International Journal of Engineering Research and Technology.
ISSN 0974-3154 Volume 11, Number 12 (2018), pp. 2089-2098

http://www.irphouse.com

Finding the Novel Effect of the Asiatic Acid as a Potential Anti-neuroinflammatory Agent Using Microglia Cell System and In Silico Molecular Docking Technology

Dong-Chan Kim[1,*]

[1]Department of Biomedical Laboratory Science,
Gimcheon University, Gimcheon City, 39528, South Korea.

ORCID:0000-0002-9361-8023
**Corresponding Author*

ABSTRACT

In present study is focusing on the investigation about the anti-neuroinflammatory effect of asiatic acid, a well known natural flavonoid, to the iNOS(inducible nitric oxide synthase)-mediated nitric oxide production in BV2 microglia and to find its biochemical interacting mechanism on iNOS protein using computational docking technology. For this, asiatic acid, a major active ingredient of the *centella asiatica* (*Umbelliferae*), were used as ligand for molecular interaction. The 3D crystallographic structure of molecular target iNOS was obtained from PDB database (PDB ID: 1M9T). Tetrahydrobiopterin, a iNOS protein ligand was taken as the standard for comparative docking analysis. Asiatic acid showed maximum binding affinity with a molecular target iNOS with the binding energy of −8.90 kcal/mol as compared to the tetrahydrobiopterin (−7.00 kcal/mol). In the cell biological assay study, asiatic acid significantly reduced iNOS-mediated nitric oxide production in BV2 microglia. These results strongly indicated that asiatic acid could be one of the potential drug candidate to protect iNOS-mediated neuroinflammation and brain diseases.

INTRODUCTION

Asiatic acid has been implicated in neuroprotection from neurodegenerative diseases both in vitro and in vivo[1]. In primary neurons, asiatic acid reduced cell death and apoptosis in a concentration-dependent manner. In addition, asiatic acid also displayed the capacity for inducing nerve differentiation[2]. These results suggest that asiatic acid could serve as a potential therapy for neurodegenerative diseases. However, the role of asiatic acid in neuroinflammation and the mechanism of neuroprotection mediated by this compound still remain to be elucidated.
Recent pharamcological approach using computer aided drug discovery (CADD) have become very important resource to identify the potential drug candidates for various kinds of brain diseases[3]. In silico drug screening technology offers the proper advantage of identifying lead compounds from several potentially useful hit compounds. In silico molecular docking technology is a tool in structural molecular biology and structure-based drug discovery and fast methods to do so[4,5]. Besides, the purpose of ligand-protein docking is to predict molecular recognition, binding modes and predicting binding affinity(kcal/mol)[6]. Scientists in the pharmacology field have employed the new drug development technologies to determine successfully useful potential binding sites and used the experimental results to indentify, improve and develop drugs that fit better into the binding pocket (active site) in the target proteins[5].

Nitric Oxide (NO) is produced from L-arginine in microglia by iNOS enzymes. iNOS is induced by microbial activating products, such as lipopolysaccharide (LPS) and inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ)[7]. NO production is enhanced in respond to LPS stimuli and mediates the neuroinflammation. Because of the importance of NO derived from iNOS in inflammatory response, there were several research efforts to find a selective iNOS blocker[8,9,10]. Thus, the compounds down-regulating the activity of iNOS are suggested to be potential as anti-neuroinflammatory agents.

Asiatic acid (Fig. 1b) is a well known natural antioxidant. It is found in *centella asiatica* (*Umbelliferae*), and previous study proved its potential therapeutic qualities and anti-oxidation effects[11]. But, the biochemical molecular interacting mechanism about the anti-neuroinflammtory effect of asiatic acid on the iNOS protein has not been well studied. Using in silico molecular docking technique and BV2 microglia cell based assay system, we investigated the potential effect of asiatic acid as a novel regulator for the iNOS-mediated nitric oxide production in microglia. In this study, the structural 3D model of the asiatic acid on the iNOS protein active site has been performed, which may expedite further development of more natural iNOS-protectors to control activated microglia-induced neuronal diseases.

MATERIALS AND METHODS

Cell culture

The immortalized murine BV-2 cell line of reactive microglia cells[12] were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Gibco, USA), streptomycin, and penicillin as described previously[13]. Under a humidified 5% CO_2/95% air atmosphere and at 37°C, cells were split twice a week and plated in 10 cm^2 Petri dishes (Corning, Acton, MA, USA) at a density of 5 × 10^5 cells for BV-2 cell line. For the experiments, cells were plated on 6-well dishes (2 × 10^6 cells/well).

iNOS-mediated NO(nitric oxide) production assay

To stimulate TLR-4, BV-2 cells were washed with phosphate-buffered saline (PBS) twice, replenished with a serum-free DMEM (Gibco, USA) and LPS (Sigma, St. Louis, MO) was added to the culture medium. Asiatic acid (product # : 546712, Sigma, St. Louis, MO), were added to cells 10 min before LPS (100 ng/ml) treatment. NO produced by the BV-2 cells was determined by assaying the levels of Nitrite using the Griess reagent (Sigma, St. Louis, MO). The significance level of the asiatic acid effect were set at $*p \leq 0.05$ versus LPS alone. All data were represented as the means ± SEM (Standard Error of the Means).

Molecular docking analysis

The three-dimensional structure of iNOS protein (PDB ID: 1M9T)(Fig. 1c) was downloaded from the RCSB protein Data Bank. The chemical structure of tetrahydrobiopterin(Pubchem CID=1125)(Fig. 1a) and asiatic acid (Pubchem CID=119034)(Fig. 1b) were obtained from PubChem compound database. It was prepared by ChemBioDraw and MOL SDF format of this ligand was converted to PDBQT file using PyRx tool[14] to generate atomic coordinates. For docking analysis, PDB coordinates of the target protein, tetrahydrobiopterin, and asiatic acid molecule were optimized by Discovery Studio version 4.5 software[15]. These coordinates had minimum energy and stable conformation. The active sites are the coordinates of the ligand in the original target protein grids, and these active binding sites of target protein were analyzed also using the Discovery Studio version 4.5 and NX-QuickPharm program(Neuronex Inc., South Korea)[16]. A computational ligand-target docking approach was used to analyze structural complexes of the iNOS(target) with tetrahydrobiopterin and asiatic acid (ligand) in order to understand the structural basis of this protein target specificity. Docking was carried out by PyRx, AutoDock Vina, and NX-QuickPharm option based on scoring functions. The energy of interaction of tetrahydrobiopterin and asiatic acid with the iNOS protein is assigned "grid point." At each step of the simulation, the energy of interaction of ligand and protein was evaluated using atomic affinity potentials computed on a grid. The significance level of the asiatic acid binding affinity on the iNOS was set at *, $p \leq 0.05$ versus tetrahydrobiopterin. All data were represented as the means ± SEM (Standard Error of the Means).

Figure 1

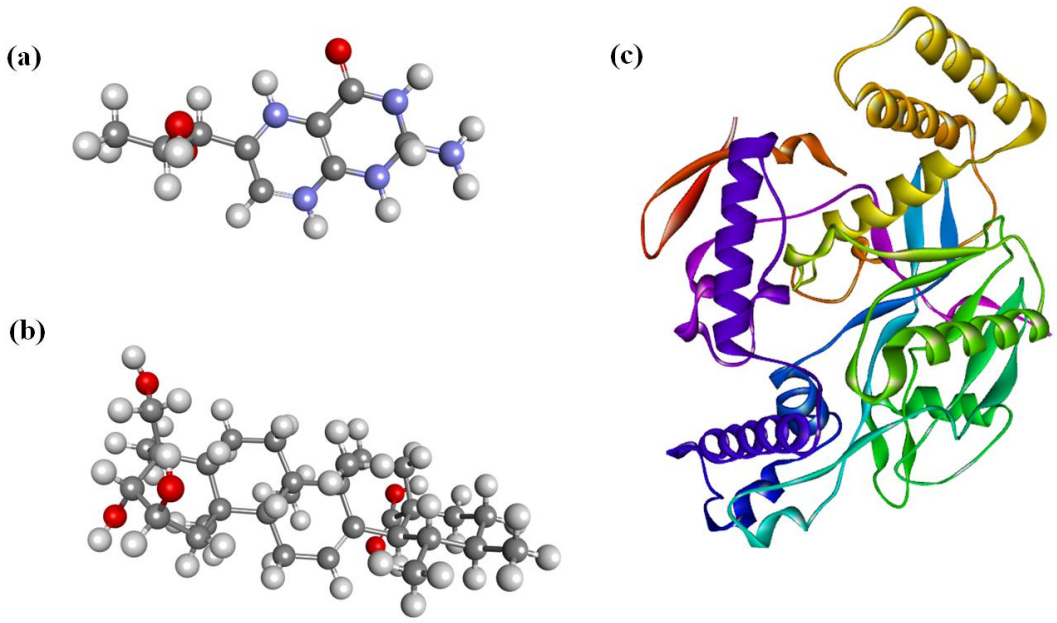

Figure 1. Ball and stick chemical structure of the (a) tetrahydrobiopterin and (b) asiatic acid. Three-dimensional (3D) structure of the iNOS protein A domain (PDB ID = 1M9T)

RESULTS

Asiatic acid binds in high affinity to the iNOS active site

PyRx Autodock 4 docking analysis was applied to investigate the molecular binding interactions of asiatic acid and tetrahydrobiopterin molecules, respectively with iNOS protein (Fig. 2a) and to elucidate the possible molecular mechanism. As shown in Fig. 2c and Fig. 3b, asiatic acid interacted with 7 amino acid residues (TRP188, CYS194, PRO344, VAL346, PHE363, TRP366, TRP457) and tetrahydrobiopterin interacted with 6 amino acid residues (LYS248, GLY247, GLN304, GLY307, HIS493, ILE494)(Fig. 2b and Fig. 3a). The average molecular binding affinity (docking energy) scores of the tetrahydrobiopterin and asiatic acid on the iNOS target were -6.61 kcal/mol (tetrahydrobiopterin, SEM ±0.23) and -8.10 kcal/mol (asiatic acid, SEM ±0.42) (Table 1). The numbers of the binding modes of both ligands to the iNOS active site were nine (n=9), respectively. Based on the present molecular docking data, asiatic acid appeared as a strong binder to the iNOS target protein than the tetrahydrobiopterin.

Figure 2

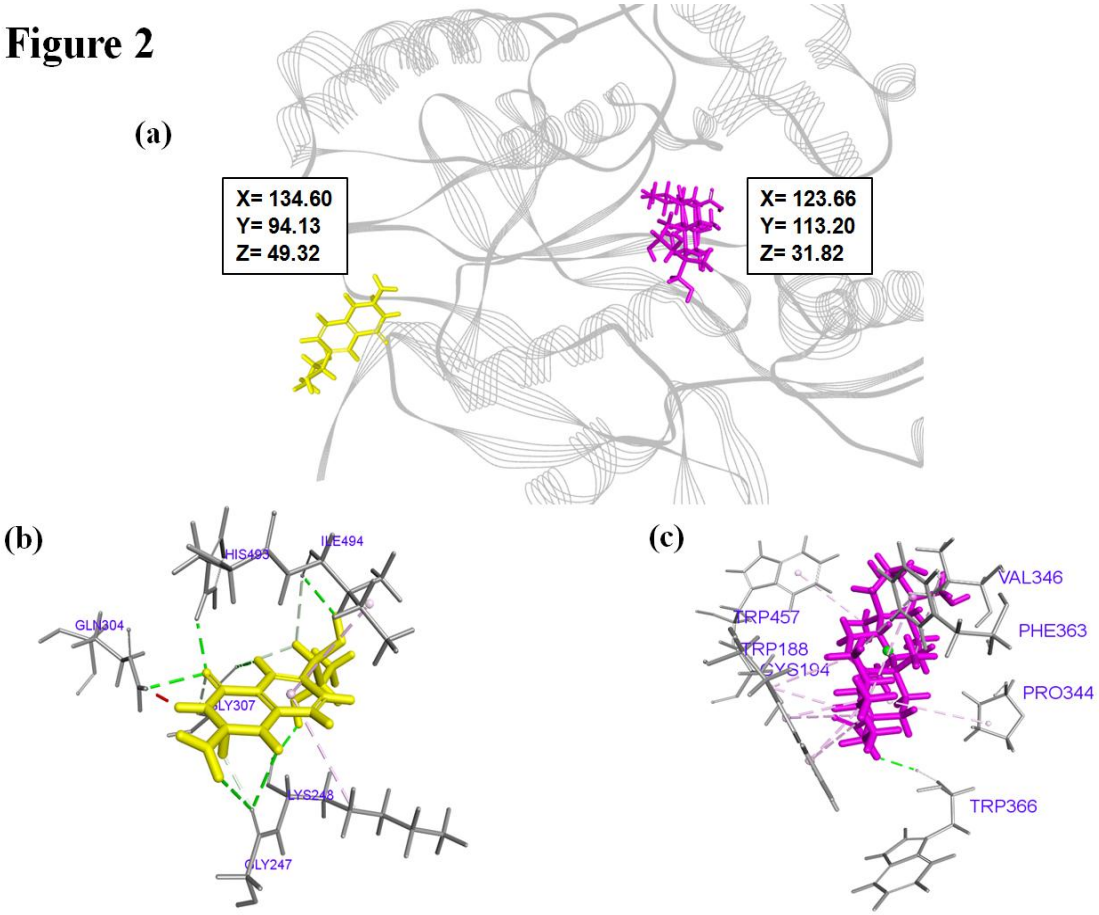

Figure 2. (a) 3D molecular docking pattern and binding position of the tetrahydrobiopterin (yellow color stick) and the asiatic acid (purple color stick) on the iNOS protein (grey color ribbon) active site. (b) 3D pattern of the interacting amino acids with tetrahydrobiopterin in the iNOS active site. (c) 3D pattern of the interacting amino acids with asiatic acid in the iNOS active site.

Figure 3

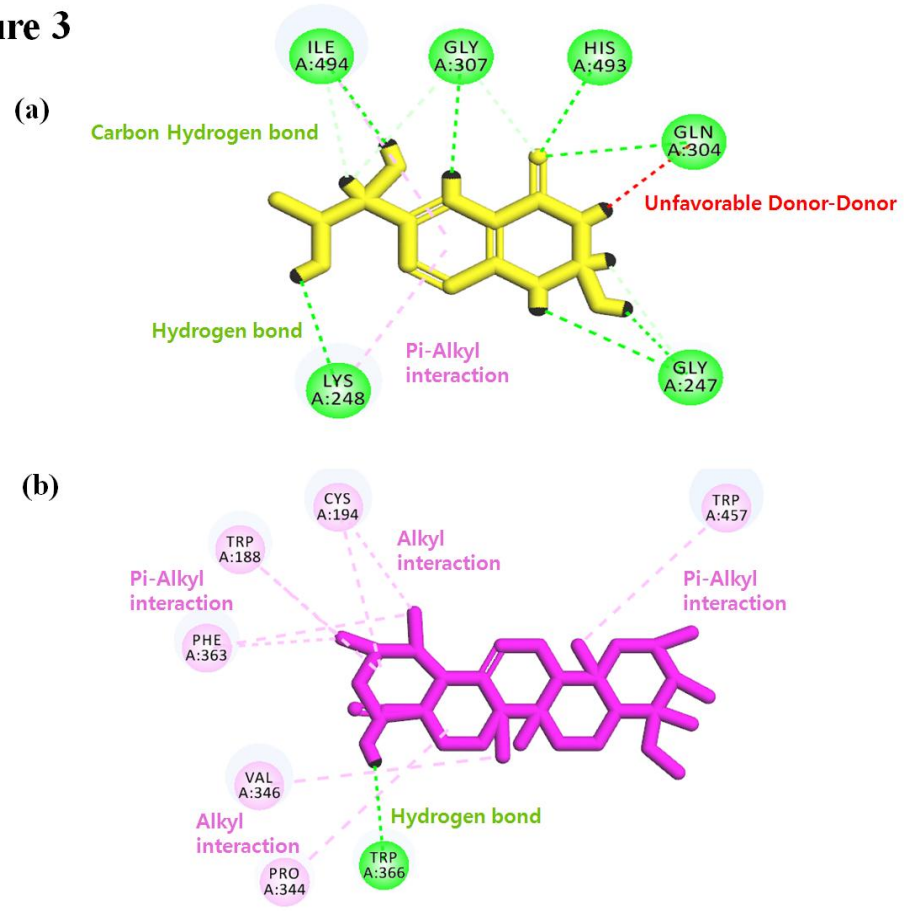

Figure 3. (a) The name of the chemical bonds (or interactions) and 2D pattern of the interacting amino acids with tetrahydrobiopterin in the iNOS active site. (c) The name of the chemical bonds (or interactions) and 2D pattern of the interacting amino acids with asiatic acid in the iNOS active site.

Table 1. Binding affinity (Docking Energy) of the tetrahydrobiopterin and asiatic acid to the iNOS protein active site.

	Max Binding Affinity (kcal/mol)	Average Binding Affinity (kcal/mol)	SEM (n = # of binding mode)
tetrahydrobiopterin on the iNOS protein	-7.00	-6.61	±0.23 (n=9)
asiatic acid on the iNOS protein	-8.90	-8.10*	±0.42 (n=9)

Effect of asiatic acid on the iNOS-mediated NO production on the BV2 microglia

The microbial neurotoxin LPS is the well-known target of innate recognition and induces a robust neuroinflammatory response by microglial cells and induces stimulation of the NF-κB signaling pathways and NO synthesis. To elucidate the effect of asiatic acid on the iNOS-mediated microglial activation, BV-2 cells were incubated with LPS in the absence or presence of asiatic acid (Fig. 4). Asiatic acid significantly reduced LPS-induced NO production in a concentration-dependent manner (Fig. 4), indicating that asiatic acid effectively affects TLR-4 and iNOS cell signaling.

Figure 4

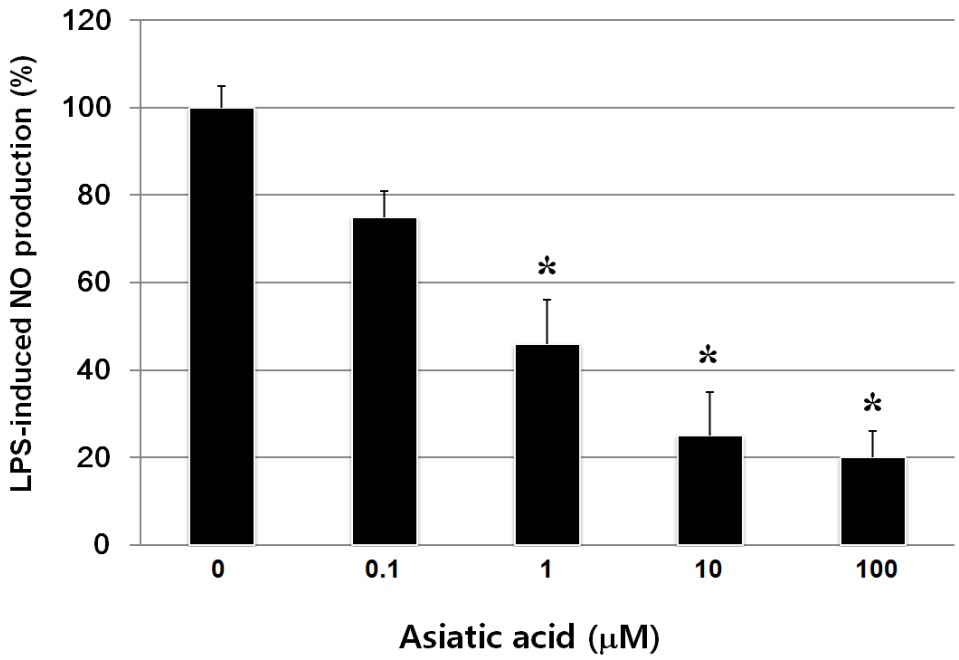

Figure 4. Effects of asiatic acid on the NO production induced by LPS in BV-2 microglial cells. Data represent means ± SEM of three independent experiments. Significant differences between LPS and asiatic acid + LPS are presented. *$p < 0.05$.

DISCUSSION

BV2 cells retain most of their morphological, phenotypical and functional properties described for primary microglial cells, which make them a useful in vitro model to search for anti-neuroinflammatory natural products and to investigate mechanisms of microglial activation. Thus BV2 cells were used to evaluate the role of asiatic acid in

LPS-stimulated neuroinflammation. Microglial activation results in the generation of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α 25, and the consecutive over-production of NO regulated by iNOS expression has been implicated in the development of inflammatory diseases. Microglia act as resident brain macrophages that become inflammatory activated in most brain pathologies[17]. In normal state, microglia protect neurons, but may accidentally kill neurons when attempting to limit infections or damage, and this may be more common with degenerative disease as there was no significant selection pressure on the aged brain in the past[17]. Neuroinflammation is one of the main mechanisms involved in the progression of dangerous neurodegenerative diseases, such as Alzheimer, Parkinson, multiple sclerosis, amyotrophic lateral sclerosis and others[18]. Natural herb derived flavonoids, possess neuroprotective potential probably related to their ability to regulate the neuroinflammatory responses involved in neurodegenerative diseases[19,20]. Asiatic acid (or asiatic acid enriched herb extracts) can reduce the pro-inflammatory cytokine(COX-2 and IL-6) expressions, down-regulate inflammatory inducers and prevent brain damage[21,22]. The aim of the present study was to evaluate the effect of asiatic acid, one of the most abundant flavonoids in herbs and fruits, on iNOS-mediated NO production in BV2 microglia. Several previous studies have revealed that anti-neuroinflammatory effects of natural flavonoids in neurodegenerative disorders[18,23,24]. It was noteworthy that asiatic acid pretreatment significantly protected LPS-TLR4-iNOS-signal pathway mediated neuroinflammation in microglia cell system(Fig. 4). In accordance with the previous reports[25,26], our data also demonstrated that asiatic acid effectively protected iNOS-mediated nitric oxide production and neuroinflammation (Fig. 4).

Asiatic acid has a polyphenol structure(fig. 1b) favorable to bind with center position of the iNOS protein (Fig 3b and Table 1). The computational docking of iNOS target with asiatic acid using auto docking procedure revealed that all the lowest energy complexes of iNOS are stabilized by intermolecular Pi-Alkyl / Alkyl interactions than tetrahydrobiopterin does(Fig. 3b). The calculated final docked maximum binding affinity of asiatic acid to iNOS protein is −8.90 kcal/mol and tetrahydrobiopterin (standard) is −7.00 kcal/mol (Table 2). Therefore, docking results revealed that this asiatic acid compound can enter the substrate-binding region of the iNOS active site and interacts effectively than tetrahydrobiopterin. The interacting numbers of the chemical bonds and binding patterns of the ligand among the iNOS amino acids might be critical factors for regulating target iNOS protein activity.

CONCLUSION

These results demonstrated clearly that asiatic acid accurately and effectively interact with iNOS protein target. Therefore, asiatic acid might play an important role in inhibiting iNOS-mediated nitric oxide production and neuroinflammation. These data also suggest that computer aided drug design process using PyRx, Discovery Studio 4.5, and NX-QuickPharm tools is highly reliable and can be a good example for indentifying the action mechanism between the iNOS and its interacting ligands. In

conclusion, asiatic acid can play an important role in altering the progression of neuroinflammatory diseases by its protective effect against iNOS-mediated oxidative brain stress.

ACKNOWLEDGEMENT

This research was supported by Gimcheon university research fund, (gc-17007).

REFERENCES

[1] M. Ahmad Rather, A. Justin Thenmozhi, T. Manivasagam, J. Nataraj, M.M. Essa, S.B. Chidambaram, Asiatic acid nullified aluminium toxicity in in vitro model of Alzheimer's disease, Front Biosci (Elite Ed) 10 (2018) 287-299.

[2] H. Jiang, G. Zheng, J. Lv, H. Chen, J. Lin, Y. Li, G. Fan, X. Ding, Identification of Centella asiatica's Effective Ingredients for Inducing the Neuronal Differentiation, Evid Based Complement Alternat Med 2016 (2016) 9634750.

[3] D. Menshykau, Emerging technologies for prediction of drug candidate efficacy in the preclinical pipeline, Drug Discov Today 22 (2017) 1598-1603.

[4] S.P. Leelananda, S. Lindert, Computational methods in drug discovery, Beilstein J Org Chem 12 (2016) 2694-2718.

[5] A. Speck-Planche, M. Cordeiro, Advanced In Silico Approaches for Drug Discovery: Mining Information from Multiple Biological and Chemical Data Through mtk-QSBER and pt-QSPR Strategies, Curr Med Chem 24 (2017) 1687-1704.

[6] R.M. Jayadeepa, A. Ray, D. Naik, D.N. Sanyal, D. Shah, Review and research analysis of computational target methods using BioRuby and in silico screening of herbal lead compounds against pancreatic cancer using R programming, Curr Drug Metab 15 (2014) 535-543.

[7] Y. Kitamura, Y. Matsuoka, Y. Nomura, T. Taniguchi, Induction of inducible nitric oxide synthase and heme oxygenase-1 in rat glial cells, Life Sci 62 (1998) 1717-1721.

[8] J.R. Sheu, Z.C. Chen, M.J. Hsu, S.H. Wang, K.W. Jung, W.F. Wu, S.H. Pan, R.D. Teng, C.H. Yang, C.Y. Hsieh, CME-1, a novel polysaccharide, suppresses iNOS expression in lipopolysaccharide-stimulated macrophages through ceramide-initiated protein phosphatase 2A activation, J Cell Mol Med 22 (2018) 999-1013.

[9] B. Jang, A. Ishigami, Y.S. Kim, E.K. Choi, The Peptidylarginine Deiminase Inhibitor Cl-Amidine Suppresses Inducible Nitric Oxide Synthase Expression in Dendritic Cells, Int J Mol Sci 18 (2017).

[10] W. Shang, X. Wei, W. Ying, Malate-aspartate shuttle inhibitor aminooxyacetic acid blocks lipopolysaccharides-induced activation of BV2 microglia, Int J Physiol Pathophysiol Pharmacol 9 (2017) 58-63.

[11] R. Arora, R. Kumar, A. Agarwal, K.H. Reeta, Y.K. Gupta, Comparison of three different extracts of Centella asiatica for anti-amnesic, antioxidant and anticholinergic activities: in vitro and in vivo study, Biomed Pharmacother 105 (2018) 1344-1352.

[12] V. Bocchini, R. Mazzolla, R. Barluzzi, E. Blasi, P. Sick, H. Kettenmann, An immortalized cell line expresses properties of activated microglial cells, J Neurosci Res 31 (1992) 616-621.

[13] J.M. Kim, P. Lee, D. Son, H. Kim, S.Y. Kim, Falcarindiol inhibits nitric oxide-

mediated neuronal death in lipopolysaccharide-treated organotypic hippocampal cultures, Neuroreport 14 (2003) 1941-1944.

[14] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, J Comput Chem 31 (2010) 455-461.

[15] R. Shahin, I. Mansi, L. Swellmeen, T. Alwidyan, N. Al-Hashimi, Y. Al-Qarar'h, O. Shaheen, Ligand-based computer aided drug design reveals new tropomycin receptor kinase a (TrkA) inhibitors, J Mol Graph Model 80 (2018) 327-352.

[16] D. Kim, S. Kim, K. Hwang, C. Kim, p-Coumaric Acid Potently Down-regulates Zebrafish Embryo Pigmentation: Comparison of in vivo Assay and Computational Molecular Modeling with Phenylthiourea, Biomedical Science Letters 23 (2017) 9.

[17] C. Cunningham, Microglia and neurodegeneration: the role of systemic inflammation, Glia 61 (2013) 71-90.

[18] C. Spagnuolo, S. Moccia, G.L. Russo, Anti-inflammatory effects of flavonoids in neurodegenerative disorders, Eur J Med Chem (2017).

[19] W. Zuo, H. Wu, K. Zhang, P. Lv, F. Xu, W. Jiang, L. Zheng, J. Zhao, Baicalin promotes the viability of Schwann cells in vitro by regulating neurotrophic factors, Exp Ther Med 14 (2017) 507-514.

[20] S.J. Jeon, M.O. Kim, F. Ali-Shah, P.O. Koh, Quercetin attenuates the injury-induced reduction of gamma-enolase expression in a middle cerebral artery occlusion animal model, Lab Anim Res 33 (2017) 308-314.

[21] S.M. Tsao, M.C. Yin, Antioxidative and antiinflammatory activities of asiatic acid, glycyrrhizic acid, and oleanolic acid in human bronchial epithelial cells, J Agric Food Chem 63 (2015) 3196-3204.

[22] K.J. Yun, J.Y. Kim, J.B. Kim, K.W. Lee, S.Y. Jeong, H.J. Park, H.J. Jung, Y.W. Cho, K. Yun, K.T. Lee, Inhibition of LPS-induced NO and PGE2 production by asiatic acid via NF-kappa B inactivation in RAW 264.7 macrophages: possible involvement of the IKK and MAPK pathways, Int Immunopharmacol 8 (2008) 431-441.

[23] D. Zhou, H. Wei, Z. Jiang, X. Li, K. Jiao, X. Jia, Y. Hou, N. Li, Natural potential neuroinflammatory inhibitors from Alhagi sparsifolia Shap, Bioorg Med Chem Lett 27 (2017) 973-978.

[24] D.Y. Chuang, A. Simonyi, J. Cui, D.B. Lubahn, Z. Gu, G.Y. Sun, Botanical Polyphenols Mitigate Microglial Activation and Microglia-Induced Neurotoxicity: Role of Cytosolic Phospholipase A2, Neuromolecular Med 18 (2016) 415-425.

[25] E. Bahar, J.Y. Kim, H. Yoon, Quercetin Attenuates Manganese-Induced Neuroinflammation by Alleviating Oxidative Stress through Regulation of Apoptosis, iNOS/NF-kappaB and HO-1/Nrf2 Pathways, Int J Mol Sci 18 (2017).

[26] S. Kanimozhi, P. Bhavani, P. Subramanian, Influence of the Flavonoid, Quercetin on Antioxidant Status, Lipid Peroxidation and Histopathological Changes in Hyperammonemic Rats, Indian J Clin Biochem 32 (2017) 275-284.