CHEMICAL ENGINEERING TRANSACTIONS
VOL. 75, 2019
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Sauro Pierucci, Laura Piazza

ISBN 978-88-95608-72-3; **ISSN** 2283-9216
DOI: 10.3303/CET1975056

Epigenetic-Induced Production of Promising Food Supplements Ingredients with Antioxidant and Anti-Acetylcholinesterase Activities

Laura C. B. Dominguete[a], Paulo V. M. Lima[b], Isabela V. Nunes[b], Denise Sande[c], Ubiraci G. de P. Lana[d], Ivanildo E. Marriel[d], Jacqueline A. Takahashi[b,*]

[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
[c]Centro Universitário de Belo Horizonte, Belo Horizonte, MG, Brazil
[d]Embrapa Milho e Sorgo, Sete Lagoas, MG, Brazil
jat@qui.ufmg.br

Fungal metabolites with antioxidant and acetylcholinesterase inhibitory activities can be developed as food supplements helpful for treatment of neurodegenerative diseases, like Alzheimer's disease. Fungal biosynthesis depends on the expression of several enzymes that can change chromatin's conformation, provoking activation or silencing of gene transcription. In this sense, the use of epigenetic modulators, able to alter chromatin's conformation, has been a modern technique used to induce the expression of silenced genes in fungi. In the present study the metabolic profile and the antioxidant and anti-acetylcholinesterase activities of the extracts of *Penicillium flavigenum* (PF) and *P. tanzanicum* (PT) grown in the presence of epigenetic modulators were analyzed. The fungi were grown in liquid media, with or without (control) the epigenetic modulators azacytidine (AZA), hydralazine (HYDRA), procainamide (PRO), suberoyl hydroxamic acid (SAHA) and sodium butyrate (BUT) (500 µM). The modulators changed the expression of exogenous metabolites produced by the fungi with overexpression of some compounds in comparison to the controls, according to the HPLC chromatographic profiles. The treatment PF-SAHA presented the higher content of total phenolic compounds (27.86 mg GAE/g) (together with PF-HYDRA and PF-BUT) and the higher total antioxidant content (25.70 mg AAE/g). Besides, PF-SAHA and PF-PRO extracts presented higher anti-acetylcholinesterase activity compared to others extracts. AZA was one of the most effective treatments for increasing expression of metabolites with antioxidant activity by *P. tanzanicum* in at least half of the assays. Treatments PT-AZA, PT-HYDRA and PT-BUT showed statistically similar anti-acetylcholinesterase activity, which were superior to the activity of the fungal control (15%). In overall, fermentative conditions using PF-SAHA and PF-PRO showed to be the most favorable to produce new bioactive metabolites to be used as food supplements for palliative treatment or prevention of Alzheimer's disease, while complete cure is not possible so far.

1. Introduction

Alzheimer's is the main type of dementia that afflicts the elderly, being characterized by cognitive decline and memory, affecting the basic skills of the individual (Alzheimer's Association, 2017). The current treatment of Alzheimer's disease relies in symptomatic targets and consists mainly in the use of inhibitors of acetylcholinesterase, enzyme that degrades the acetylcholine neurotransmitter, found in reduced quantity in patients with this pathology (Bahloul et al., 2016). Since high levels of oxidative stress are also observed in Alzheimer's patients, recent approaches have suggested the benefits of antioxidant substances in the treatment of Alzheimer's disease (Wang et al., 2016).
Fungi are a good source of natural food antioxidants with several advantages such as low cost of production (Alberti et al., 2017). In addition, the market of natural additives is growing due to the health hazards caused

Paper Received: 22 April 2018; Revised: 21 July 2018; Accepted: 17 November 2018

Please cite this article as: Dominguete L., Lima P., Nunes I., Sande D., Gomes de Paula Lana U., Marriel I., Takahashi J.A., 2019, Epigenetic-induced Production of Promising Food Supplements Ingredients with Antioxidant and Anti-acetylcholinesterase Activities, Chemical Engineering Transactions, 75, 331-336 DOI:10.3303/CET1975056

by certain synthetic substances (Hilares et al., 2018) and to the modern new health habits of a large part of the population (Vieito et al., 2018). Filamentous fungi produce antioxidants such as carotenoids, phenolic compounds and flavonoids (Tavares et al., 2018), as well as acetylcholinesterase inhibitors (Lima et al., 2018a). Nevertheless, many genes responsible for production of bioactive metabolites in fungi are silenced in laboratory conditions (Akone et al., 2016) which lacks of biotic environmental stress. A modern technique to overcome this problem is the introduction of epigenetic modulators, as the DNA methyltransferases (DNMT) and the histone-desacetilases (HDACs) in the culture medium to activate the expression and biosynthesis of natural products by fungi (VanderMolen et al., 2014). The aim of this work was to evaluate the modifications in the metabolic profiles and consequent biosynthesis of antioxidant and acetylcholinesterase inhibitors by *Penicillium flavigenum* and *P. tanzanicum* cultivated with epigenetic modulators.

2. Materials and Methods

2.1 Materials

Reagents utilized for bioassays and epigenetic modifyers 5-azacytidine (AZA), hydralazine (HYDRA), procainamide (PRO), suberoyl hydroxamic acid (SAHA) and sodium butyrate (BUT) were purchased from Sigma (St. Louis, USA), Neon (São Paulo, Brazil) and Dinâmica (Indaiatuba, Brasil). Culture media ingredients were obtained from Himedia (Mumbai, Índia), Spectrum Chemical (New Brunswick, EUA), Synth (São Paulo, Brazil) and Vetec (Rio de Janeiro, Brazil). Water purification was accomplished using a Milli-Q apparatus (Direct-Q Millipore). *Penicillium flavigenum* and *P. tanzanicum* were isolated from soil. They were stored at collection of the Biotechnology and Bioassays Laboratory (UFMG, Brazil). Their identification were made by molecular biology (GenBank Codes MH862182.1 and NR_158820.1, respectively).

2.2 Fungus cultivation and preparation of extracts

Culture medium (200 mL) containing (g/L) peptone (5), sucrose (30), potassium phosphate dibasic (1), sodium nitrate (2.5), magnesium sulfate heptahydrate (0.5), ferrous sulfate heptahydrate (0.001), potassium chloride (0.5) and cupric sulfate pentahydrate (0.005) was distributed in Erlenmeyer flasks and sterilized. Then the epigenetic modifyers were added to each flask (500 µM) followed by inoculation of a spore suspension of each fungal species. Three controls were prepared: (i) culture medium (blank), (ii) culture medium and epigenetic modifyer and (iii) culture medium plus fungus. Experiments were run in duplicate. After 28 days at 25 °C, broth was extracted with ethyl acetate (60 mL, three times). Organic fractions were pooled and solvent was completely removed obtaining the crude extracts.

2.3 Analysis by High Performance Liquid Chromatography (HPLC)

Extracts were profiled by HPLC using the conditions described in Table 1 on a Shimadzu 20A with two pumps LC-20AT, detector UV-vis SPD-20A and Kromasil C18 column 250 X 4.6 mm x 5 µm. For data acquisition was used a LC solution Software. Solutions of each extract were prepared at 1 mg / mL in methanol (HPLC grade), filtered with membranes 47 mm diameter and 0.45 µm pore size and injected into the equipment. The wavelengths selected for HPLC analysis were 340 nm (*P. flavigenum*) and 410 nm (*P. tanzanicum*).

Table 1: HPLC conditions for fungal extracts chromatographic profiling

	P. flavigenum			*P. tanzanicum*	
Time (min)	Formic acid (%)	Methanol (%)	Time (min)	Formic acid (%)	Methanol (%)
0.01	50	50	0.01	33	67
5.00	40	60	15.00	10	90
20.00	10	90	35.00	4	96
30.01	0	100	37.00	0	100
63.00	0	100	50.00	0	100

*aqueous solution 0.1% (v/v).

2.4 Antioxidant assays

Antioxidant activity was evaluated *in vitro* by different methodologies. Total antioxidant capacity (TAC) was performed according Umamaheswari and Chatterjee (2008) with adaptations using phosphomolybdate method in triplicate and L-ascorbic acid as standard. Activity was determined at 695 nm in spectrophotometer. TAC was expressed in mg ascorbic acid equivalent (AAE) per g of extract. Total phenolic compounds content was determined by Folin Ciocalteu method (Gao et al., 2000). After 20 min of incubation the readings were performed in spectrophotometer at 760 nm by interpolation of results from gallic acid analytical curve (positive standard) in methanol. Final results were expressed in mg of gallic acid equivalent (GAE) per g of extract.

Reducing power ability was also evaluated according to Umamaheswari and Chatterjee (2008), with ascorbic acid as positive standard. In this assay, absorbance reading was performed at 700 nm and the results were expressed as % of ferric reducing power (ascorbic acid was considered as 100%). Total flavonoid content was performed based in the method used by Azieana et al. (2017) in triplicate. Quercetin was used as the standard and absorbance readings were performed at 695 nm. The result was expressed in mg of quercetin equivalent (QE) per g of extract.

2.5 Acetylcholinesterase (AChE) inhibition assay

Ellman method (1961) with modifications was used to determine quantitatively the inhibition of AChE by the fungal extracts. Extract dissolved in methanol (25 µL) was mixed with acetylthiocholine iodide solution (25 µL), 5,5'-dithiobis (2-nitro-benzoic acid) solution (125 µL) and Tris/HCl buffer pH 8.0 containing albumin bovin serum (50 µL). Absorbance of samples and controls was measured on a microplate reader spectrophotometer (Multiscan GO, ThermoFisher) at a wavelength of 406 nm and read every 1 min for 8 times. After this, the solution containing the enzyme AChE (25 µL) was added and absorbance was measured again every 1 min for 10 times. The enzyme used was dissolved in Tris/HCl buffer solution and eserine was prepared (1 mg/mL in dimethyl sulfoxide, DMSO). The test was done in quintuplicate and inhibition percentages were obtained using the equation: % inhibition = 100 − (sample reaction speed/negative control reaction speed × 100). DMSO was used as negative control.

2.6 Statistical analysis

The data were statistically analyzed by Analysis of Variance (ANOVA) and Tukey's test to compare the means of each test performed. Program used was GraphPad Prism, Version 5.

3. Results and Discussion

3.1 Extracts profiles by High Performance Liquid Chromatography (HPLC)

In general, the modulators influenced the expression of secondary metabolites by the fungi in different ways. For *P. flavigenum*, PRO and AZA increased the expression of metabolites with retention time (Rt) 49 min, while HYDRA affected the substance with Rt 37 min and SAHA those with Rt 8 and 49 min. SAHA also induced production of new metabolites (absent in basal metabolism) with Rt 10 and 10.5 min (Figure 1A). On the other hand, BUT did not cause significant change in the HPLC profile. The results indicate that most of the modulators activated and/or stimulated the expression of genes responsible for secondary metabolites production. The capacity to activate genes has also been reported previously for SAHA towards *Gibellula formosana* (Asai et al., 2012) and other fungal species (VanderMolen et al., 2014).
Regarding *P. tanzanicum* was observed an enhanced expression of metabolites in the treatments with SAHA (Rt 6, 10 and 18 min), AZA (Rt 10 and 19 min), HYDRA (Rt 6, 10 and 16.5 min), PRO (Rt 10 and 16.5 min) and also BUT (15, 18 and 19 min) (Figure 1B).

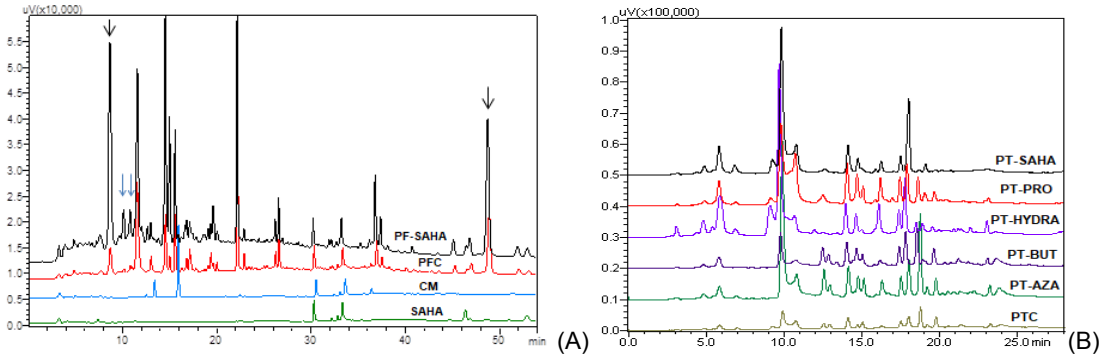

Figure 1: (A) HPLC profiles of metabolites produced by P. flavigenum (PF) under epigenetic modulation with SAHA (black), in comparison with control fungal metabolites (red). Culture medium (blue) and SAHA (green) profiles are shown for comparison purposes. (B) HPLC profiles of metabolites produced by P. tanzanicum (PT) under epigenetic modulation with SAHA, PRO, HYDRA, BUT and AZA, in comparison with fungal metabolites produced under basal conditions without epigenetic modulation (PTC).

3.2 Antioxidant activity

In general, results showed positive influence of the epigenetic modulators in the production of antioxidant metabolites by both fungal species. SAHA, HYDRA and BUT enhanced the production of phenolic compounds (statistically comparable effects) in relation to the control (grown without epigenetic modifier) of *P. flavigenum*. When grown under modulation with SAHA, *P. flavigenum* also produced the pool of metabolites with the higher total antioxidant activity (25.70 mg EAA/g) while metabolites expressed in the presence of PRO presented higher reducing ferric activity (29.64%) and higher level of flavonoids (236 mg EQ/g) (Table 2). Other works have demonstrated the ability of this fungal species to produce antioxidant compounds, such as the *P. flavigenum* (CML3827) with 18.8 mg GAE/g of extract of total phenolic content (Tavares et al., 2018) and the *P. flavigenum* (CML2965) producing a polyketide with antioxidant activity (Souza et al., 2016).

Table 2: Antioxidant activity determined for extracts of P. flavigenum (PF) under epigenetic modulation (AZA, BUT, HYDRA, PRO, SAHA) in comparison to the extract control (PFC). Data in the same line followed by different letters are statistically different according to ANOVA and Tukey's test (p < 0.05).

	PF-AZA	PF-BUT	PF-HYDRA	PF-PRO	PF-SAHA	PFC
Total Phenolic Compounds (mg GAE/g)	18.23 ± 2.33 **b**	33.37 ± 3.38 **a**	27.75 ± 2.59 **a**	13.88 ± 2.15 **b**	27.86 ± 7.27 **a**	15.94 ± 0.83 **b**
Total Antioxidant Activity (mg AAE/g)	19.25 ± 1.28 **bc**	13.45 ± 1.48 **d**	16.58 ± 0.88 **cd**	20.69 ± 2.18 **b**	25.70 ± 1.74 **a**	19.97 ± 1.34 **bc**
Reducing Ferric Power (%)	13.08 ± 1.11 **b**	15.08 ± 2.09 **ab**	14.25 ± 5.98 **b**	29.64 ± 13.16 **a**	11.92 ± 1.96 **b**	8.02 ± 1.47 **b**
Total Flavonoids (mg QE/g)	171.97± 2.50 **d**	205.14 ± 2.93 **b**	200.91 ± 1.71 **b**	236.36 ± 6.95 **a**	182.21± 3.03 **c**	134.33 ± 2.85 **e**

P. tanzanicum cultivated under the effect of the modulator AZA showed the highest total content of phenolic compounds (30.96 mg EAG/g), while PRO and HYDRA generated the extracts with the higher total antioxidant activities (69.23 and 52.08 mg EAA/g, respectively). As for ferric reducing power and total flavonoids content, SAHA showed to be the most effective modulator (23.24% and 252.33 mg QE/g, respectively) (Table 3).
It is interesting to point that, in all treatments, total flavonoid levels were higher than in the controls, except for *P. tanzanicum* treated with PRO. Therefore, epigenetic modifyers showed their role in activating genes responsible by flavonoids biosynthesis.

Table 3: Antioxidant activity determined for extracts of P. tanzanicum (PT) under epigenetic modulation (AZA, BUT, HYDRA, PRO, SAHA) in comparison to the extract control (PTC). Data in the same line followed by different letters are statistically different according to ANOVA and Tukey's test (p < 0.05).

	PT-AZA	PT-BUT	PT-HYDRA	PT-PRO	PT-SAHA	PTC
Total Phenolic Compounds (mg GAE/g)	30.96 ± 1.45 **a**	15.96 ± 0.82 **c**	16.88 ± 1.77 **c**	16.69 ± 1.56 **c**	8.92 ± 1.84 **d**	20.73 ± 1.15 **b**
Total Antioxidant Activity (mg AAE/g)	28.71 ± 2.01 **c**	18.66 ± 1.46 **d**	52.08 ± 3.81 **b**	69.23 ± 8.12 **a**	25.97 ± 2.87 **cd**	29.51 ± 1.55 **c**
Reducing Ferric Power (%)	13.67 ± 1.67 **b**	12.01 ± 1.47 **b**	13.85 ± 0.70 **b**	11.40 ± 1.18 **b**	23.24 ± 3.33 **a**	9.26 ± 2.78 **b**
Total Flavonoids (mg QE/g)	209.00 ±1.58 **bc**	215.23 ± 1.94 **b**	198.47± 9.75 **c**	181.72 ± 4.23 **d**	252.33 ± 4.64 **a**	176.81 ± 2.24 **d**

3.3 Inhibition of acetylcholinesterase

The effect in acetylcholinesterase inhibition for all extracts resulting from treatments of *P. flavigenum* and *P. tanzanicum* are presented in Figure 2 (A and B, respectively). The presence of SAHA and PRO led *P. flavigenum* to produce metabolites more active (31% and 30%, respectively), being statistically equal to each other and more active than the control extract (PFC, 12%). On the other hand, the treatments AZA, HYDRA and BUT for *P. tanzanicum* were more efficient in increasing the biosynthesis of acetylcholinesterase inhibitors (30%, 25%, 24%, respectively), being statistically equal to each other and more active than the others, including fungal control (PTC, 13%).
Observing the effect of the epigenetic modulators in the secondary metabolism of both fungal species, both types, DNA-methyltransferase inhibitors and inhibitors of histone desacetilases influenced the production of

acetylcholinesterase inhibitors in different levels. It is described in the literature the positive synergism when fungi are fed with two epigenetic modulators simultaneously (SAHA and PRO) (Lima et al., 2018b).

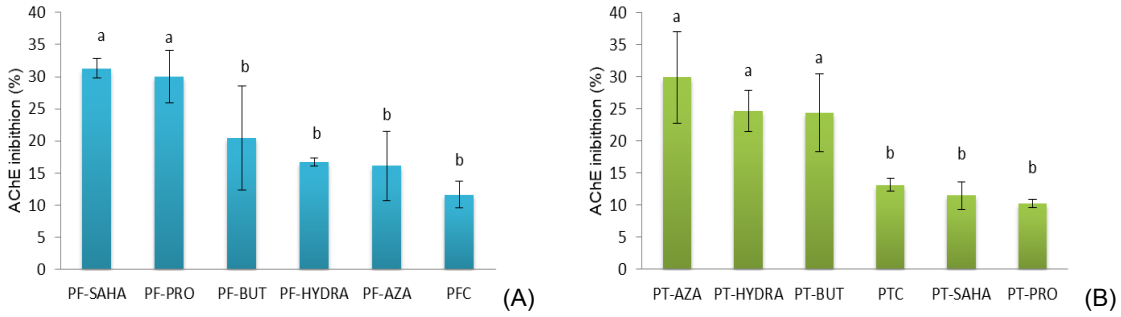

Figure 2: Percentage of acetylcholinesterase inhibition obtained for extracts of P. flavigenum (A) and P. tanzanicum (B). PF and PT refers to P. flavigenum and P. tanzanicum respectively, which are followed the letters of their respective treatments C (control, that means extract produced without epigenetic modulation), AZA (5-azacitidin), BUT (sodium butirate), HYDRA (hydralazine), PRO (procainamide), and SAHA (suberoyl hydroxamic acid). Different letters mean significantly different values (ANOVA, Tukey test, $p < 0.05$).

4. Conclusions

The growth of *P. flavigenum* and *P. tanzanicum* in the presence of epigenetic modulators was successful to produce pools of secondary metabolites with antioxidant and inhibitor of acetylcholinesterase, either by increasing the amount of bioactive compounds already produced or by inducing the expression of metabolites silenced at basal conditions. In general, this approach was very successful to increase the production of flavonoids being an interesting technique for industrial production of antioxidants by fungi. The experiments point that the metabolites produced by *P. flavigenum* in the presence of SAHA and PRO have the higher potential for the treatment of Alzheimer's disease.

Acknowledgments

We acknowledge CAPES, CNPq, FAPEMIG, VALE, EMBRAPA and National Institute of Science and Technology - INCT BioNat, grant # 465637/2014-0, Brazil for grants and scholarships. JAT acknowledges CNPq Fellowship 304922/2018-8 and FAPEMIG for financial help (Process APQ-02604-16).

References

Akone S.H., Mándi A., Kurtán T., Hartmann R., Lin W., Daletos G., Proksch P., 2016, Inducing secondary metabolite production by the endophytic fungus *Chaetomium* sp. through fungal-bacterial co-culture and epigenetic modification, Tetrahedron, 72, 6340–6347.

Alberti F., Foster G.D., Bailey A.M., 2017, Natural products from filamentous fungi and production by heterologous expression, Applied Microbiology and Biotechnology, 101, 493–500.

Alzheimer's Association, 2017, 2017 Alzheimer's disease facts and figures, Alzheimer's & Dementia, 13, 325–373.

Asai T., Chung Y.-M., Sakurai H., Ozeki T., Chang F.-R., Wu Y.-C., Yamashita, K., Oshima, Y., 2012, Highly oxidized ergosterols and isariotin analogs from an entomopathogenic fungus, *Gibellula formosana*, cultivated in the presence of epigenetic modifying agents, Tetrahedron, 68, 5817–5823.

Azieana J., Zainon M.N., Noriham A., Rohana M.N., 2017, Total Phenolic and Flavonoid Content and Antioxidant Activities of Ten Malaysian Wild Mushrooms, Open Access Library Journal, 4, 1–9.

Bahloul N., Bellili S., Aazza S., Chérif A., Faleiro M.L., Antunes M.D., Miguel M.G., Mnif W., 2016, Aqueous extracts from tunisian diplotaxis: phenol content, antioxidant and anti-acetylcholinesterase activities, and impact of exposure to simulated gastrointestinal fluids, Antioxidants, 5, 1–15.

Ellman G.L., Courtney K.D., Andres V., Featherstone R.M., 1961, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochemical Pharmacology, 7, 88–90.

Gao X., Ohlander M., Jeppsson N., Björk L., Trajkovski V., 2000, Changes in antioxidant effects and their relationship to phytonutrients in fruits of sea buckthorn (*Hippophae rhamnoides* L.) during maturation, Journal of Agricultural and Food Chemistry, 48, 1485–1490.

Hilares R.T., Souza R.A., Marcelino P.F., Da Silva S.S., Dragone G., Mussatto S.I., Santos J.C., 2018, Sugarcane bagasse hydrolysate as a potential feedstock for red pigment production by *Monascus ruber*, Food Chemistry, 245, 786–791.

Lima G.D.S., Andrade G.F., Da Silva M.A.N., De Sousa E.M.B., Takahashi J.A., 2018a, Novel kojic acid-based functionalized silica nanoparticles for tyrosinase and ache inhibition and antimicrobial applications, Chemical Engineering Transactions, 64, 175–180. DOI: 10.3303/CET1864030.

Lima M.T.N.S., dos Santos L.B., Bastos R.W., Nicoli, J.R., Takahashi J.A., 2018b, Antimicrobial activity and acetylcholinesterase inhibition by extracts from chromatin modulated fungi, Brazilian Journal of Microbiology, 49, 169–176.

Souza P.N. da C., Grigoletto T.L.B., de Moraes, L.A.B., Abreu L.M., Guimarães L.H.S., Santos C., Galvão L.R., Cardoso P.G., 2016, Production and chemical characterization of pigments in filamentous fungi, Microbiology, 162, 12–22.

Tavares D.G., Barbosa B.V.L., Ferreira R.L., Duarte W.F., Cardoso P.G., 2018, Antioxidant activity and phenolic compounds of the extract from pigment-producing fungi isolated from Brazilian caves, Biocatalysis and Agricultural Biotechnology, 16, 148–154.

Umamaheswari M., Chatterjee T.K., 2008, *In vitro* antioxidant activities of the fractions of *Coccinia grandis* L. leaf extract, African Journal of Traditional, Complementary and Alternative Medicines, 5, 61–73.

VanderMolen K.M., Darveaux B.A., Chen W.L., Swanson S.M., Pearce C.J., Oberlies N.H., 2014, Epigenetic manipulation of a filamentous fungus by the proteasome-inhibitor Bortezomib induces the production of an additional secondary metabolite, RSC Advances, 4, 18329–18335.

Vieito C., Fernandes É., Vaz Velho M., Pires P., 2018, The effect of different solvents on extraction yield, total phenolic content and antioxidant activity of extracts from pine bark (*Pinus pinaster* subsp. *atlantica*), Chemical Engineering Transactions, 64, 127–132. DOI: 10.3303/CET1864022.

Wang Y., Wang H., Chen H.Z., 2016, AChE inhibition-based multi-target-directed ligands, a novel pharmacological approach for the symptomatic and disease-modifying therapy of Alzheimer's disease, Current Neuropharmacology, 14, 364–375.