products during storage. It also could be a promising method of decontamination in industries and storage units, in order to avoid contamination and ensure food security to the consumer.

Acknowledgements

5. References

Alves, J.R.C.1995: Nitretação em plasma pulsado: equipamento, preparação e caracterização das camadas nitretadas. Universidade Federal de São Carlos (UFScar). São Paulo.

Choi, JH. Han, I.; Baik, HK.; Lee, MH. and D.W. Han, 2006: Analysis of sterilization effect by pulsed dielectric barrier discharge. Journal of Electrostatics 64, 17–22.

Dasan, B.G., Mutlu, M. and I.H. Boyaci, 2016: Decontamination of Aspergillus flavus and Aspergillus parasiticus spores on hazelnutsvia atmospheric pressure fluidized bed plasma reactor. International Journal of Food Microbiology216, 50–59.

Fraternale, D., Giamperi, L. and D. Ricci. 2003: Chemical Composition and antifungal activity of essential oil obtained from in vitro plants of Thymus mastichina L. Journal of Essential Oil Research 15, 278–281.

Fridman, G. Brooks, A. Balasubramania, N. Fridman, A. and A. Gutsol, 2007: Comparison of direct and indirect effects of non-thermal atmospheric-pressure plasma on bacteria. Plasma Processes and Polymers 4, 370–375.

Geyter, N. and R. Morent, 2012. Nonthermal plasma sterilization of living and nonliving surfaces. Annual Review of Biomedical Engineering. 14, 255–274.

Guo, J., Huang, K., and J. Wang, 2015: Bactericidal effect of various nonthermalplasma agents and the influence of experimental conditions in microbial inactivation: a review. Food Control 50, 482–490.

IARC - International Agency for Research of Cancer. 1993: Toxins derived from Fusarium graminearum, F. culmorum and F. crookwellense: zearalenona, deoxynivalenol, nivalenol and fusarenon-X. Monographs on the Evaluation of Carcinogenic Risks to Humans 56, 397-444.

Laroussi, M., 2005. Low temperature plasma-based sterilization: overview and state-of-the-art. Plasma Processes and Polymers 2, 391–400.

Mendis, D.A., Rosenberg, M., and F. Azam, 2000: A note on the possible electrostatic disruption of bacteria. IEEE Transactions on Plasma Science. 28, 1304–1306.

Mir, S.A., Shah, M.A.,and M.M. Mir, 2016: Understanding the role of plasma technology in food industry. Food and Bioprocess Technology 9, 734–750.

Misra, N.N., Pankaj, S.K., Frias, J.M., Keener, K.M. and P.J. Cullen, 2015: The effects of nonthermal plasma on chemical quality of strawberries. Postharvest Biology and Technology 110, 197–202.

Misra, N.N.; Keener, K.M.; Bourke, P.; Mosnier, J. and P.J. Cullen, 2014: In-package atmospheric pressure cold plasma treatment of cherry tomatoes. Journal of Bioscience and Bioengineering 118,177–182.

Niemira, B.A., 2012: Cold plasma decontamination of foods. Annual Review of Food Science and Technology 3, 125–142.

Pankaj, S.K., Bueno-Ferrer, C. and N.N. Misra, 2014: Applications of cold plasma technology in food packaging. Trends in Food Science and Technology 35, 5–17.

Savi, G.D. and V.M. Scussel, 2014: Effects of ozone gas exposure on toxigenic fungi species from Fusarium, Aspergillus, and Penicillium genera. Ozone: Science & Engineering, 36, 144-152.

Scholtz, V., Pazlarova, J., Souskova, H., Khun, J. and J. Julak, 2015: Nonthermal plasma-A tool for decontamination and disinfection. Biotechnology Advances 33, 1108–1119.

Scussel, V.M., 2017: "FungosemGrãosArmazenados."In Armazenagemdegrãos, edited by I. Lorini, L.H. Miike, and V.M. Scussel. Campinas: Biogeneziz.

Selcuk, M., Oksuz, L. and P. Basaran, 2008: Decontamination of grains and legumes infected with Aspergillus spp. and Penicillum spp. by cold plasma treatment. Bioresource Technology 99, 5104–5109.

Computer-Aid Molecular Docking Technology in Cereal Mycotoxin Analysis

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Abstract

Computer-aid molecular docking is a simulative process that receptors and ligands recognize each other through energy matching and geometric matching. It is widely used in bioactive compounds simulative screening and preliminary exploring the bioactivity and toxicity of molecular, which plays important guiding role in toxicity and bioactivity study of molecular entities. In our study, we used the computer-aid molecular docking software-discovery studio 3.1 client to test the mechanism of aflatoxins such as aflatoxin B1, B2, M1, M2, G1, G2 and the results of our experiment help to illustrate the pathway of aflatoxin's toxication. We also used this technology to test the preliminary toxicity of zearalenone (ZEN) and its two degradation products: α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL), which indicates that these three products possessed significant estrogenic activity. The order of the estrogenic activity is: α-zearalenol > zearalenone >β-zearalenol.

Key words: computer-aid molecular docking, aflatoxin, zearalenone, toxicity

1. Introduction

Over the last few decades, computer-aid molecular docking technology has grown significantly in the development of new drug molecules. As a powerful technique, it relives the tension in drug discovery such as time-consuming, high-cost and low success rates. Additionally, with rapid development of biological structures and computer technology, this technology is widely used in mycotoxin toxicity pathway research (Shoichet et al., 2002; Powers and Setzer, 2015).

Using direct docking methods or virtual high-throughput screening, affinity of molecules to targets can be estimated based on compounds' conformation and complementarity with residues in binding site. Through analysis of binding free energies, further filtering and optimization of possible molecules subsequently follow, a limited number of lead molecules are selected for *in vitro* bioactivity tests. Automatic docking is aimed at the determination of the optimal position and orientation of molecule in binding pocket of particular protein target (Verdonk et al., 2011; Śledź and Caflisch, 2017). Quality of protein–ligand interactions are usually qualified by ligand efficiency (LE) and average binding energy per non-hydrogen atom of the ligand. While virtual high-throughput screening is performed to evaluate libraries of molecules for binding affinity to the protein target. This HTS strategy can shortlist compounds that are most likely to bind to the selected target with the highest affinity. A plethora of software have been developed for molecular docking including Dock, GOLD, and AutoDock, et. In addition, other docking strategies such as flexible ligand docking, fragment docking and fragment growing have been used in high-throughput docking campaigns (Macalino et al., 2015; Leelanada and Lindert, 2016).

In our research, we used the computer-aid molecular docking software-Discovery Studio 3.1 client (Accelrys, USA) to test the mechanism of aflatoxins and illustrate the pathway of aflatoxin's toxication. We also used this technology to test the preliminary toxicity of zearalenone and its two degradation products: α-zearalenol and β-zearalenol, which indicates that these three products possessed significant estrogenic activity.

2. Materials and Methods

The 2D structure of Aflatoxin B1 and Oltipraz was generated by ChemDraw Ultra 12.0 (CambridgeSoft, Cambridge, MA, USA). A homology model of protein receptor was constructed from crystal structure of PDB: obtained from the RCSB protein Data Bank,

Water molecules were removed and H atoms were added to the structure. 3D structures of the compounds were generated and optimized by the Discovery Studio 2.1 package (Accelrys, San Diego, CA, USA). The receptor-grid files were carried out using a grid-receptor generation program using default settings after ensuring that the ligands and the protein are in correct form. The GOLD program in the Discovery Studio software was used to perform the docking simulations, which allows full flexibility of the ligand.

The structures of the aflatoxins，zearalenone, α-zearalenol and β-zearalenol were drawn in chem3D with standard lengths and angles. The Gasteiger–Huckel charge, with a distance-dependent dielectric function, and AM1 docking calculations were applied for the minimization of

the molecules. To modify the structure of receptor, missing atoms, bonds, and contacts were checked, hydrogen atoms were added to the enzyme structure, and water molecules were removed. Intercalation models were optimized using the CHARMm forcefield with the added parameters. After performing the docking simulation, the scores of the docked conformers were ranked the best binding modes in the cavity was picked out.

3. Results and Discussion

3.1. Aflatoxin toxicity analysis

Aflatoxins are among the most potent natural hepato-carcinogenic products, which are produced mainly by the fungi *Aspergillus flavus* and *A. parasiticus*. Twelve aflatoxins analogues including aflatoxin B1, B2, G1, M1, P1, Q1, H1, GM, B2a and aflatoxicol have been separated and identified. The basic structures of aflatoxins are dihydrofuran, coumarin and aflatoxin B1(AFTB1), which is the analogue of dihydrofuran oxynaphthalene, contains two furan rings (the basic toxic structure) and one cumarin (Eaton and Gallagher, 1994; Koudande, 2013).
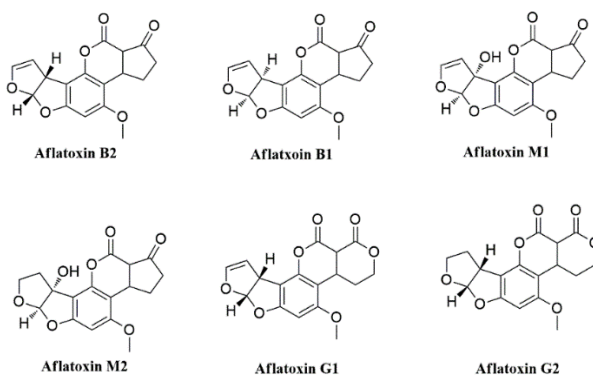

Fig. 1 The chemical structures of aflatoxin's metabolic products

The interaction between AFTB1 and receptor proteins

In our research, we chose some kinds of proteins which play significant roles in cell apoptosis, estrogen metabolism, immunosuppression and digestive system function as the potential targets of the toxic pathway of aflatoxin b1, including Caspase-1, cell division protein kinase 2, serine/threonine protein kinase chk1, progesterone receptor, androgen receptor, estrogen receptor, alpha-thrombin, prostaglandin g/h synthase 2, estradiol 17-beta-dehydrogenase 1, macrophage migration inhibitory factor and estrogen sulfotransferase. We made AFTB1 molecular docked with the above proteins by molecular docking software, the results was shown in **Tab 1.** As the results shown, estrogen sulfotransferase was proved to be the best dock receptor of AFTB1 and the score were 130.22 and -10.9013 by Libdock and CDocker, respectively.
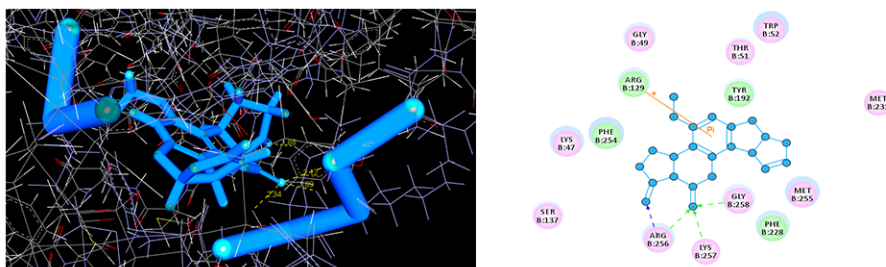

Fig. 2 The interaction between AFTB1 and estrogen sulfotransferase

Tab. 1 AFTB1 targets predicted by Libdock and CDocker

Protein	PDB	Libdock score	CDocker score
caspase-1	3D6F	828.002	/
cell division protein kinase 2	1HCK	972.643	-0.549952
serine/threonine-protein kinase chk1	1IA8	100.060	/
progesterone receptor	1A28	986.966	/
androgen receptor	5DIU	113.528	-209.024
estrogen receptor	1QKM	110.000	-221.885
alpha-thrombin	1ABI	822.093	/
prostaglandin g/h synthase 2	1PD2	117.653	/
estradiol 17-beta-dehydrogenase 1	1BHS	986.731	/
macrophage migration inhibitory factor	1MFI	908.723	-968.253
estrogen sulfotransferase	1HY3	130.022	-109.013
sex hormone-binding globulin	1D2S	111.558	-162.633

The interaction between AFTB1 and estrogen sulfotransferase

We made AFTB1 docked with estrogen sulfotransferase, the docking result was shown in **Fig. 2**. AFTB1 could perfectly docked into the formed cavity of estrogen sulfotransferase protein and there was formed cation-π interaction between the benzene ring and arginine residue (ARG B:129). The carbonyl group in coumarin formed hydrophobic interactions with tyrosine residue（TYR B:192） and the oxygen atom in furan ring formed hydrophobic interactions with arginine residue (ARG B:256).

The interaction between AFTB2 and estrogen sulfotransferase

We made AFTB2 docked with estrogen sulfotransferase, the docking result was shown in **Fig. 3**. AFTB1 could perfectly docked into the formed cavity of estrogen sulfotransferase protein and there was formed cation-π interaction between the benzene ring and arginine residue (ARG B: 129). The carbonyl group in coumarin formed hydrophobic interactions with arginine residue （ARG B:256）， allysine residue (LYS B:257) and glycine residue (GLY B:258).
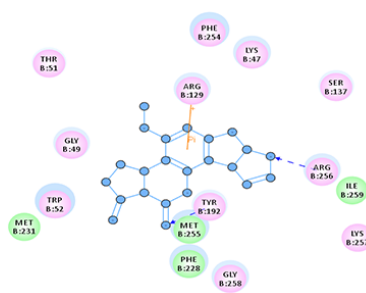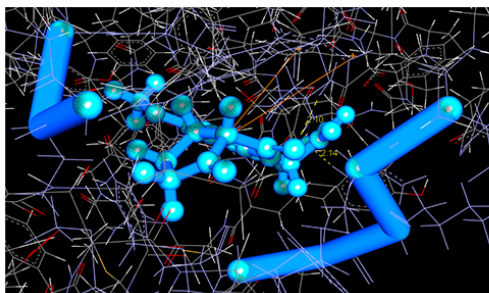
Fig. 3 The interaction between AFTB2 and estrogen sulfotransferase

The interaction between AFTBM1 and estrogen sulfotransferase

We made AFTB M1 docked with estrogen sulfotransferase, the docking result was shown in **Fig. 4**. AFTB1 could perfectly docked into the formed cavity of estrogen sulfotransferase protein and there was formed cation-π interaction between the benzene ring and arginine residue (ARG B:129). The phenolic hydroxy group furan ring hydrophobic interactions with arginine residue （ARG B:256), allysine residue (LYS B:257) and glycine residue (GLY B:258). The oxygen atom in furan ring formed

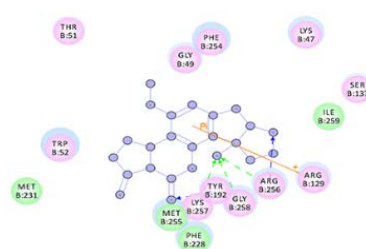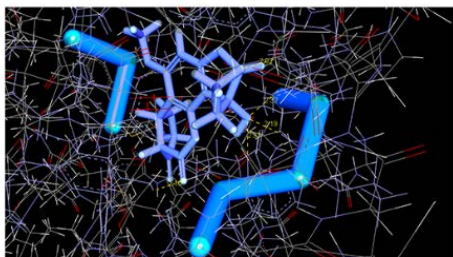hydrophobic interactions with arginine residue (ARG B:256).

Fig 4. The interaction between AFTB M1 and estrogen sulfotransferase

The interaction between AFTB M2 and estrogen sulfotransferase

We made AFTB M2 docked with estrogen sulfotransferase, the docking result was shown in **Fig. 5.** There was formed cation-π interaction between the benzene ring and arginine residue (ARG B:129). The phenolic hydroxy group furan ring hydrophobic interactions with arginine residue（ARG B:256），lyrosine residue（TYR B:192），and glycine residue (GLY B:258). The oxygen atom in furan ring formed hydrophobic interactions with arginine residue（ARG B:256），the carbonyl group in coumarin formed hydrophobic interactions with tyrosine residue (TYR B:192).

The interaction between AFTB G1 and estrogen sulfotransferase

We made AFTB G1 docked with estrogen sulfotransferase, the docking result was shown in **Fig. 6.** There was formed cation-π interaction between the benzene ring and arginine residue (ARG B:129). The inside carbonyl group in coumarin formed hydrophobic interactions with arginine residue (ARG B:256), allysine residue (LYS B:257) and glycine residue (GLY B:258). The outside carbonyl group in coumarin formed hydrophobic interactions with allysine residue (LYS B:47), The oxygen atom in furan ring formed hydrophobic interactions with arginine residue(ARG B:256).
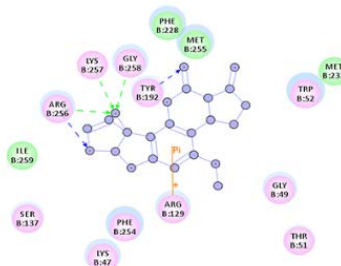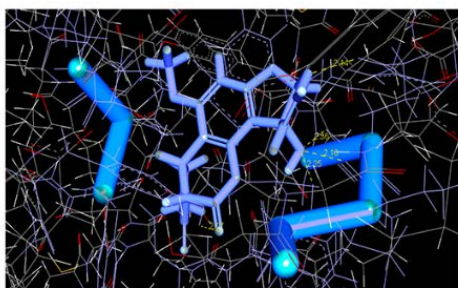

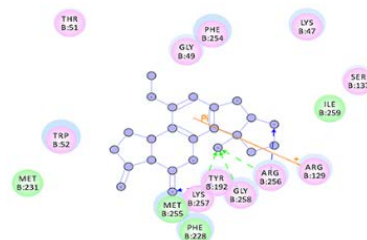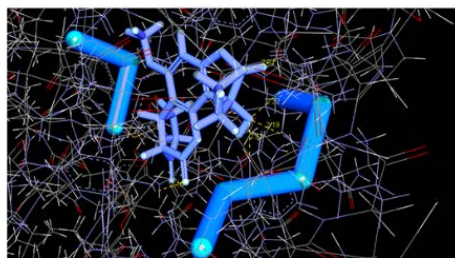**Fig. 5** The interaction between AFTB M2 and estrogen sulfotransferase

Fig. 6. The interaction between AFTB M2 and estrogen sulfotransferase

The interaction between AFTB G2 and estrogen sulfotransferase

We made AFTB G1 docked with estrogen sulfotransferase, the docking result was shown in **Fig. 7.** There was no formed cation-π interaction between the benzene ring and any residue. The inside carbonyl group in coumarin formed hydrophobic interactions with arginine residue (ARG B:256), allysine residue (LYS B:257) and glycine residue (GLY B:258). The outside carbonyl group in coumarin formed hydrophobic interactions with allysine residue (LYS B:47).
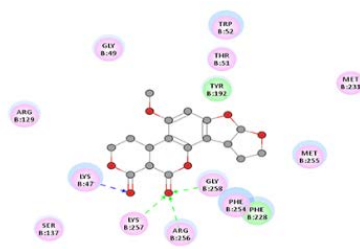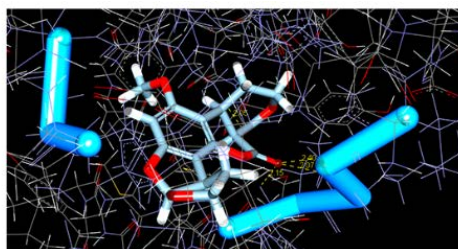

Fig.7 The interaction between AFTB G2 and estrogen sulfotransferase

The Binding energy and docking score between ligand and receptors

The results of binding energy and docking score between aflatoxin B1, B2, M1, M2, G1, G2 and estrogen sulfotransferase were shown in **Tab 2**. The binding energy between AFTB1, AFTB2, AFTB M1, AFTB M2, AFTB G1, AFTB G2 with estrogen sulfotransferase was -10.9013 kcal/mol, -20.2356 kcal/mol, -8.5654 kcal/mol, - 8.2156 kcal/mol, -19.5298 kcal/mol, -14.1768 kcal/mol. AFTB M1 was proved to be the best binding ligand with estrogen sulfotransferase receptor.

Tab 2 the Binding energy and docking score between ligand and receptors

Ligand	Binding energy	Number
Aflatoxin M2	-8.21584	10
Aflatoxin M1	-8.5654	10
Aflatoxin B1	-10.9013	10
Aflatoxin G2	-14.1769	10
Aflatoxin G1	-19.5298	10
Aflatoxin B2	-20.2356	10

3.2. Estrogenic effect in zearalenone

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-resorcylic acid lactone, abbreviated as ZEN] is a mycotoxin that is produced by *Fusarium roseum* and is usually isolated from moldy corn (Reed et al, 2004). Zeranol a synthetic tetrahydro-derivative of ZEN, has been used as a growth promoter

for food-producing animals (Caldwell et al., 1970). Earlier studies have shown that ZEN and ZOL have strong estrogenic effects, and each of them was reported to have a similar dose–response curve pattern in stimulating uterine weight gains in neonatal rats or immature mice (Urry et al, 1966). The most commonly ZOL are α-ZOL and β-ZOL, which are shown in **Fig. 8.**
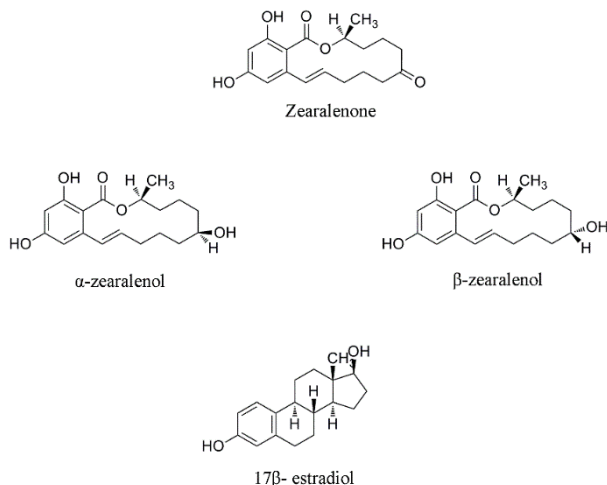

Zearalenone

α-zearalenol

β-zearalenol

17β- estradiol

Fig. 8 Chemical structure of ZEN, α-ZOL, β-ZOL and β-estriol

The interaction between ZEN and α-estrogen receptor protein

In order to prove the estrogen effect of ZEN in molecular stage, we used the molecular docking software to simulate the binding situation between ZEN and α-estrogen receptor protein, as **Fig. 9** shown, ZEN can perfectly docked into the cavity of estrogen receptor protein crystal structure, and the hydroxy group of benzene ring formed strong hydrophobic interactions with contiguous alanine residue (ALA350) and glutamic acid residue (GLU353), which strengthen the binding ability between the ligand sand the receptors.
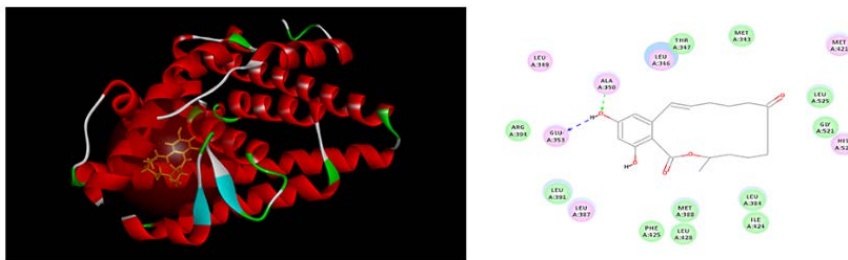

Fig. 9 The interaction between ZEN and α-estrogen receptor protein

The interaction between ZEN and β-estrogen receptor protein

We used the molecular docking software to simulate the binding situation between ZEN and β-estrogen receptor protein, as **Fig 10** shown, the 3-hydroxy group in benzene ring of ZEN formed strong hydrophobic interactions with contiguous histidine residue (HIS475) and glycine residue (GLY472), which strengthen the binding ability between the ligand sand the receptors.
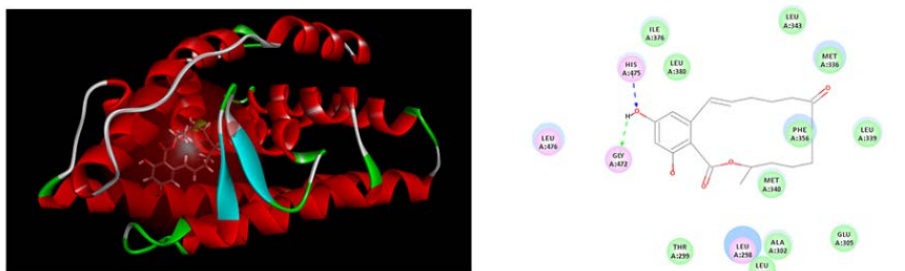
Fig. 10 The interaction between ZEN and β-estrogen receptor protein

The interaction between ZOL and estrogen receptor protein

We have proved that ZEN molecule had favorable binding ability with α-estrogen receptor protein and β-estrogen receptor protein, which demonstrated that ZEN possessed strong estrogen effect. In the following study, we would prove whether ZOL had the similar estrogen effect.

We used the molecular docking software to simulate the binding situation between α-ZOL, β-ZOL and α- estrogen receptor protein, β-estrogen receptor protein. From Fig 11, It was proved that α-ZOL can perfectly docked into the cavity of estrogen receptor protein crystal structure, 3-hydroxy group in benzene ring of α-ZOL formed strong hydrophobic interactions with contiguous arginine residue (ARG394) in α- estrogen receptor protein and glycine residue (GLY472) in β-estrogen receptor protein; aliphatic hydroxyl group in aliphatic chain formed strong hydrophobic interactions with methionine (MET421) in α- estrogen receptor protein and glutamic residue (GLU305) in β-estrogen receptor protein. The binding ability between β-ZOL and estrogen receptor protein was relatively weak and there only one existed hydrophobic interaction according to the docking results (Fig. 12)
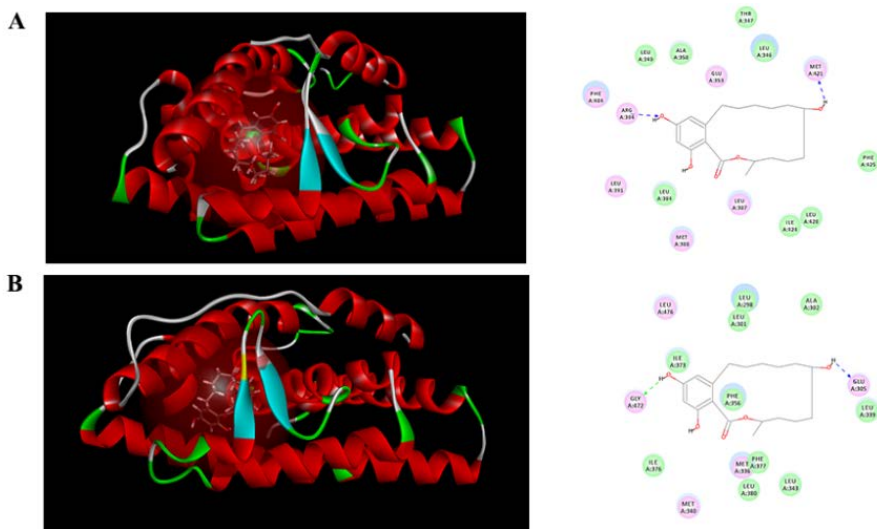

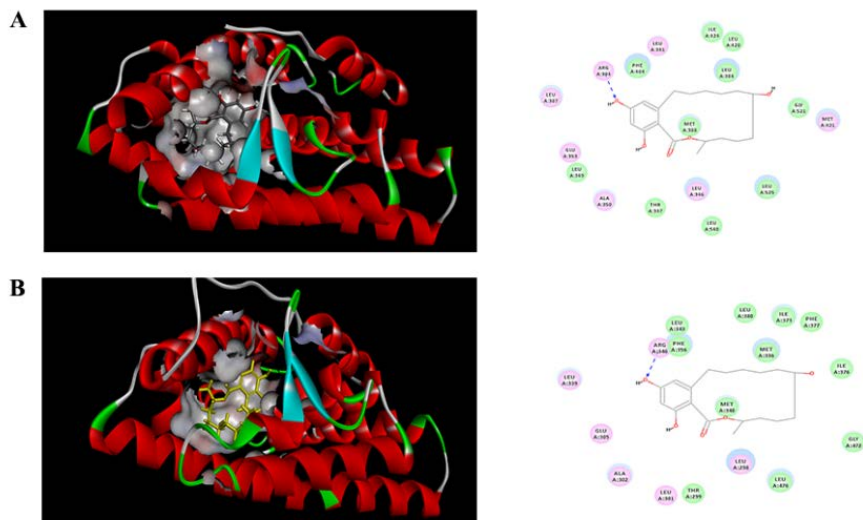**Fig. 11** The interaction between α-ZOL and estrogen receptor protein

Fig. 12 The interaction between β-ZOL and estrogen receptor protein

The binding ability and docking score between ligands and recepor

The results of binding energy and docking score between 17β- estradiol (positive control), ZEN, α-ZOL, β-ZOL and estrogen receptor protein were shown in Tab 3. The order of the estrogenic activity is: α-zearalenol > zearalenone >β-zearalenol.

Tab3 the Binding energy and docking score between ligand and receptors

Ligand	Estrogen receptor protein	Binding energy （kcal/mol）	Libdocks score
17β- estradiol	α- estrogen receptor protein	-26.8334	98.9602
17β- estradiol	β-estrogen receptor protein	-26.8334	108.235
ZEN	α- estrogen receptor protein	-38.9894	113.57
ZEN	β-estrogen receptor protein	-38.9894	106.956
α-ZOL	α- estrogen receptor protein	-41.937	94.0647
α-ZOL	β-estrogen receptor protein	-41.937	72.6476
β-ZOL	α- estrogen receptor protein	-27.6144	115.28
β-ZOL	β-estrogen receptor protein	-27.6144	107.458

Acknowledgements

References

Leelananda, S.P. and S. Lindert, 2016. Computational methods in drug discovery. Beilstein Journal of Organic Chemistry 12, 2694-2718.

Caldwell, R. W., Tuite, J., Stob, M. and R. Baldwin,1970. Zearalenone production by Fusarium species. Applied Microbiology 20, 31-34.

Eaton, D. L. and E. P. Gallagher, 1994. Mechanisms of aflatoxin carcinogenesis. Annual Review of Pharmacology 34, 135-172.

Koudande, D. O, 2013. A review on aflatoxin contamination and its implications in the developing world: a sub-Saharan African perspective. Critical Reviews In Food Science and Nutrition 53, 349-365.

Macalino, S.J., Gosu, V., Hong, S. and S. Choi, 2015. Role of computer-aided drug design in modern drug discovery. Archives of Pharmacal Research 38, 1686-1701.

Powers, C. N. and W. N. Setzer, 2015. A molecular docking study of phytochemical estrogen mimics from dietary herbal supplements. Powers and Setzer in Silico Pharmacology 3, 1-63.

Reed, K. F. M., Sprague, M., Mcfarlane, N. M. and J. R. Walsh, 2004. Zearalenone and its presence in pasture. Animal Production in Australia 25, 140-143.

Shoichet, B.K., S. L. Mcgovern, S.L., Wei,B and J. Irwin, 2002. Lead discovery using molecular docking. Current Opinion in Chemical Biology 6, 439-446.

Śledź, P. and A. Caflisch, 2017. Protein structure-based drug design: from docking to molecular dynamics 48, 93-102.

Urry, W.H., Wehrmeister, H.L., Hodge, E. B. and P. H. Hidy,1966. The structure of zearalenone. Tetrahedron Letters 7, 3109-3114.

Verdonk, M. L., Giangreco, I. R., Hall, J., Korb, O., Mortenson, P. N. and C. W. Murray, 2011. Docking performance of fragments and druglike compounds. Journal of Medicinal Chemistry 54, 5422-5431.

Insects and mycobiota in *Phaseolus vulgaris* L. grains sold in retail stores

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Abstract

In Brazil beans are an important protein source and the great variety of *Phaseolus* and *Vigna* beans grains are sold in retail markets. The objective of this study was to isolate fungi from insects and *Phaseolus vulgaris* (var. Pinto) from 15 samples of different retail stores in São Paulo. The samples were placed in Petri dishes containing culture medium of potato-dextrose-agar and incubated at 25°C for 7 days. Fungi were identified in several insects: *Callosobruchus maculatus* (yeasts - 50%), *Sitophilus* spp. (*Chaetomium* spp. – 3.1%; *Rhizopus stolonifer*- 3.1%; Non Sporulating Fungi (NSF) – 12.5% and *Eurotium chevalieri* - 9.4%, *Acanthoscelides obtectus* (*Penicillium* spp. – 18.5% and yeasts – 18.5%) and *Zabrotes subfasciatus* (*Alternaria alternata* – 13.6 % and *Penicillium* spp. – 41 %). No fungi were observed in the parasitoid *Dinarmus basalis*. In grain samples, the following fungi were found: *Penicillium* spp. (6%), *E. chevalieri* (5%), *R. stolonifer* (0.3%), *Aspergillus flavus* (3 %), NSF (8 %), Yeasts (2.6%), *Phoma* spp. (1.6%) and *Alternaria alternata* (3.6%).

Keywords: bean, grain, fungi, insects

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most widely consumed legumes in the world (Barros and Prudencio, 2016). Bean growers are looking for new storage options that maintain the quality of seed beans for use in planting and also for beans produced for the retail market. The most frequent causes of losses in storage beans are: insects, fungi and rodents. This causes the decrease in quality, as taste and the appearance of grain (Bragantini, 2005). The stored beans are mainly attacked by *Acanthoscelides obtectus* (Say), *Zabrotes subfasciatus* and *Callosobruchus maculatus* (Botelho, 2002). Insects are vectors for fungi and cause physical damage to the grain. The control of both effects is important in the safety and quality of stored grains (Aquino and Potenza, 2013). This study analyzed the insects and mycobiota in samples of *Phaseolus vulgaris* (pinto beans) purchased in several retail markets in São Paulo.

2. Materials and methods

Fifteen 1-kg samples of *Phaseolus vulgaris* (pinto bean) were purchased in retail markets of São Paulo. Samples were sieved and the insects collected using aspirator. Samples were held at 27 ± 2º C and 70 ± 5% relative humidity for 45 days, sieved and the emerged insects collected. For the fungal isolation, the samples were submitted to direct plating on potato dextrose agar (PDA) and