterase from electric eel, type V-S (Sigma, St. Louis, USA) and eserine (Sigma, St. Louis, USA).

Microorganisms used in this study were stored in the collection of the Biotechnology and Bioassays Laboratory (LaBB) of the Department of Chemistry, at Federal University of Minas Gerais, which were identified by a random numerical code, ranging from 500 to 750. Fungi were fermented in submerse liquid at room temperature (25 °C) in a total volume of 200 mL composed by peptone (5 g/L), sucrose (30 g/L), potassium phosphate dibasic (1 g/L); sodium nitrate (2.5 g/L), magnesium sulfate heptahydrate (0.5 g/L); ferrous sulfate heptahydrate (0.001 g/L), potassium chloride (0.5 g/L) and cupric sulfate pentahydrate (0.005 g/L), for 28 days. After that time, the content of each fungus fermentation was filtered and mycelia was separated from broth (liquid portion). Then, the mycelia were frozen and the broths were tested for total antioxidant activity by colorimetric method of phosphomolybdate and for inhibition of AChE by spectrophotometric method proposed by Ellman et al. (1961). Among the broths tested, that from *Hypocrea lixii* presented the most promising pool of bioactive metabolites.

2.2 Phosphomolybdate assay for total antioxidant capacity

To determine total antioxidant capacity (TAC) of broths the method proposed by Umamaheswari and Chatterjee (2008) was used. L-Ascorbic acid was used as standard. A stock solution of ascorbic acid (500

µL/mL) was prepared in distilled water, from which dilutions were made ranging from 3 µg/mL to 20 µg/mL. In test tubes, 300 µL of broth were mixed with 3 mL of working reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were covered with aluminum foil and incubated for 90 min at 95° C. Subsequently, 200 µL of the contents of each tube were pipetted to 96 wells microplate for reading in a microplate reader (Multiscan GO, ThermoFisher), at 695 nm. Total antioxidant capacity was expressed in µg ascorbic acid equivalent (AAE) per mL of broth. The test was done in triplicate.

2.3 Acetylcholinesterase (AChE) inhibition assay

Ellman method (1961) was used to determine quantitatively the inhibition of AChE by the fungal broths. An amount of 25 µL of a standard solution of ATCI (15 mM); 125 µL of DTNB solution or Ellman's reagent (3 mM); 50 µL of solution Tris/HCl buffer (50 mM) pH 8.0 containing albumin bovin serum (0.1% w/v) and 25 µL of different broths containing fungal metabolites were added in microplate wells. The reaction of tiocolin with Ellman's reagent produces 5-thio-2-nitrobenzoate, a compound that can be detected and quantified at a wavelength of 406 nm. Absorbance of samples and controls was measured using a microplate reader spectrophotometer (Multiscan GO, ThermoFisher), and read every 60s, 8 times (totaling 8 minutes). After completion of this reading, were added 25 µL of solution containing the enzyme AChE (0.222 U/mL) and absorbance was measured again every 60 s, 8 times again. The enzyme used was dissolved in Tris/HCl buffer solution and eserine was prepared at a concentration of 1 mg/mL in dimethyl sulfoxide (DMSO). The test was done in quadruplicate. Inhibition percentages were calculated comparing the speeds of reaction promoted by sample with speed reaction of negative control of samples (distilled water) using the following calculation: % inhibition = 100 − (sample reaction speed/ negative control reaction speed × 100). The negative control used of eserine was DMSO. For results presentation, inhibition of the samples was calculated in percentages in relation to the activity of eserine (positive control) which was considered as 100%.

3. Results and Discussion

3.1 Total antioxidant activity

Total antioxidant activity of twenty-five 28 days-old liquid media (broths), determined by phosphomolybdate method, after static fermentation at 25 °C, are presented in Figure 1.

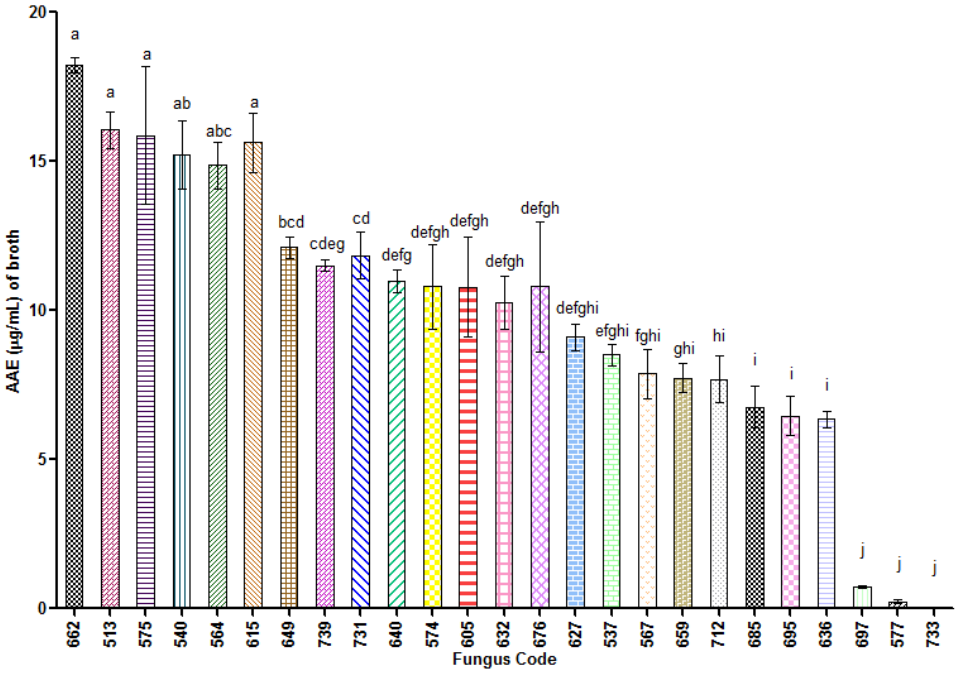

Figure 1: Total antioxidant activity (± standard deviation) in µg ascorbic acid equivalent (AAE)/mL of broth, of twenty-five broths of some filamentous fungi screened. Fungi are presented by different numerical codes. Columns with different letters are significantly different (ANOVA, Tukey test, $p < 0.05$).

The fermented broth by *Hypocrea lixii* (662) showed the highest activity, with 18.22 µg AAE/mL of broth, jointly with the fungi species codified as 513 (16.04 µg AAE/mL broth), 575 (15.86 µg AAE/mL broth), 615 (15.61 µg

AAE/mL broth), 540 (15.20 µg AAE /mL broth) and 564 (14.85 µg AAE/mL of broth). Antioxidant activity of fungal metabolites has been already reported in the literature. For instance, Smith et al. (2015) demonstrated that extracts of filamentous fungi *Pleurotus* spp. and *Monascus purpureus* obtained after submerged liquid fermentation, presented high antioxidant activity which could be explored in supplements, nutraceuticals, or as parcial substitutes of synthetic antioxidants, such as butylated hydroxytoluene (BHT). In the only study found in the literature that investigates the antioxidant potential of *Hypocrea lixii*, its extract presented high antioxidant activity (80.13%) in DPPH test, compared with the standard BHT (Bhimba et al., 2012). This positive result reported by Bhimba et al. (2012) for *H. lixii* extract by DPPH test (complementary to the methodology of total antioxidant capacity), confirms that this fungal species presents compounds with potent antioxidant activity. It is worth mentioning that since the material used in the present work was the broth and not the fungal extract, the secondary metabolites responsible for total antioxidant activity are diluted in relation to extract. For this reason, the activity obtained (18.22 µg AAE/mL) makes this fungal species very promising for several applications related to oxidative stress, either in foodstuff, cosmetics or as pharmaceuticals products.

3.2 Acetylcholinesterase (AChE) inhibition ability

Since there is a close correlation between antioxidant activity and acetylcholinesterase inhibition, the broths most active in antioxidant activity were screened for AChE inhibition. The results obtained were expressed in comparison to activity of eserine, a standard inhibitor used as positive control in this work, and are presented in Figure 2. The fungi were identified by numeric codes. *Hypocrea lixii*, numerical code 662, presented the highest AChE inhibitory activity (65.4%), jointly with fungi 552 (63.6%), 570 (61.8%), 550 (56.4%) and 660 (54.5%). The remaining broths showed activities below 50% such as 515 (49%), 741 (47.3%), 686 (36.4%), 742 (25.45%). The broth of fungus 569 was inactive (0%).

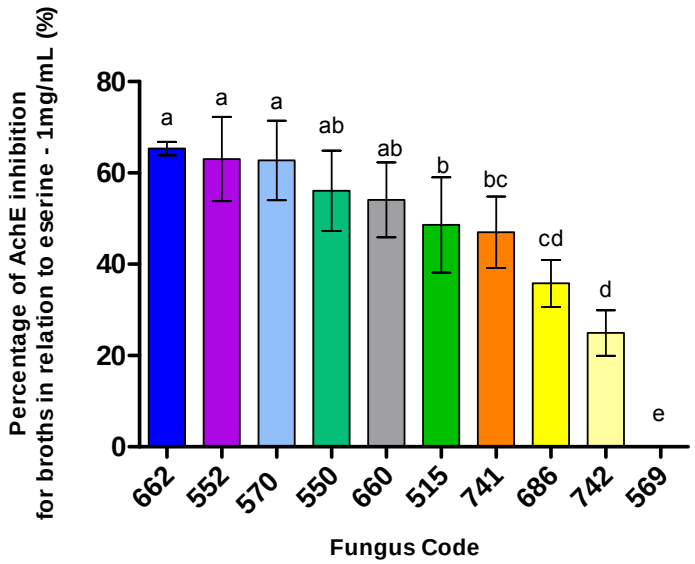

Figure 2: Percentage media (± standard deviation) of AChE inhibition for broths of filamentous fungi in relation to eserine (positive control). Fungi are presented by different numerical codes. Columns with different letters are significantly different (ANOVA, Tukey test, $p < 0.05$).

The better result of antioxidant and acetylcholinesterase screenings was for the same fungal species, *H. lixii*, a very interesting outcome since it is more usual to have different hits in different assays. The confluence of results is a good indicative of the promising role of this fungal species in the development of new anti-Alzheimer medicines.

There were not found previous studies on AChE inhibition ability of *H. lixii*, however the literature points that a fungus from the same genus, *Hypocrea vinosa*, is able to inhibit tyrosinase (Ohkawa et al., 2010).

The most frequent development of findings such as the one related herein is the isolation of the active metabolites aiming at new drug development. However, this process is also very time consuming and costly. Taking the example of medicinal plants, a faster way to take advantage of species such as *H. lixii* could be submit the broth to freeze-drying and use this material for *in vivo* assays, without the isolation of bioactive metabolites step. This might bring about new medicines or nutraceutics to help Alzheimer's patients to have better living conditions in the urgency that this kind of patients deserves.

A concern to implement the use of fungal whole extracts as medicines relies in the possible toxic effects of the extract components. However, good experiences have been reported, like *Agaricus blazei*, very commonly used in its natural form with different indications. For instance, powdered formulations of *A. blazei* were studied as a food supplement, presenting positive results, such as high antioxidant activity, high concentration of phenolic compounds and tocopherols, as well as presenting essential nutrients such as carbohydrates, proteins and unsaturated fatty acids (Carneiro et al., 2013). In addition, other study points out others biological activities for *A. blazei* cultivated under submerged fermentation, such as immunoactivity, antitumor activity and immunomodulatory capabilities (Wang et al., 2017).
Regarding specifically to *H. lixii*, it is known that the genus *Hypocrea* is the reproductive sexual form of Trichoderma, belonging both to the same organism, and *Trichoderma reesei* was considered a safe microorganism (Nevalainen et al., 1994). This can be considered a positive signal to encourage using *H. lixii* broth for therapeutic purposes.

4. Conclusions

Among the fungal studied, the fermented broth of *Hipocrea lixii* stood out as a very promising source of metabolites with antioxidant activity (18.22 µg AAE/mL broth). Literature previous study corroborate and broads the scope of biological potential of this fungal species. *H. lixii* broth also presented significant inhibition of AChE (65.4%), being the most active species in both screenings. Therefore, the fermented broth of *H. lixii* has an outstanding potential for further applications in prevention or treatment of Alzheimer's disease.

Acknowledgments

We acknowledge CAPES, CNPq, FAPEMIG, VALE and EMBRAPA for financial resources, grants and scholarships.

References

Abirami, A., Nagarani G., Siddhuraju P., 2014, *In vitro* antioxidant, anti-diabetic, cholinesterase and tyrosinase inhibitory potential of fresh juice from *Citrus hystrix* and *C. maxima* fruits, FSHW, 3, 16-25.

Abraham S., Basukriadi A., Pawiroharsono S., Sjamsuridzal W., 2015, Insecticidal Activity of Ethyl Acetate Extracts from Culture Filtrates of Mangrove Fungal Endophytes, Mycobiology, 43, 137-149.

Aly A.H., Debbab A., Proksch P., 2011, Fungal endophytes: unique plant inhabitants with great promises, Appl Microbiol Biotechnol, 90, 1829-1845.

Bhimba, B.V., Franco D.A.A.D., Mathew J.M., Jose G.M., Joel E.L., Thangaraj M., 2012, Anticancer and antimicrobial activity of mangrove derived fungi *Hypocrea lixii* VB1, CJNM, 10, 77-80.

Carneiro A.A.J., Ferreira I.C.F.R., Dueñas M., Barros L., Silva R., Gomes E., Santos-Buelga C., 2013, Chemical composition and antioxidant activity of dried powder formulations of *Agaricus blazei* and *Lentinus edodes*, Food Chem., 138, 2168-2173.

Dufossé L., Fouillaud M., Caro Y., Mapari S.A.S., Sutthiwong N., 2014, Filamentous fungi are large-scale producers of pigments and colorants for the food industry, Curr. Opin. Biotech. 26, 56-61.

Ellman G.L., Courtney K.D., Andres V., Featherstone R.M., 1961, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem Pharmacol.,7, 88-95.

Hajlaoui H., Mighri H., Aouni M., Gharsallah N., Kadri A., 2016, Chemical composition and *in vitro* evaluation of antioxidant, antimicrobial, cytotoxicity and anti-acetylcholinesterase properties of Tunisian *Origanum majorana L.* essential oil, Microb. Pathog., 95, 86-94.

Lakioti E.N., Moustakas K.G., Komilis D.P.,Domopoulou A.E., 2017, Karayannis V.G. Sustainable solid waste management: socio-economic considerations, Chemical Engineering Transactions, 56, 661-666, DOI:10.3303/CET1756111.

Li S., Tan H., Wang N., Zhang Z., Lao L., Wong C., Feng Y., 2015, The Role of Oxidative Stress and Antioxidants in Liver Diseases, Int. J. Mol. Sci., 16, 26087-26124.

Mendiola-Precoma J., Berumen L.C., Padilla K., Garcia-Alcocer G., 2016, Therapies for Prevention and Treatment of Alzheimer's Disease, Biomed. Res. Int., DOI: 10.1155/2016/2589276.

Nevalainen H., Suominen P., Taimisto K., 1994, On the safety of *Trichoderma reesei*, J. Biotech., 37, 193-200.

Nisa H., Kamili A.N., Nawchoo I.A., Shafi S., Shameem N., Bandh S.A., 2015, Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: A review, Microb. Pathog., 82, 50-59.

Ohkawa Y., Miki K., Suzuki T., Nishio K., Sugita T., Kinoshita K., Takahashi K., Koyama K., 2010, Antiangiogenic metabolites from a marine-derived fungus, *Hypocrea vinosa*, J. Nat. Prod., 73, 579-582.

Saravanakumar K., Vivek R., Boopathy N.S., Yaqian L., Kathiresan K., Chen J., 2015, Anticancer potential of bioactive 16-methylheptadecanoic acid methyl ester derived from marine *Trichoderma*, J. Appl. Biomed., 13, 199-212.

Smith H., Doyle S., Murphy R., 2015, Filamentous fungi as a source of natural antioxidants, Food Chem., 185, 389-397.

Suresha B.S., Srinivasan K., 2013, Antioxidant properties of fungal metabolite nigerloxin *in vitro*, Appl. Biochem. Microbiol., 49, 587-591.

Umamaheswari M.; Chatterjee T.K., 2008, *In vitro* antioxidant activities of the fractions of *Coccinia grandis L.* leaf extract, AJTCAM, 5, 61-73.

Wang H., Yang L., Yu H., Chen M., Wang H., Lu T., 2017, Characteristics of fucose-containing polysaccharides from submerged fermentation of *Agaricus blazei* Murill, J. Food Drug Anal., DOI:10.1016, http://dx.doi.org/10.1016/j.jfda.2017.07.006.

Zhang, H.Y., Zheng, C.Y., Wang, Z.F., Tang, L.L. Gao, X. Tang., X.C., 2008, Potential therapeutic targets of huperzine A for Alzheimer's disease and vascular dementia. Chem. Biol. Interact, 175, 396-402.

Zhao J., Wang W., Zhao C., Luo M., Mu F., Fu Y., Zu Y., Yao M., 2013, *Hypocrea lixii*, novel endophytic fungi producing anticancer agent cajanol, isolated from pigeon pea (Cajanus cajan [L.] Millsp.), J. Appl. Microbiol, 115, 102-113.