Final scientific report
FK125302

Natural compounds play an important role in drug discovery by providing large chemical diversity and covering an alternative chemical space compared with synthetic derivatives. The aim of the project FK125302 was screening for biologically active natural compounds with preferable physicochemical and pharmacokinetic properties.

Although the study initially focused on naturally occurring diarylheptanoid derivates, it was later expanded to other phenolic compounds such as gingerols, shogaols, phenolic amides, phenylpropanoids and lignans.

Altogether thirty-eight compounds were isolated from five plants by column and flash chromatography, as well as semi-preparative High Performance Liquid Chromatography (HPLC): seven diarylheptanoids from the leaves of *Corylus maxima* Mill., two lignans, five phenylpropanoids and a small aromatic aldehyde from the rhizomes of *Alpinia galanga* (L.) Willd., seven gingerol and shogaol derivatives from the rhizomes of ginger (*Zingiber officinale* Roscoe), six phenolic amides from the twigs of *Celtis occidentalis* L., three diarylheptanoids, three flavonoids and four small cinnamic acid derivatives from the rhizomes of *Alpinia officinarum* Hance. Structures were determined by Orbitrap Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy.

The isolated compounds were tested for gastrointestinal (GI) membrane and blood–brain barrier (BBB) permeability with the Parallel Artificial Membrane Permeability Assay (PAMPA). Aqueous stability at different physiologically occurring pH values was determined by Ultra High Performance Liquid Chromatography with Diode Array Detection (UHPLC-DAD), degradation products were identified by UHPLC-Orbitrap MS.

Results were published in the form of four research articles, all in first quartile (Q1) journals with a total impact factor of 20.596.

1. Membrane Permeability and Aqueous Stability Study of Linear and Cyclic Diarylheptanoids from *Corylus maxima*

The isolation of diarylheptanoids from the leaves of *C. maxima* started in the first year of the study based on our previous HPLC-MS studies on the plant that indicated the presence of linear diarylheptanoid derivatives [1]. The minor amounts of this type of molecules in in the

extracts, as well as their instability made the process unexpectedly time-consuming. In addition, quantity of the isolated compounds was in the milligram range, not allowing all the previously planned experiments to be conducted (e.g. investigation of plasma protein binding or metabolic stability). Nonetheless, the results of the study on the diarylheptanoids of *C. maxima* were suitable for publishing in the high-ranked journal, *Pharmaceutics* (Q1, IF 6.525 10.3390/pharmaceutics14061250). Main conclusions are summarized below.

Altogether seven diarylheptanoids were identified and isolated from *Corylus maxima* for the first time: two linear diarylheptanoid glycosides: hirsutanonol-5-*O*-β-D-glucopyranoside (**co-1**) and platyphyllonol-5-*O*-β-D-xylopyranoside (**co-4**), a linear aglycone: platyphyllenone (**co-5**), a cyclic diarylheptanoid glycoside: alnusonol-11-*O*-β-D-glucopyranoside (**co-6**) and three cyclic aglycones: alnusone (**co-7**), giffonin F (**co-8**) and carpinontriol B (**co-9**). Structures of the compounds were elucidated by UHPLC-Orbitrap MS and NMR spectroscopy. Cyclic diarylheptanoid derivatives were reported in *C. maxima* for the first time.

Aqueous stability of the isolated compounds at three physiologically occurring pH values (1.2, 6.8 and 7.4) as well as their passive diffusion across biological membranes (GIT and BBB) were investigated together with characteristic constituents of *C. maxima*, oregonin (**co-2**), hirsutenone (**co-3**), quercitrin (**co-10**) and myricitrin (**co-11**) (**Table 1**). Degradation products were identified by UHPLC-Orbitrap MS.

Our results indicate that among the investigated compounds, solely the cyclic diarylheptanoid aglycone alnusone (**co-7**) has both good aqueous stability and satisfactory membrane penetration ability. The other cyclic diarylheptanoid aglycones were found to be stable in aqueous environment, but the ones with larger molar mass and higher polarity, giffonin F (**co-8**) and carpinontriol B (**co-9**), were not able to cross the lipid membranes in the PAMPA-GI and PAMPA-BBB models.

All the linear diarylheptanoids (compounds **co-1**, **co-2**, **co-3**, **co-4**, **co-5**) and the cyclic diarylheptanoid alnusonol-11-*O*-β-D-glucopyranoside (**co-6**) demonstrated significant pH-dependent decomposition in aqueous media. The linear compounds **co-3** and **co-4** were labile only at the highest investigated pH value, while **co-1**, **co-2**, **co-5** and the cyclic derivative **co-6** significantly decomposed both at pH 6.8 and 7.4. Among the linear diarylheptanoid derivatives, **co-1**, **co-2** and **co-6** were the most unstable at pH 7.4. Based on our PAMPA results, among them, only platyphyllenone (**co-5**) can be considered to be able to cross the lipid membranes of the GIT and the BBB by passive diffusion.

The flavonoid glycosides quercitrin (**co-10**) and myricitrin (**co-11**) showed poor membrane penetration ability in both the PAMPA models. In addition, myricitrin was found to be unstable in aqueous medium, its concentration significantly decreased at pH 7.4.

Table 1. Results of the aqueous stability studies: compound concentration after 4 hours of incubation at 37°C compared to the initial value (%) (n=3), and the PAMPA experiments: logP_e values (n=9).

	Aqueous stability			logP_e PAMPA-BBB (n=9)	logP_e PAMPA-GI (n=9)	*c*log*P*
	pH=1.2 (n=3)	pH=6.8 (n=3)	pH=7.4 (n=3)			
hirsutanonol-5-*O*-β-D-glucopyranoside (**co-1**)	97.03±2.74	95.95±1.52*	63.26±1.93*	n.d.	n.d.	1.3
oregonin (**co-2**)	98.67±2.43	91.22±3.01*	59.93±2.85*	n.d.	n.d.	1.9
hirsutenone (**co-3**)	99.63±0.37	96.22±2.87	83.80±2.41*	n.d.	n.d.	3.9
platyphyllonol-5-*O*-β-D-xylopyranoside (**co-4**)	100.47±1.7	98.78±0.91	89.79±2.00*	n.d.	n.d.	2.6
platyphyllenone (**co-5**)	99.45±1.05	94.98±2.10*	90.40±1.52*	-5.24±0.25	-4.92±0.07	4.5
alnusonol-11-*O*-β-D-glucopyranoside (**co-6**)	100.92±2.92	74.03±1.39*	61.76±0.58*	n.d.	n.d.	1.6
alnusone (**co-7**)	99.86±0.50	101.55±2.14	100.47±1.87	-4.66±0.14	-4.90±0.17	4.2
giffonin F (**co-8**)	99.97±1.01	102.68±2.45	100.92±2.02	n.d.	n.d.	2.8
carpinontriol B (**co-9**)	102.75±1.09	101.51±1.75	103.28±1.81	n.d.	-5.49±0.30	1.6
quercitrin (**co-10**)	100.91±0.53	102.35±1.85	100.75±0.96	n.d.	n.d.	0.9
myricitrin (**co-11**)	96.23±2.46	99.94±0.55	48.44±6.15*	n.d.	n.d.	0.6

Abbreviations: n.d.: not detected in the acceptor phase; PAMPA-GI: Parallel Artificial Membrane Permeability Assay for the gastrointestinal tract; PAMPA-BBB: Parallel Artificial Membrane Permeability Assay for the blood-brain barrier

* $p < 0.05$ compared with the initial solutions

The recent study might corroborate the therapeutic potential of naturally occurring cyclic diarylheptanoids in terms of physicochemical and pharmacokinetic properties in addition to their beneficial biological effects that have already been revealed. In order to be able to draw definite conclusions, a higher number of molecules should be evaluated. However, our results justify further investigations on naturally occurring cyclic diarylheptanoids as potential therapeutic agents bringing the ones with lower molecular weight and higher lipophilicity into focus.

2. Stability Study of *Alpinia galanga* Constituents and Investigation of Their Membrane Permeability by ChemGPS-NP and the Parallel Artificial Membrane Permeability Assay

The rhizome of *Alpinia galanga* was included in the study together with that of *Alpinia officinarum*. Although diarylheptanoids were detected in sufficient amounts for isolation purposes only in the latter species, stability issues occurring during the PAMPA experiments of *A. galanga* extracts were found to be interesting to explore. Results of the study were published in the in the journal *Pharmaceutics* (Q1, IF 6.525 10.3390/pharmaceutics14091967). Main conclusions are summarized below.

Alpinia galanga Willd., greater galangal, has been used for thousands of years as a spice as well as in traditional medicine. Its central nervous system (CNS) stimulant activity and neuroprotective effects have been proved both in animal models and human trials [2, 3]. However, the compounds responsible for these effects have not been identified yet. In addition, the mechanism of the nervous system stimulant activity of the extracts has not been clarified yet. Therefore, the aim of our study was the identification and isolation of the main constituents of the rhizome extracts with good BBB permeability.

The ethyl acetate extract of *A. galanga* was tested for the prediction of the passive intestinal absorption by the PAMPA-GI method. All the main compounds of the extract were detected in the acceptor side, except for galanganol A (**a-5**) and B (**a-6**). In addition, the instability of three constituents was also shown by the study. On this basis, all these main compounds were isolated in order to determine their structure, stability, and capability to cross the blood–brain barrier. Isolation was carried out by the use of column and flash chromatographic and semi-preparative HPLC methods.

The isolated compounds were identified by Orbitrap Mass Spectrometry and NMR spectroscopy as *p*-OH-benzaldehyde (**a-1**), *trans*-*p*-coumaryl-alcohol (**a-2**), *p*-coumaryl-aldehyde (**a-4**), galanganol A (**a-5**), galanganol B (**a-6**), *trans*-*p*-acetoxycinnamyl alcohol (**a-7**), 1′*S*-1′-acetoxychavicol acetate (ACA) (**a-9**), and 1′*S*-1′-acetoxyeugenol acetate (AEA) (**a-10**). Five compounds were found to be unstable in aqueous media: an isomer of *trans*-*p*-coumaryl-alcohol (**a-3**), *trans*-*p*-acetoxycinnamyl alcohol (**a-7**), an isomer of *trans*-*p*-acetoxycinnamyl alcohol (**a-8**), ACA (**a-9**), and AEA (**a-10**).

The compounds were tested for their capability to cross the blood–brain barrier by the PAMPA-BBB method. Based on their measured log P_e values, *p*-OH-benzaldehyde (**a-1**), *trans*-*p*-coumaryl-alcohol (**a-2**), *p*-coumaryl-aldehyde (**a-4**), *trans*-*p*-acetoxycinnamyl

alcohol (**a-7**), ACA (**a-9**), and AEA (**a-10**) were found to be able to penetrate the BBB via passive diffusion (BBB+), suggesting that they contribute to the positive effects of greater galangal extracts in the central nervous system. Although compounds **a-7**, **a-9**, and **a-10** proved to be unstable, based on the approximate log P_e values, it can be assumed that these components also have good BBB penetration capability. The other two compounds, galanganol A (**a-5**) and galanganol B (**a-6**), had log P_e values lower than −6.0, indicating that they are unable to enter the central nervous system by passive diffusion across the BBB.

The results of the ChemGPS-NP framework investigation for the compounds' ability of passive diffusion also support the outcomes of the PAMPA-BBB experiments; a passive permeation could be expected for all compounds investigated, except for galanganol A (**a-5**) and B (**a-6**) (**Figure 1**).

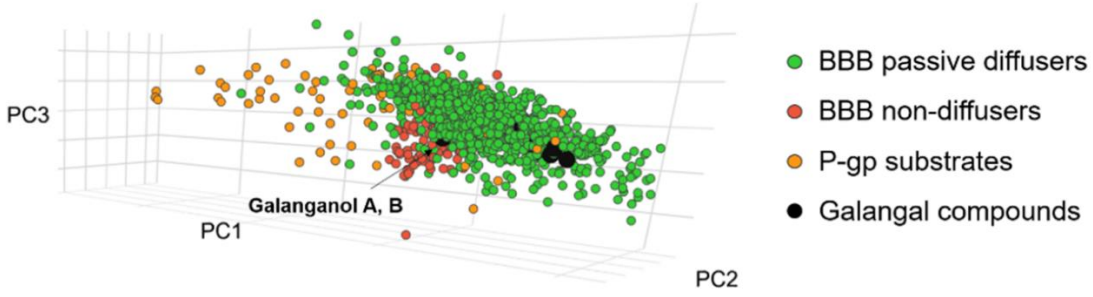

Figure 1. Localization of the galangal compounds in chemical space compared to known passive and P-gp substrates, as well as non-diffuser molecules, using ChemGPS-NP.

Examination of the chemical space position of galangal compounds in relation to known psychostimulants in the ChemGPS-NP framework revealed that all the molecules in proximity are from the group of NET/SERT inhibitors. The highest number of vicinal compounds was shown by *p*-coumaryl-alcohol. As ACA and AEA did not show much proximity to either compound, the importance of further investigation of smaller compounds and degradation products became more pronounced.

3. Blood-brain barrier permeability study of ginger constituents

Ginger (*Zingiber officinale* Roscoe) was included in the study in the first year of the project with the initial aim of the isolation of the minor diarylheptanoid constituents. These diarylheptanoids turned out to be extremely minor, meanwhile, the blood–brain barrier permeability of the main gingerol and shogaol derivatives was also unexplored. Therefore, the

easier and more exciting way was chosen with the isolation and PAMPA-BBB study of the main compounds. The results justified the choice: a research article was published in the *Journal of Pharmaceutical and Biomedical Analysis* (Q1, IF 3.773 10.1016/j.jpba.2019.112820.) in January 2020, which has received 26 citations since then (according to scopus.com). Main conclusions are summarized below.

Seven gingerol and shogaol derivatives were isolated from *Zingiber officinale* by the use of flash chromatographic and semi preparative HPLC methods: ([6]-gingerol **(z-1**), [8]-gingerol (**z-2**), [10]-gingerol (**z-3**), [6]-shogaol (**z-4**), [10]-shogaol (**z-5**), 1-dehydro-[6]-gingerdione (**z-6**) and 1-dehydro-[10]-gingerdione (**z-7**)). Taken in consideration the promising positive effects of ginger and its constituents in diseases affecting the central nervous system (anxiety, Alzheimer's disease, Parkinson's disease and epilepsy) [4-8], the compounds were tested for their capability to cross the blood-brain barrier by the PAMPA-BBB method. Based on their measured logP_e values, [6]-gingerol (**z-1**), [8]-gingerol (**z-2**), [6]-shogaol (**z-4**) were found to be able to penetrate the BBB via passive diffusion, suggesting them to contribute to the aforementioned positive effects of ginger extracts in the central nervous system. The other four compounds [10]-gingerol (**z-3**), [10]-shogaol (**z-5**), 1-dehydro-[6]-gingerdione (**z-6**) and 1-dehydro-[10]-gingerdione (**z-7**) had logP_e values lower than −6.0 indicating them being unable to enter the central nervous system by passive diffusion through the BBB. For the latter constituents the measured membrane retention exceeded 95% in all cases, suggesting the compounds being "stuck" in the membrane. This might be attributed to the relatively high lipophilicity of the non-penetrating constituents compared to the penetrating ones, since the calculated log$P$ values of the compounds showed relatively strong negative correlation with the measured logP_e data (**Figure 2**).

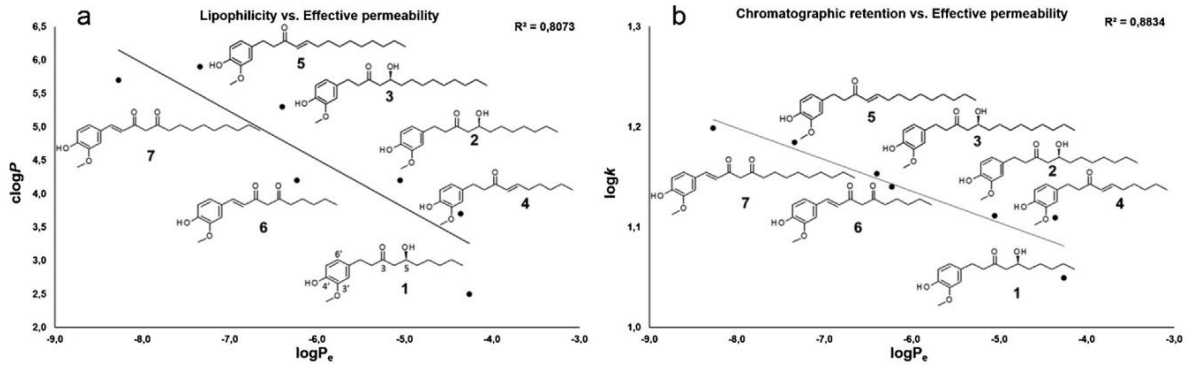

Figure 2. (a) Negative correlation between the calculated logP and the measured logP_e values of the isolated ginger constituents. (b) Negative correlation between the experimental log$k$ and the logP_e values of the isolated ginger constituents.

4. UHPLC-DPPH method reveals antioxidant tyramine and octopamine derivatives in *Celtis occidentalis*

Since many of the investigated diarylheptanoids were found to be unstable or challenging to isolate, in the third year of the project an additional plant species with unexplored phytochemistry was included in our studies: *Celtis occidentalis* L. An UHPLC-DPPH method was applied for the rapid screening for antioxidant constituents in the twigs methanolic extract, which revealed six constituents (unfortunately not diarylheptanoids). The results were published in the *Journal of Pharmaceutical and Biomedical Analysis* (Q1, IF 3.773 10.1016/j.jpba.2020.113612). Main conclusions are summarized below.

The aforementioned antioxidant compounds were isolated by the means of flash chromatography and semi-preparative HPLC and identified by Orbitrap-MS and NMR spectroscopy as *N-trans-p*-coumaroyloctopamine (**ce-1**), *N-trans*-feruloyloctopamine (**ce-2**), *N-trans*-caffeoyltyramine (**ce-3**), 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide (**ce-4**), *N-trans-p*-coumaroyltryramine (**ce-5**) and *N-trans*-feruloyltyramine (**ce-6**). All the six compounds are reported in *Celtis occidentalis* for the first time, *N-trans-p*-coumaroyloctopamine (**ce-1**) and *N-trans*-feruloyloctopamine (**ce-2**) have not been identified in *Celtis* species yet, while 2-*trans*-3-(4-hydroxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-oxoethyl] prop-2-enamide (**ce-4**) is considered to be a new compound (**Figure 3**).

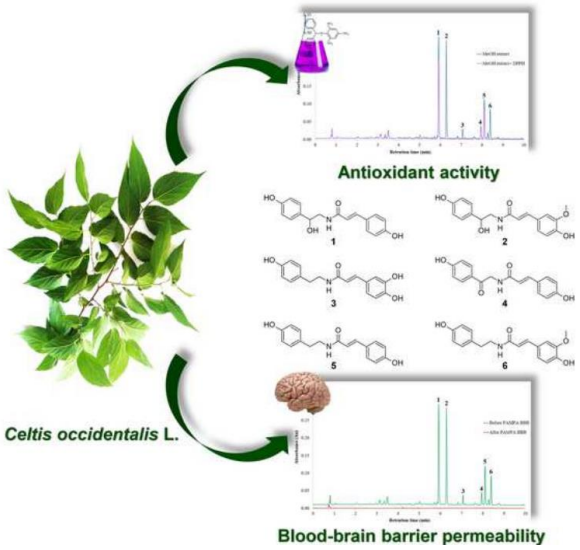

Figure 3. UHPLC-DPPH method reveals antioxidant tyramine and octopamine derivatives in *Celtis occidentalis*

The isolation of the compounds made the determination of their individual free radical scavenging activity possible: *N-trans*-caffeoyltyramine (**ce-3**) was shown to possess the highest antioxidant activity followed by *N-trans*-feruloyltyramine (**ce-1**) and *N-trans*-feruloyloctopamine (**ce-2**), all exceeding the activity of the whole extract, suggesting antagonistic interactions between the constituents.

Since literature data together with the aforementioned results suggests potential positive effects of the compounds in the central nervous system [9], BBB penetration capability was investigated by coupling UHPLC separation to the PAMPA-BBB assay. Based on the results it could be concluded that no compounds in the *Celtis* extract are able to cross the BBB via passive diffusion.

5. Isolation and membrane permeability study of *Alpinia officinarum* constituents

In order to prepare extracts rich in diarylheptanoids from the rhizome of *Alpinia officinarum* L., ultrasonic extraction was carried out with solvents of increasing polarity (chloroform, ethyl acetate and methanol). Preliminary investigations by UPLC-DAD-MS indicated the presence diarylheptanoid and flavonoid derivatives in all the three extracts.

16 Flash chromatographic fractions were prepared from the ethyl acetate extract of the rhizome and tested in the PAMPA-BBB model, screening for constituents with good membrane permeability. 15 main compounds were detected on the acceptor side of the PAMPA model together with 14 minor constituents. Our primary aim was the isolation and NMR identification of the main compounds, since minor constituents are unlikely play significant role in the biological effects of the plant extracts. Until now, three diarylheptanoids: 1-(4-hydroxy-3-methoxyphenyl)-7-(3,4-dihydroxyphenyl)heptane-3,5-diol (**ao-1**), 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-phenylheptan-3-one (**ao-2**) and 7-(4-hydroxy-3-methoxyphenyl)-1-phenylheptan-3,5-diol (**ao-3**) three flavonoids: pinobanksin (**ao-4**), galangin (**ao-5**) and pinocembrin (**ao-6**), and four small cinnamic acid derivatives: 3-methoxy-4-hydroxybenzoic acid (**ao-7**), *p*-methoxy-benzoic acid (**ao-8**), 4-hydroxybenzaldehyde (**ao-9**) and *p*-hydroxy vanillin (**ao-10**). The isolation and NMR identification of additional compounds is in progress. Thereafter investigation of the membrane permeability and aqueous stability of the isolated compounds is planned. Publication of the results is expected next year.

6. Summary

The principal investigator has to admit that the work summarized in the Final scientific report slightly differs from the original workplan.

Many obstacles could be listed that came in our way, including the non-functioning HPLC-MS instrument at the Department or the COVID-19 pandemic.

Nonetheless, I believe, the main reason for this difference between the planned and the implemented is, on the one hand, the inexperience of the principal investigator. By the time of the preparation of the research plan, I just left the pharmaceutical industry for the academic sphere and my mindset had not followed yet. I missed to consider the increasing number of my educational tasks as well as the different working conditions when creating the research plan. On the other hand, I could blame my scientific curiosity and the irresistible desire to explore the unexplored.

However, I would say that I realized soon enough that among the given circumstances, sticking to the original research plan was not feasible. Isolation of diarylheptanoids in amounts sufficient for all the experiments planned would have been extremely time-consuming. It was too much risk to take. Therefore, I decided to choose the safer way by expanding the diversity of the investigated molecules and reducing the number of experiments.

As a result, 5 plants were investigated, 38 constituents isolated, 11 BBB+ natural compounds identified, 4 research articles published in Q1 journals with a total of 20.596 impact factors.

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principal investigator

Budapest, 2022. 10. 23.

References

1. Riethmüller, E.; Tóth, G.; Alberti, Á.; Végh, K.; Burlini, I.; Könczöl, Á.; Balogh, G.T.; Kéry, Á. First Characterisation of Flavonoid- and Diarylheptanoid-Type Antioxidant Phenolics in *Corylus maxima* by HPLC-DAD-ESI-MS. J. Pharm. Biomed. Anal. 2015, 107, 159–167.

2. Saha, S.; Banerjee, S. Central Nervous System Stimulant Actions of *Alpinia galanga* (L.) Rhizome: A Preliminary Study. Indian J. Exp. Biol. 2013, 51, 828–832

3. Srivastava, S. Selective Enhancement of Focused Attention by *Alpinia galanga* in Subjects with Moderate Caffeine Consumption. Open Access J. Clin. Trials 2018, 10, 43–49.

4. Zeng G., Zhang Z., Lu L., Xiao D., Zong S., He J. (2013) Protective Effects of Ginger Root Extract on Alzheimer Disease-Induced Behavioral Dysfunction in Rats. Rejuvenation Res. 16(2):124-133.

5. Lim S., Moon M., Oh H., Kim H. G., Kim S. Y., Oh M. S. (2014) Ginger improves cognitive function via NGF-induced ERK/CREB activation in the hippocampus of the mouse. J Nutr Biochem. 25:1058-1065.

6. Azam F., Amer A. M., Abulifa A. R., Elzwawi M. M. (2014) Ginger components as new leads for the design and development of novel multi-targeted anti-Alzheimer's drugs: A computational investigation. Drug Des Devel Ther. 8:2045-2059.

7. Park G., Kim H. G., Ju M. S., Ha S. K., Park Y., Kim S. Y., Oh M. S. (2013) 6-Shogaol, an active compound of ginger, protects dopaminergic neurons in Parkinson's disease models via anti-neuroinflammation. Acta Pharmacol Sin. 34:1131-1139.

8. Vishwakarma S. L., Pal S. C., Kasture V. S., Kasture S. B. (2002) Anxiolytic and antiemetic activity of *Zingiber officinale*. Phytother Res. 16:621-626.

9. D.K. Kim, K. Lee (2003) Inhibitory effect of Trans-*N*-*p*-coumaroyl tryamine from the twigs of Celtis chinensis on the acetylcholinesterase Arch. Pharm. Res., 26. 735-738