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In silico methods for metabolomic and toxicity prediction of zearalenone, α-zearalenone and β-zearalenone

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ABSTRACT

Zearalenone (ZEA), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (ZEA's metabolites) are co/present in cereals, fruits or their products. All three with other compounds, constitute a cocktail-mixture that consumers (and also animals) are exposed and never entirely evaluated, nor *in vitro* nor *in vivo*. Effect of ZEA has been correlated to endocrine disruptor alterations as well as its metabolites (α-ZEL and β-ZEL); however, toxic effects associated to metabolites generated once ingested are unknown and difficult to study. The present study defines the metabolomics profile of all three mycotoxins (ZEA, α-ZEL and β-ZEL) and explores the prediction of their toxic effects proposing an *in silico* workflow by using three programs of predictions: MetaTox, SwissADME and PASS online. Metabolomic profile was also defined and toxic effect evaluated for all metabolite products from Phase I and II reaction (a total of 15 compounds). Results revealed that products describing metabolomics profile were: from O-glucuronidation (1z and 2z for ZEA and 1 ab, 2 ab and 3 ab for ZEA's metabolites), S-sulfation (3z and 4z for ZEA and 4 ab, 5 ab and 6 ab for ZEA's metabolites) and hydrolysis (5z and 7 ab for ZEA's metabolites, respectively). Lipinsky's rule-of-five was followed by all compounds except those coming from O-glucuronidation (HBA>10). Metabolite products had better properties to reach blood brain barrier than initial mycotoxins. According to *Pa* values (probability of activation) order of toxic effects studied was carcinogenicity > nephrotoxic > hepatotoxic > endocrine disruptor > mutagenic (AMES TEST) > genotoxic. Prediction of inhibition, induction and substrate function on different isoforms of Cytochrome P450 (CYP1A1, CYP1A2, CYP2C9 and CYP3A4) varied for each compounds analyzed; similarly, for activation of caspases 3 and 8. Relying to our findings, the metabolomics profile of ZEA, α-ZEL and β-ZEL analyzed by *in silico* programs predicts alteration of systems/pathways/mechanisms that ends up causing several toxic effects, giving an excellent sight and direct studies before starting *in vitro* or *in vivo* assays contributing to 3Rs principle; however, confirmation can be only demonstrated by performing those assays.

1. Introduction

Mycotoxins are low-molecular-weight toxic compounds synthetized by different types of molds belonging mainly to the genera *Aspergillus, Penicillium, Fusarium* and *Alternaria* (Berthiller et al., 2013; Juan et al., 2020; Pascari et al., 2019). Effects associated are diverse according to the chemical structure which provides a great variety in ADME/T characteristics (absorption, distribution, metabolism, and excretion/toxicity) and still to elucidate for most of them.

Zearalenone (ZEA) is a *Fusarium* mycotoxin of primary concern. It is commonly found in cereals like barley, sorghum, oats, wheat, millet, and rice (Juan et al., 2017a, 2017b; Stanciu et al., 2017; Bakker et al., 2018; Oueslati et al., 2020). When ingested and metabolized, two major metabolites, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), can be found in various tissues; nonetheless, their presence is starting to be commonly found in food and feed as natural contaminants (EFSA et al., 2011, 2017). Once ingested by the consumer, further metabolite products from all three mycotoxins (ZEA, α-ZEL and β-ZEL) can be generated by Phase I and II reactions, although their effect is unknown. Studies of these compounds contribute to metabolomics profile for following the compound transformation (metabolic changes) whose identification and quantification will help to elucidate the complete toxic effects. It can help to understand global metabolic disturbances.

Effects associated to ZEA, α-ZEL and β-ZEL have been studied *in vitro* and *in vivo* and estrogenic effect, oxidative stress, cytotoxicity, DNA damage, among others have been reported (Eze et al., 2019; Frizzell

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et al., 2011; Agahi et al., 2020; Juan-García et al., 2020). On the other hand, the entire implication of these compounds in producing toxic effects are unknown, same as with its metabolite products originated in Phase I and II reactions. So that, there are many indirect or side effects associated yet not studied and their involvement in pathways, cascade or routes still need to be discovered. Nowadays, the development of computational and informatics programs facilitates to predict experimental approaches in toxicology which need to be confirmed with further assays. These systems use chemical structures, parameters and descriptors which by comparison with other studied compounds, can give as a result empirical knowledge of their effect to prevent against exposure or even to promote the development of therapeutics to avoid or decrease toxic effects, concerning drugs.

Combination of compounds is a routine practice in medicine for palliate diseases achieving successful results. Previous to this practice it is necessary to evaluate the potential effects that this might cause. For toxic compounds there have been developed several mathematical methods implemented in informatics programs for assessing the effect of compounds combination and effects contributing to computational toxicology: Chou and Talalay by using isobolograms, Simple Addition of Effect, Factorial Analysis of Variance by using simple 2-way ANOVA, Bliss Independence Criterion, Loewe's Additivity Law, Highest Single Agent (HSA) Model (Gaddums non-interaction), etc. (Kifer et al., 2020). For mycotoxins' mixture assessment, Choy and Talalay method has been widely used in predicting potential effects (synergism, addition and antagonism) (Juan-García et al, 2016, 2019a, 2019b; Agahi et al., 2020) even with strong differences in chemical structures as well as in the variety of fungi *spp*. producer.

The global research scenario for new therapies and development of new drugs for common diseases, or as it is happening nowadays in the global world pandemic SARS-COVID-19 for health side-effects, the use of virtual screening techniques for helping in the discovery of new strategies and without using or avoiding long-term biological assays, is a good alternative. All these strategies end-up in exploring profile of effects by application of computer programs. One of this alternative programs is PASS online (Prediction of Activity Spectra for Substances) an *in silico* approach that reveals biological activities of compounds, their mechanisms of action and connected side-effects (Lagunin et al., 2000). The available PASS online version predicts over 4000 kinds of biological activity, including pharmacological effects, mechanisms of action, toxic and adverse effects, interaction with metabolic enzymes and transporters, influence on gene expression, etc. as described on its web page (www.pharmaexpert.ru/passonline) (Lagunin et al., 2000). Prediction is based on the analysis of structure activity-relationships for more than 250,000 biologically active substances including drugs, drug-candidates, leads and toxic compounds (Lagunin et al., 2000).

The support of new compounds discoveries and knowledge of its toxicity is given by other on-line programs which work with different parameters, some of them are: SwissADME, Meta-Tox, GUSAR, ROSC-Pred, etc. Each program is focused in providing different predictions, and for example while MetaTox predicts the Phase I and II metabolite products that can be generated from one compound (Rudik et al., 2017), SwissADME is a computational program that allows to compute physicochemical descriptors as well as ADME parameters, pharmacokinetic properties, drug-like nature and medicinal chemistry friendliness of one or multiple small molecules (Daina et al., 2017).

To escape long-term biological assays and implementing the computational programs for testing compounds and their predicted metabolites, here it is presented an *in silico* working procedure and the prediction of the entire potential effects of three mycotoxins (zearalenone (ZEA), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL)) and its Phase I and II metabolite products, by using three *in silico* programs described for computational toxicology: MetaTox, SwissADME and PASS online; all available on-line.

2. Materials and methods

Mycotoxins herein studied for this predictive *in silico* study displayed endocrine disruptor effects associated and correspond to: zearalenone (ZEA) (MW: 318,37 g/mol), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (MW: 320,38 g/mol) (Fig. 1).

2.1. Procedure followed (workflow)

Firstly, prediction of Phase I and II metabolites products was obtained by MetaTox software (http://way2drug.com/mg2/) with a molecular sketcher based on Marvin JS chemical editor. This editor is used for input and visualization of molecular structure (in canonical SMILE) of each mycotoxin, obtaining a metabolomic profile. "No-limit" in metabolite likeness and "all" reactions in predicting metabolites for drawn structure were selected (Rudik et al., 2017). Secondly, all compounds predicted from reactions and mycotoxins were evaluated through i)SwissADME by obtaining physicochemical descriptors (http://www.swissadme.ch/index.php) (Daina et al., 2017; Cheng et al., 2012; Yang et al., 2018) and following the Lipinski's rule of five (RO5) (see section 2.2. below) and ii)SwissSimilarity which provides an identification number HMDB (Human Metabolome Database version 4.0, https://hmdb.ca/) with a score associated (Zoete et al., 2016). Afterwards, all compounds were predicted as active compounds or inactive compounds according to probability of activation values (Pa) and probability of inactivation values (Pi), respectively; as well as their biological activities through PASS online software (http://www.pharmaexpert.ru/passonline/info.php) (Workflow 1). Lastly, potential toxic effects were predicted for Pa > Pi with PASS online software.

2.2. In silico software: MetaTox, SwissADME and PASS online

Three *in silico* softwares available online for studying prediction of toxicity and biological activities were used: MetaTox, SwissADME and PASS online.

MetaTox is a software based in generating metabolites and calculating probability of their formation where metabolism pathway generation is integrated with the prediction of acute toxicity. Metabolomics' profile is predicted by the formation from nine classes of reactions (aliphatic and aromatic hydroxylation, N and O-glucuronidation, N-, S- and C-oxidation, and N- and O-dealkylation) that are catalyzed by five human isoforms of cytochromes P450s (1A2, 2C19, 2C9, 2D6, 3A4) and by human UDP glucuronosyltransferase without differentiation into isoforms. The calculation of probability for generated metabolites is based on analyses of "structure-biotransformation reactions" and "structure-modified atoms" relationships using a Bayesian approach (Rudik et al., 2017).

SwissADME is a web tool that enables to predict the computation of key physicochemical properties, pharmacokinetics, mycotoxin-likeness and medicinal chemistry friendliness (for one or multiple molecules), (Daina et al., 2017; Cheng et al., 2012; Yang et al., 2018). This predictive *in silico* model shows statistical significance, predictive power, intuitive interpretation, and straight forward translation to molecular design. This program uses Lipinski's rule-of-five (RO5) for the lead compounds. The compounds were then filtered through that rule (RO5) to predict their mycotoxins likeliness. Lipinski's descriptors evaluate the molecular properties for compound pharmacokinetics in the human body, especially for oral absorption. The rule states molecules to have: molecular weight (MW) $\leq$500, number of hydrogen bond donors (HBD) $\leq$5, number of hydrogen bond acceptors (HBA) $\leq$10, cLogP $\leq$5 and number of rotable bounds (n-ROTB) $\leq$10. Molar reactivity in the range of 40–130 and topological polar surface area (TPSA) were also considered. Targets of p-glycoprotein (P-gp) efflux and isoforms of cytochrome P450 that metabolize the majority of toxic compounds (CYP3A4, CYP2C9, CYP2C19, CYP1A1 and CYP1A2) were investigated.

The biological prediction of activity spectra for mycotoxins and

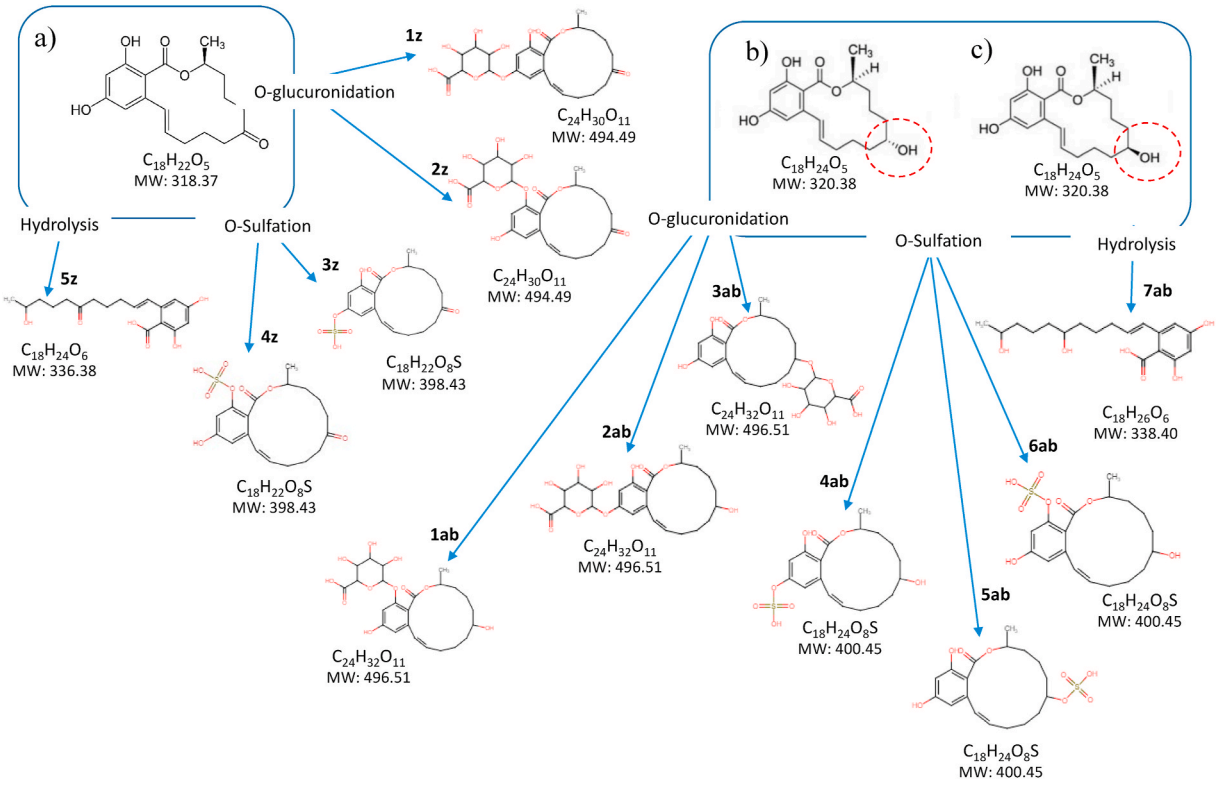
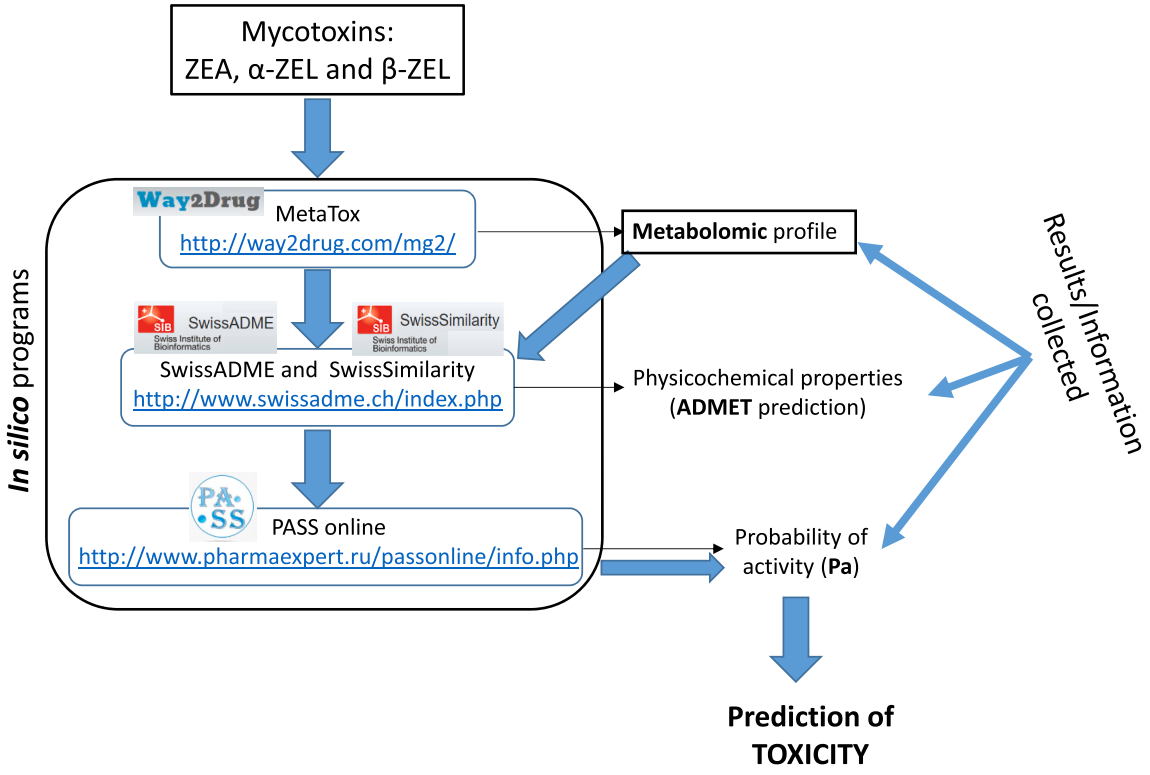


Fig. 1. Metabolomic profile and chemical structures of mycotoxins predicted by MetaTox: ZEA (a), α-ZEL (b) and β-ZEL (c).



Workflow 1. Procedure followed to predict the toxic effect of mycotoxins and its metabolite products by using different in silico programs.

metabolite products were obtained by PASS online (available in www.pharmaexpert.ru/passonline) (Lagunin et al., 2000). This software was used to evaluate the general biological potential of all compounds and provided simultaneous prediction of several types of biological activity based on their chemical structure. It also estimated the predicted activity spectrum of mycotoxins as probable activity (Pa, probability to be active) and probable inactivity (Pi, probability to be inactive). Both probabilities, Pa and Pi values, vary from 0.000 to 1.000; nevertheless,

values are expressed as percentage of probability (%).

Among all toxic effects for all three mycotoxins and products of Phase I and II reactions provided from PASS, prediction was evaluated for: carcinogenesis, endocrine disruption, nephrotoxicity, mutagenicity (with and without AMES test), genotoxicity and hepatotoxicity. Biological activities prediction inhibiting, inducing or as substrate was evaluated for different isoforms of Cytochrome P450 and caspases 3 and 8. All predictions of probabilities were expressed as percentage of probability (%).

3. Results

3.1. Meta-Tox for predicting metabolite products: describing the metabolomics profile

Metabolite prediction included in MetaTox uses dictionaries of biotransformation based on preliminary prediction of possible classes of biotransformation describing also the metabolomics profile of the compounds. Mycotoxins' canonical SMILE structure were used to predict metabolite products in MetaTox. Fig. 1 collects chemical structure of mycotoxins and metabolite products predicted by MetaTox (five from ZEA (from 1z to 5z) and 7 for each ZEA's metabolite (from 1 ab to 7 ab)). Metabolite products predicted for ZEA were from: reaction of O-glucuronidation (metabolites 1z and 2z), reaction of S-sulfation (metabolites 3z and 4z) corresponding to Phase II products and one from reaction of hydrolysis (5z) corresponding to Phase I products. For α-ZEL and β-ZEL, products were equal for each one with a total of seven products for each isoform and corresponding to same reactions as ZEA: O-glucuronidation (metabolites: 1 ab, 2 ab and 3 ab), S-sulfation (metabolites: 4 ab, 5 ab and 6 ab) and hydrolysis (metabolite 7 ab) reactions. A total of 12 compounds were proposed as predicted metabolites products form Phase I and II reactions.

3.2. SwissADME for physicochemical descriptors of zearalenone, α-zearalenol, β-zearalenol and phase I and II metabolite products

Target of mycotoxins in organs and systems are wide and unknown for most of them; however, they are able to activate several routes or pathways. ZEA, α-ZEL and β-ZEL were analyzed through SwissADME online sever for molecular properties to validate them as potential inducers/activators of toxic mechanisms. All three mycotoxins were filtered through Lipinski's RO5 to predict their mycotoxin likeliness (Table 1). All three mycotoxins and metabolite products were studied and only metabolites coming from O-glucuronidation of ZEA (metabolites 1z and 2z) or α-ZEL and β-ZEL (metabolites 1 ab, 2 ab and 3 ab) violated Lipinski's rule because of HBA (hydrogen bond acceptor) (Table 1). It is also reported the human metabolome database identification number (HMDB ID) and the score of similarity predicted provided from SwissSimilarity. All compounds had one or more HMDB ID with score >50% (Table 1). To notice that values were the same for metabolite products coming from the same metabolization reaction.

Probability for ADMET and toxicity profile for all compounds was evaluated. Table 2 reports values for mycotoxins, while Table 3 for metabolite products of Phase I and II's reactions of all three mycotoxins. Results reveal that ZEA mycotoxin has very low prediction for BBB crossing (28.22%) and similar tendency was obtained for α-ZEL and

Table 2
Probability of ADMET and toxicity profile for ZEA, α-ZEL and β-ZEL.

	ZEA	α-ZEL and β-ZEL
	Probability (%)	Probability (%)
Absorption & Distribution		
BBB	28.22	31.47
HIA	97.61	97.50
P-gp substrate	85.50	84.12
Caco-2 permeability	48.84	59.94
LogPapp (cm/s)	−5.67	−5.39
Metabolism		
CYP450 2C9 substrate	57.95	60.44
CYP450 2D6 substrate	86.69	83.54
CYP450 3A4 substrate	55.40	57.08
CYP450 1A2 inhibitor	68.95	76.60
CYP450 2C9 inhibitor	84.90	89.37
CYP450 2D6 inhibitor	91.60	90.07
CYP450 2C19 inhibitor	75.95	72.46
CYP450 3A4 inhibitor	79.60	76.82
Toxicity		
AMES toxicity	90.0	85.00
Carcinogens	90.0	66.04
Rat acute toxicity (LD_{50}, mol/kg)	1.88	1.94

BBB: blood-brain barrier; HIA: human gastrointestinal absorption; P-gp: P-glycoprotein.

Table 1
Lipinski's molecular descriptors for ZEA, ZEA's metabolites (α-ZEL and β-ZEL) and its products of reaction (from O-glucuronidation, O-sulfation and hydrolysis) from SwissADME and SwissSimilarity.

	HMDB ID	MW(≤500)	HBD (≤5)	HBA(≤10)	cLog P (<5)	MR (≤10)	n-ROTB(≤10)	TPSA
ZEA	31,752 (99.6%)	318.37	2	5	3.58	88.40	0	83.83
O-Glucuronidation								
Metabolite 1z*	34,753 (74.1%)	494.49	5	11*	1.14	121.13	3	180.05
Metabolite 2z*	60,634 (84.3%)							
O-Sulfation								
Metabolite 3z	33,623 (99.6%)	398.43	2	8	3.06	98.60	2	135.58
Metabolite 4z	31,752 (87.6%)							
Hydrolysis								
Metabolite 5z	31,752 (52.4%)	336.38	4	6	3.10	92.16	10	115.06
α-ZEL and β-ZEL	41,838 (99.8%) 41,824 (99.7%)	320.38	3	5	3.37	89.36	0	86.99
O-Glucuronidation								
Metabolite 1 ab*	34,753 (86.8%)	496.51	6	11*	0.94	122.09	3	183.21
Metabolite 2 ab*	60,634 (75.6%)							
Metabolite 3 ab*	31,752 (53.9%)							
O-Sulfation								
Metabolite 4 ab	33,623 (91.5%)	400.45	3	8	2.85	99.56	2	138.74
Metabolite 5 ab	31,752 (90.4%)							
Metabolite 6 ab	41,838 (91.1%)							
Hydrolysis								
Metabolite 7 ab	41,824 (50.6%)	338.40	5	6	2.89	93.12	10	118.22

HMDB ID = Human Metabolome Database Identification; MW = Molecular weight; g/mol (acceptable range: <500); HBD = Hydrogen bond donor (acceptable range: ≤5); HBA = Hydrogen bond acceptor (acceptable range: ≤10); cLogP = High lipophilicity (expressed as LogP, acceptable range: <5); MR = Molar refractivity (acceptable range: 40–130); n-ROTB: number of rotatable bounds; TPSA = Topological polar surface area; Å2. *Denotes violation of Lipinski's RO5.

Table 3
Probability of ADMET and toxicity profile of products predicted by MetaTox from ZEA α-ZEL and β-ZEL.

	Metabolomic profile of ZEA					Metabolomic profile of α-ZEL and β-ZEL						
Reaction	O-Glucuronidation		S-Sulfation		Hydrolysis	O-Glucuronidation			S-Sulfation			Hydrolysis
Metabolites	1z	2z	3z	4z	5z	1 ab	2 ab	3 ab	4 ab	5 ab	6 ab	7 ab
Probability (Prob)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)	Prob (%)
Absorption & Distribution												
BBB	37.65	37.65	97.05	97.05	79.17	31.47	50.00	37.65	97.00	97.04	97.00	79.17
HIA	72.33	70.65	95.94	96.20	96.75	97.50	68.84	71.40	95.72	96.97	95.90	97.43
P-gp substrate	89.04	78.58	82.69	75.15	75.38	84.12	78.48	80.17	81.59	80.54	73.72	73.06
Caco-2 permeability	81.87	87.20	76.48	80.89	62.41	59.94	86.25	86.09	66.51	55.72	70.99	61.16
LogPapp (cm/s)	−7.85	−8.24	−6.58	−6.97	−6.51	−7.96	−7.57	−7.42	−6.29	−6.14	−6.69	−6.16
Metabolism												
CYP450 2C9 substrate	100	100	79.13	59.58	59.92	60.44	79.88	80.22	58.95	61.28	61.74	61.90
CYP450 2D6 substrate	87.97	88.12	86.54	86.41	86.75	83.54	87.85	87.74	85.62	86.69	85.35	86.83
CYP450 3A4 substrate	63.85	64.20	60.69	61.92	50.71	57.08	64.36	63.41	62.04	60.19	63.23	51.50
CYP450 1A2 inhibitor	57.71		74.19		73.02	76.60	53.79	57.71	69.70	72.83	69.70	64.06
CYP450 2C9 inhibitor	92.01		82.74		84.24	89.37	92.95	92.01	82.61	81.81	82.61	79.70
CYP450 2D6 inhibitor	92.29		87.55		90.45	90.07	91.41	92.29	87.62	86.89	87.62	90.48
CYP450 2C19 inhibitor	74.09		77.83		82.96	72.46	79.05	74.09	75.21	76.29	75.21	74.04
CYP450 3A4 inhibitor	73.18		84.7		64.02	76.82	73.89	73.18	75.62	78.53	75.62	61.88
Toxicity												
AMES toxicity	68.00	66.00	73.00	66.00	79.00	85.00	67.00	70.00	68.79	76.79	60.79	74.00
Carcinogens	65.75	65.74	88.57	88.57	77.10	66.04	61.54	65.74	62.12	64.01	62.12	75.52
Rat acute toxicity (LD50, mol/kg)	2.65	2.22	2.50	2.03	2.36	1.94	2.36	2.45	2.77	2.37	2.3	2.27

BBB: blood-brain barrier; HIA: human gastrointestinal absorption; P-gp: P-glycoprotein.

β-ZEL (31.47%). However, high gastrointestinal absorption was reported for all three mycotoxins (HIA >97%, Caco-2 permeability >48% and P-glycoprotein substrate >84%) (Table 2). The results indicate moderate to high absorption by the gastrointestinal tract, but unlikely to penetrate into the brain on its current form unless metabolized (Table 3). Distribution (P-gp substrate) was favored with probability >84%. For metabolism prediction, several cytochrome P450 (CYP450) isoenzymes were evaluated showing similar pattern for all three mycotoxins. Probability of ZEA as substrate in CYP450 went from 55.40% (isoform 3A4) to 86.69% (isoform 2D6); while as inhibitor of CYP450 from 68.95% (isoform 1A2) to 91.60% (isoform 2D6). For α-ZEL and β-ZEL, as substrates of CYP450 probability went from 60.44% (isoform 2C9) to 83.54% (isoform 2D6); while as substrate from 72.46% (isoform 2C19) to 90.07% (isoform 2D6) (Table 2). For toxicity evaluation, ZEA reported higher values than α-ZEL and β-ZEL (Table 2).

For Phase I and II metabolite products of all three mycotoxins, ADMET probability values revealed that all 12 compounds (5 metabolite products from ZEA and 7 products from α-ZEL and β-ZEL) were able to pass the gastrointestinal tract (>70%), especially metabolite products originated in S-Sulfation and hydrolysis. Probability of BBB crossing was >95% for all same metabolites originated in same reaction mentioned above although quite low for O-glucuronidation metabolite products (<37%) (Table 3). Distribution (P-gp substrate) was favored for all compounds originated from all reactions (>73%). It is noticed that as long as the Phase I and II reactions take place, metabolite products become more suitable to reach BBB compartment (Table 3).

In metabolism, all ZEA's predicted products were substrate of CYP450 with probability from 100% (metabolites 1z and 2z) to 59.58% (metabolite 4z); while for α-ZEL and β-ZEL metabolites predicted products, it ranged from 51.5% (metabolite 7 ab) to 87.85% (metabolite 2 ab) (Table 3). Compounds were predicted as inhibitor for CYP450 with probabilities from 57.71% to 92.29% (metabolites 1z and 2z) for ZEA's predicted products; while from 53.79% (metabolite 2 ab) to 92.29% (metabolite 3 ab) for α-ZEL and β-ZEL's predicted products (Table 3). To notice that as inhibitors of CYP450 (for all five isoenzymes), ZEA's predicted products from O-glucuronidation (metabolites 1z and 2z) and S-sulfation (metabolites 3z and 4z) revealed the same probability; while this happened in α-ZEL and β-ZEL predicted products from S-sulfation (metabolites 4 ab and 6 ab) (Table 3).

Lastly in terms of toxicity evaluation, probability measured for AMES toxicity oscillated between 60.79% and 85% of no-AMES toxicity and carcinogenicity from 62.12 to 88.57%. Rat acute toxicity oscillated from 1.94 to 2.77 mol/kg.

3.3. Prediction of toxic effects by PASS online

Mycotoxins and products from metabolomics profile were studied by PASS online (Workflow 1). To validate them as suitable inducers/activator candidates, PASS online server was used which predicts possible effects of a compound based on its structural information. This tool compares more than 300 effects and biochemical mechanisms of compounds and gives the probability of activity (Pa) and inactivity (Pi) (Hasan et al., 2019).

Fig. 2 shows the probability for seven different toxic effects: carcinogenicity, endocrine disruptor, nephrotoxic, mutagenicity (and AMES test), genotoxicity and hepatotoxicity. It can be observed that ZEA had the highest probability in reporting carcinogenicity (78.2%); while α-ZEL and β-ZEL in genotoxicity (88.4%) (Fig. 2A). Among toxic effects studied, for all metabolite products (5 from ZEA and 7 from α-ZEL and β-ZEL), carcinogenicity reported the highest probability for all three mycotoxins followed by nephrotoxic > hepatotoxic > endocrine disruptor > mutagenic (AMES TEST) > genotoxic (Fig. 2B). Nonetheless, metabolite products from ZEA mycotoxin had the broadest range of probability in all toxic effects studied. Details of toxic effects per metabolite product from Phase I and II reactions are reported in Supplementary 1. Regarding the carcinogenicity effect predictions in rat and mouse (male and female), and the IARC classification is reported in Supplementary 2.

3.4. Prediction of biological activities by PASS online

Biological activities predicted by PASS online are reported in Figs. 3 and 4. It has been divided in one hand the most common isoforms of cytochrome P450 involved in metabolizing toxic compounds (Fig. 3); and in the other hand, cysteine proteases enzymes which are primary effectors in cell death: caspase 3 and caspase 8 (Fig. 4).

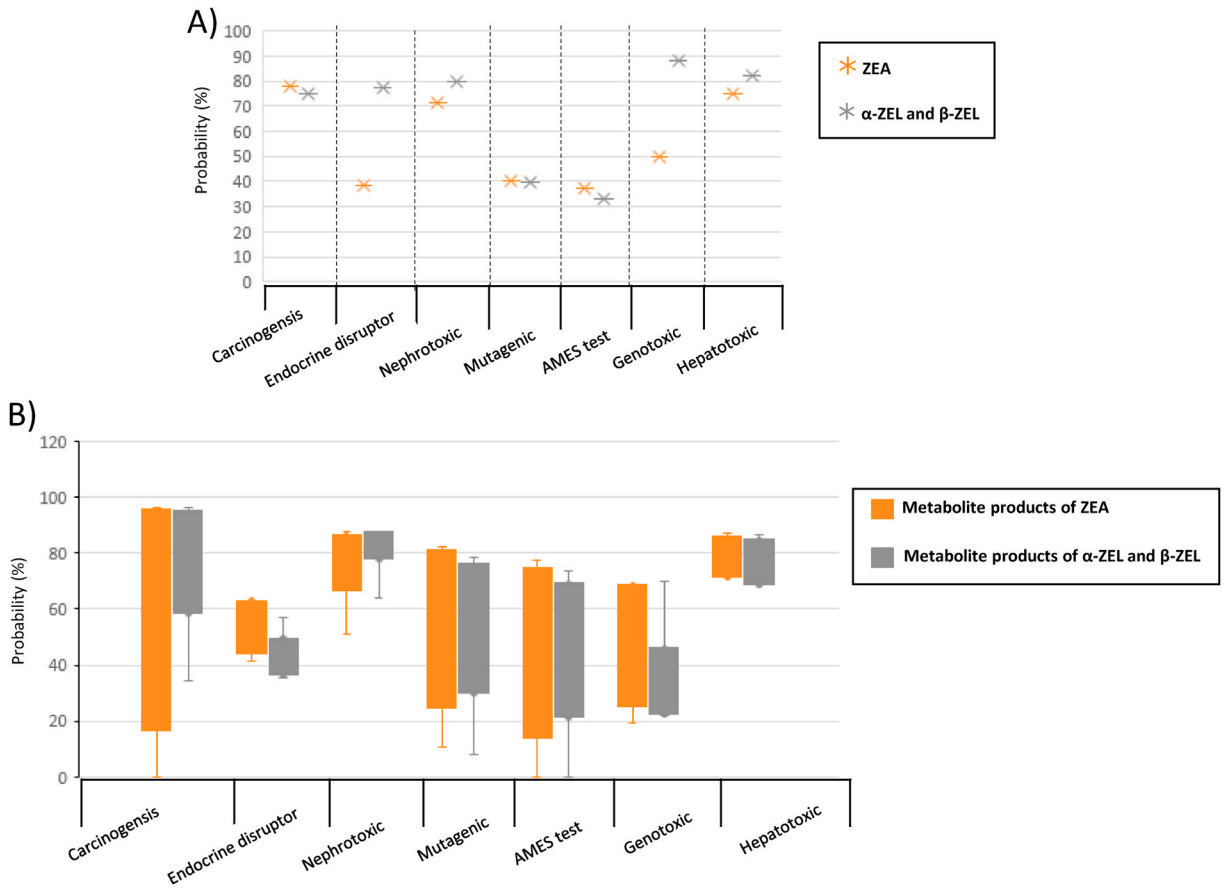


Fig. 2. Prediction of toxic effects (probability, %) for ZEA (orange star), α-ZEL and β-ZEL (grey star) (A) and all metabolite products (B, box diagram) of Phase I and II reactions obtained from those mycotoxins: ZEA (orange box) and ZEA's metabolites (grey box). Bars in (B) report the maximum and minimum value of prediction out of the box. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4.1. Cytochrome P450

Prediction effects on isoforms of Cytochrome P450 (CYP1A1, CYP1A2, CYP2C9 and CYP3A4) are reported in Fig. 3 for all three mycotoxins and compounds defined in the metabolomics profile (from Phase I and II reactions). Effects are reported for each compounds acting as substrate, inducer or inhibitor. For all CYP450 isoforms all three mycotoxins reported effect as substrates, inducers and inhibitors; however, α-ZEL and β-ZEL reported higher probability prediction than ZEA in all of them independently of its mode of action (Fig. 3).

In detail, for isoform CYP1A1, all compounds had effects on it (Fig. 3A). Metabolite products coming from α-ZEL and β-ZEL had slightly higher probability prediction as substrate (>37%) than ZEA (>35%) for all O-glucuronidation, S-sulfation and hydrolysis products; as inducers, only metabolite products coming from O-glucuronidation reported this prediction effects. Finally, as inhibitor, only metabolite 5z from hydrolysis of ZEA and 6 ab from S-sulfation of α-ZEL and β-ZEL presented such prediction both in 30% (Fig. 3A).

For isoform CYP1A2, ZEA metabolite products had effects on it as substrate, except those coming from S-sulfation; and products of S-sulfation from α-ZEL and β-ZEL had no-effect (Fig. 3B). As inducers of this isoform (CYP1A2), only metabolite products of S-sulfation from ZEA (3z and 4z) were predicted in 16%. As inhibitor none of the compounds reported prediction in this direction (Fig. 3B).

For isoform CYP2C9, ZEA, α-ZEL and β-ZEL were predicted as substrate; while only ZEA as inducer and α-ZEL and β-ZEL as inhibitor (Fig. 3C). For metabolite products coming from O-glucuronidation of these mycotoxins all were predicted as i) substrate: 54% for those coming from ZEA and >60% for those coming from α-ZEL and β-ZEL; and as ii) inducers: >38% for all those coming from ZEA and from α-ZEL and β-ZEL. Metabolite product of hydrolysis coming from ZEA (5z) was predicted only as inducer (26%); while that coming from α-ZEL and β-ZEL (7 ab) was predicted as substrate (22%), inhibitor (23%) and inducer (26%). However, no-effect was predicted for S-sulfation compounds (neither as substrate, inhibitor or inducer).

Finally, ZEA, α-ZEL and β-ZEL were predicted as substrate and inducers with probabilities >60% for isoform CYP3A4 (Fig. 3D). All metabolite products from ZEA of O-glucuronidation and S-sulfation were predicted as substrate ranging from 32% (2z) to 61% (4z); and inducers ranging from 57% (4z) to 80% (1z). No effect was predicted for its hydrolysis product (5z). Similar prediction effect was observed for metabolite products from α-ZEL and β-ZEL as substrates ranging from 38% (1 ab) to 81% (5 ab) and as inducers ranging from 58% (6 ab) to 81% (3 ab). The hydrolysis product 7 ab, was only predicted as substrate (35%) (Fig. 3D).

3.4.2. Caspases 3 and 8

Caspases are involved in cascade activation of cell death, occurring either naturally or by exposure to toxic compounds. Prediction for caspases 3 and 8 activation (stimulation) is reported in Fig. 4A and B, respectively of all 15 compounds. Prediction of activation of both caspases, 3 and 8, was higher for α-ZEL and β-ZEL (86% and 49% for caspase 3 and 8, respectively) than for ZEA (73% and 43% for caspase 3 and 8, respectively).

Caspase 3 was activated for all compounds studied and for metabolite predicted from α-ZEL and β-ZEL probability was higher than those from ZEA (Fig. 4A). Metabolite products of i) O-glucuronidation from α-ZEL and β-ZEL reported caspase activation >80% while those from ZEA <77%; ii) S-sulfation from α-ZEL and β-ZEL reported caspase

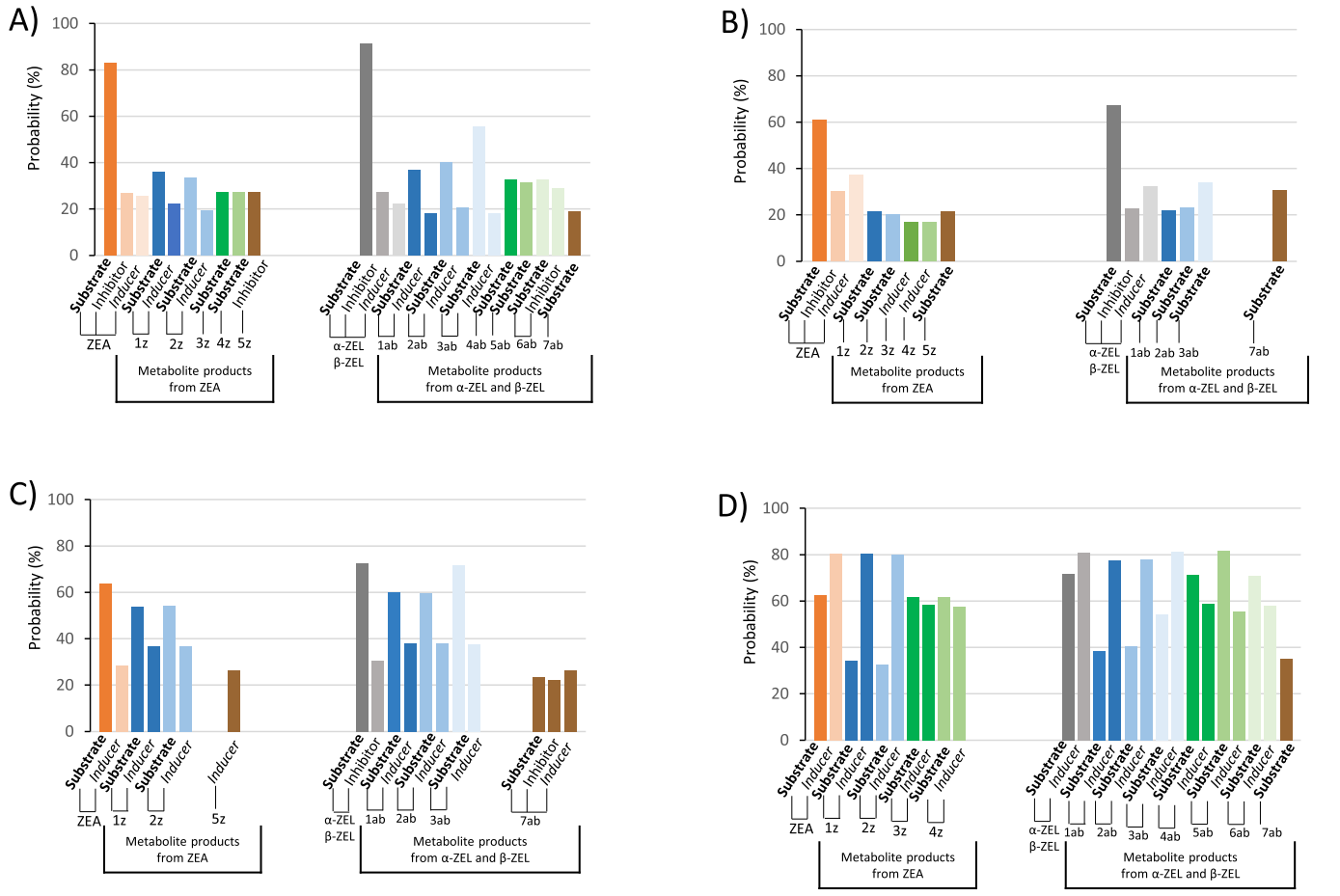


Fig. 3. Prediction of inhibition, induction and substrate function of different isoforms of Cytochrome P450 (probability, %) that metabolize the majority of xenobiotics: CYP1A1 (A); CYP1A2 (B); CYP2C9 (C) and CYP3A4 (D). Prediction is reported for each metabolite product from ZEA (from dark to light orange), α-ZEL and β-ZEL (from dark to light grey). O-glucuronidation products (from dark to light blue), S-sulfation products (from dark to light green) and hydrolysis products (in brown). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activation >36% while those from ZEA <30% and iii) hydrolysis from α-ZEL and β-ZEL reported caspase activation 33% while those from ZEA 35% (Fig. 4A).

For caspase 8, ZEA metabolite products reported prediction of activation only from those coming from O-glucuronidation and hydrolysis, from 56% to 29%, respectively (Fig. 4B); while those metabolites products coming from α-ZEL and β-ZEL reported activation of caspases from 51% (1 ab) to 60% (3 ab) for O-glucuronidation products, from 25% (6 ab) to 27% (4 ab) for S-sulfation products and 34% (7 ab) for the hydrolysis product (Fig. 4B).

4. Discussion

The present study explores the prediction of toxicity of three mycotoxins (ZEA, α-ZEL and β-ZEL) and products defining its metabolomics profile by proposing an *in silico* workflow and by using three software of computational toxicology: MetaTox, SwissADME and PASS online. All three mycotoxins are well-known to be copresent in food and feed not following good manufacture/agricultural practices, generating a public health concern as well as agricultural economic losses. Its effect as endocrine disruptor has been widely reported although the implications of its metabolite products regarding that toxic effects (or others) are unknown.

The workflow proposed, uses MetaTox to obtain the metabolite products formed during Phase I and II reactions, contributing to describe the metabolomics profile (Rudik et al., 2017); SwissADME (Daina et al., 2017) here it has been used for assessing the ADMET processes suffered by three mycotoxins (ZEA, α-ZEL and β-ZEL) and its metabolites products (1z-5z for ZEA and 1 ab-7ab for α-ZEL and β-ZEL); and PASS online, predicted the toxic effect of activation and the biological activities with probability values (Pa, probability of activation). Different parameters are used for each software program which help in predictions, but as it occurs with *in vitro* or *in vivo* studies, they must be prudently assessed (Workflow 1).

Metabolites products predicted through MetaTox for the mycotoxins studied came from two Phase II reactions: O-glucuronidation and S-sulfation. Both are detoxication reactions of first line facilitating excretion. ZEA was predicted to generate two metabolites for each type of reaction (from 1z to 4z); while for α-ZEL and β-ZEL three metabolites (from 1 ab to 6 ab) (Fig. 1 and Table 1). For Phase I reaction, only hydrolysis reaction was predicted to take place from ZEA, α-ZEL and β-ZEL, generating only one metabolite product, 7z and 7 ab for ZEA and ZEA's metabolites, respectively. In summary a total of 12 compounds defined the metabolomic profile of ZEA, α-ZEL and β-ZEL (Fig. 1 and Table 1). Coinciding with other studies, these reactions take place and generate these compounds; however, their effects are unknown; in fact, the use of these metabolite products as biomarkers have been found in the literature in biomonitoring studies (Lorenz et al., 2019; Follmann et al., 2016; Shephard et al., 2013; Wallin et al., 2015; Gerding et al., 2015) or directly detected in food and aromatic plants as masked mycotoxins (Berthiller et al., 2006, 2009; Mannani et al., 2019). However, an analysis of *in silico* prediction of toxic effects defined by the metabolomics profile is here the first time reported. EFSA has dealt in assessing the risk of ZEA, α-ZEL and β-ZEL and has indicated that metabolites

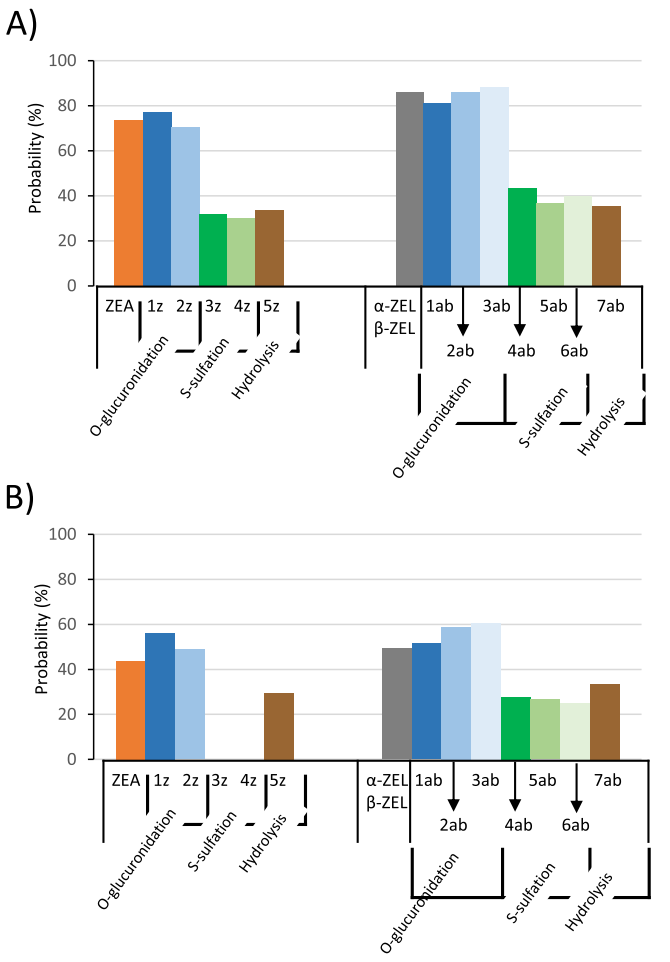


Fig. 4. Prediction of caspases activation (probability, %) implicated in cell death pathway: caspase 3 (A) and caspase 8 (B). Graphics are reported for ZEA, α-ZEL, β-ZEL and metabolites products of those generated during Phase I and II reactions: Oglucuronidation (in blue): 1z and 2z from ZEA, and 1 ab, 2 ab and 3 ab from ZEA's metabolites; S-sufation (in green): 3z and 4z from ZEA, and 4 ab, 5 ab and 6 ab from ZEA's metabolites; and hydrolysis (in brown): 5z from ZEA and 7 ab from ZEA's metabolites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

products coming from them (also reported as modified forms) might have effects (oestrogenic effect, genotoxicity, endocrine receptor, …) (EFSA, 2011 and 2014) and contribute to the exposure evaluation but the uncertainty exists as there is a lack of data which entails difficulties in defining its toxic effects (EFSA et al., 2014, 2016, 2017). Not to mention the gap in effects of its mixtures or with other mycotoxins or contaminants.

In silico analysis show that ZEA, α-ZEL and β-ZEL are poorly achieving the BBB, have good distribution and are highly favored to be absorbed gastrointestinally (Table 2). The interesting point noticed with the analysis of metabolites products of these mycotoxins, obtained from O-glucuronidation, S-sulfation and hydrolysis reactions, is that these properties change inversely, especially for achieving the BBB (see values from Tables 2 and 3) from low values to high values. There are studies coinciding and others opposite to the results predicted in here when compared with those reported by *in vitro* and *in vivo* studies. For all three mycotoxins it has been reported a good gastrointestinal absorption (rapid and extensive) as well as the formation of metabolites from hydrolysis, sulfation and glurcuronidation (Biehl et al., 1993; Frizzell et al., 2015; Pfeiffer et al., 2011; Plasencia et al., 1991); in fact, several strategies and recommendations have been also considered for the entire risk assessment (EFSA 2017; Lorenz et al., 2019). Optimal gastrointestinal absorption predicted by Lipinsky RO5 is reported in Table 1 for the metabolomics profile. It also indicates that the probability of one compound to be absorbed orally is directly related to the ADMET and toxic effects. Only metabolites coming from O-glucuronidation were not following the Lipinsky's RO5 (HBA>10), because of not passing the gastrointestinal barrier; however, mycotoxins, and metabolites from S-sulfation and hydrolysis reactions did which indicates their good distribution.

Toxic effects associated to compounds from metabolomics profile and mycotoxins seem to contribute one to another. Related to this, EFSA has indicated to assume the toxic effects of one compound as the sum of all metabolites coming from that compound (EFSA, 2011; Lorenz et al., 2019). Nonetheless, it is possible to analyze individual predictions *in silico*. The most common effect associated to ZEA as well as ZEA's metabolites is as endocrine disruptors with a ranking of oestrogenic potential effect established by EFSA as follows: α- ZEL > ZEN > β-ZEL (EFSA 2011). Besides this common and demonstrated toxic effect through *in vitro* and *in vivo* assays (EFSA 2017; Eze et al., 2019), other effects according to several parameters can be predicted (Fig. 2A) as well as for its metabolite products (Fig. 2B). According to the analysis of main effects predicted *in silico* for ZEA, α- ZEL, β-ZEL and its metabolite product defining the metabolomic profile, carcinogencity is the toxic effect predicted with high probability; however, IARC has classified ZEA (since 1993) as Group 3 (not classifiable as to their carcinogenicity to humans) based on inadequate evidence in humans and limited evidence in experimental animals (IARC 1993); to mention different behave in mice and mouse with limited evidence reported. This explains the prediction described in Fig. 2, which although carcinogenicity indicates high probability (80–90%), the evidence is not coinciding with assays carried out for evaluating such effect. This is not happening with other effects reported in Fig. 2 which coincide with studies carried out either *in vivo* or *in vitro* (especially for ZEA as it is the most studied): mutagenicity (Abbès et al., 2007; Ben Salah-Abbès et al., 2009); nephrotoxic in rats (Becci et al., 1982), genotoxic (Ouanes et al., 2003, 2005; El-Makawy et al., 2001). As mentioned before the prediction needs to be confirmed with further assays without forgetting that it is giving a valuable indication to start from.

Cytochrome P450 (CYP450) is an enzymatic complex important as mechanism of defense by the organism when in contact with contaminants. Its main function is to metabolize the majority of toxic compounds through Phase I reactions. It is constituted by several isoforms to highlight the following as the most implicated in defense: CYP3A4, CYP2C9, CYP2C19, CYP1A1 and CYP1A2 (SwissADME). Expression of different isoforms occurs by exposure to contaminants as mycotoxins; which can act as inhibitors, inducers or substrates of this enzymatic complex. Results reported in Fig. 3 reveal that the highest predictions effects were for CYP3A4 (40–80%) (Fig. 3D). When analyzing the action of mycotoxins, all three act as substrate, inducers and inhibitors ranging from 60% to 90%, from 21% to 38% and from 23% to 32%, respectively for isoforms CYP1A1 and CYP1A2 (Fig. 3); while as substrate (62%–71%) and inducers (89%) for CYP3A4. Finally, for isoform CYP2C9, ZEA act as substrate and inducer and, α- ZEL and β-ZEL as substrate and inhibitor (Fig. 3). For metabolite products, probabilities of action were marked for isoform CYP3A4. This isoform jointly CYP1A2 have been reported to play an important role in metabolism of ZEA in humans (Pfeiffer et al., 2009); while jointly with CYP2C8 denotes a high activation hydroxylation of ZEA (Bravin et al., 2009). In summary, different isoforms of CYP seem to contribute in the metabolization of all 15 compounds according to *in silico* prediction which coincides with the studies performed *in vitro* (Pfeiffer et al., 2009; Bravin et al., 2009); and more specifically with the isoform CYP3A4 which has the highest values of probability (Fig. 3D).

Apoptotic cell death has been studied for ZEA *in vitro* revealing that activation of caspase 3 and 8 occurs (Banjerdpongchai et al., 2020; Gazzah et al., 2010; Othmen et al., 2008; Agahi et al., 2020 Zhu et al., 2012); as well as for α- ZEL and β-ZEL (Abid-Essefi et al., 2009). Nothing

is known nor for its metabolite products defined in the metabolomics profile. Both caspases, implicated in the cascade activation for apoptotic cell death, have been predicted *in silico* as reported in Fig. 4. Results for ZEA coincide with those reported in the literature *in vitro* denoting a major activation for caspase 3 than caspase-8 (Barjerdpongchai et al., 2010). Among that, similar tendency was observed for all the other 14 compounds studied; and while O-glucuronidates present highest prediction of activation for both caspase-3 and 8 and all compounds, S-sulfation products from ZEA (3z and 4z) do not contribute to activation of cell death through caspase-8 (Fig. 4B). The prediction presented in this work in cell death and the *in vitro* confirmation reported for ZEA, α- ZEL and β-ZEL reveal that the apoptosis pathway of cell death is contributed by its metabolite products, which are generated during its detoxification by Phase I and II reactions.

5. Conclusions

In conclusion, the results obtained in the present study indicate that toxicity of ZEA, α-ZEL and β-ZEL mycotoxins and their metabolomics' profile can be predicted *in silico*. MetaTox was able to predict a total of 12 metabolites defining the metabolomics profile of each mycotoxin studied (5 from ZEA and 7 from α-ZEL and β-ZEL). SwissADME permitted to analyze each compound by its physicochemical properties and predict the behave of each one according to its absorption, distribution, metabolism and toxicity. Among that it was possible to assign a HMDB ID according to a score of similarity. Lastly, PASS online provided an entire prediction of all compounds based on its structural information reported in Pa values. The results indicate moderate to high absorption by the gastrointestinal tract, but unlikely to penetrate into the brain on its current form unless metabolized. Slightly better properties to reach blood brain barrier than initial mycotoxins were observed. Toxic effects associated for all compounds revealed that carcinogenicity reported the highest probability for all three mycotoxins followed by nephrotoxic > hepatotoxic > endocrine disruptor > mutagenic (AMES TEST) > genotoxic. Prediction of inhibition, induction and substrate function on different isoforms of Cytochrome P450 varied for each compounds analyzed; similarly, for activation of caspases 3 and 8.

The metabolomics profile of ZEA, α-ZEL and β-ZEL analyzed by *in silico* programs (MetaTox, SwissADME and PASS online) predicts alteration of systems/pathways/mechanisms that ends up causing several toxic effects, giving an excellent sight and direct studies before starting *in vitro* or *in vivo* assays contributing to 3Rs principle by a reduction of animal testing. This innovative proposal in the field of computer toxicology helps (and opens a new window) to investigate the chemical risk assessment, a topic of great interest amongst researchers and safety authorities; nonetheless, it is necessary to continue developing and performing assays that confirm the predictions estimated to achieve solidest conclusions.

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Compliance with ethical standards.

CRediT authorship contribution statement

Fojan Agahi: Data curation, Investigation, Methodology, Visualization, Writing - original draft. **Cristina Juan:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Guillermina Font:** Funding acquisition, Investigation, Writing - review & editing. **Ana Juan-García:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111818.

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