In vitro antigenotoxic activity, in silico ADME prediction and protective effects against aflatoxin B1 induced hepatotoxicity in rats of an Erythrina latissima stem bark extract

In Vitro Antigenotoxic Activity, *In Silic*o ADME prediction and Protective Effects against Aflatoxin B_1 Induced Hepatotoxicity in Rats of an *Erythrina latissima* Stem Bark Extract

Yancho Zarev[a, b], Tania Naessens[a], Mart Theunis[a], Esameldin Elgorashi[c], Sandra Apers[a,†], Iliana Ionkova[b], Luc Verschaeve[d], Luc Pieters[a], Nina Hermans[a], Kenn Foubert[a]

Affiliation

[a] Natural Products & Food Research and Analysis (NatuRA), Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610, Antwerp, Belgium

[b] Department of Pharmacognosy; Faculty of Pharmacy, Medical University - Sofia, Str. Dunav 2, 1000 Sofia, Bulgaria

[c] Toxicology and Ethnoveterinary Medicine; Food, Feed and Veterinary Public Health; ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South-Africa

[d] Risk assessment, Sciensano, Juliette Wytsmanstraat 14, 1050 Brussels, Belgium

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Abbreviations: ADME, absorption, distribution, metabolism, excretion; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFB_1, aflatoxin B_1; DMSO, dimethyl sulfoxide; ELBE, *E. latissima* stem bark extract; HBD, number of hydrogen bond donor atoms; HBA, number of hydrogen bond acceptor atoms; HPLC-DAD, high performance liquid chromatography - diode-array detector; γ-GT, gamma-glutamyltransferase.

Corresponding author: Prof. Dr. Luc Pieters, Natural Products & Food Research and Analysis (NatuRA), Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, 2610, Antwerp, Belgium. E-mail address: luc.pieters@uantwerpen.be

† We dedicate this paper to our colleague, Sandra Apers, who passed away much too early on February 5th, 2017.

Abstract

Stem bark of *Erythrina latissima* E. Mey (Leguminosae) contains a wide range of prenylated flavonoids able to counteract the genotoxic properties of aflatoxin B_1 (AFB_1). Thus, the hypothesis was raised that *E. latissima* stem bark extracts (ELBE) may counteract the *in vivo* hepatotoxic effects of aflatoxins, contaminants in food and feed. An HPLC-DAD method was developed and validated to determine the level of flavonoid aglycones (11.82%) and glycosides (16.17%). ADME, pharmacokinetic and drug-likeness assessment of major flavonoids of ELBE, using the web tool SwissADME, showed good oral bioavailability. The protective effect of ELBE against AFB_1 induced genotoxicity in the Vitotox assay after metabolic activation was confirmed (IC_{50} of 44.32 µg/ml), followed by evaluation of its inhibitory effect on hepatotoxicity in rats induced by the same agent. Male Wistar rats were orally treated with ELBE (20 mg/kg, 50 mg/kg and 100 mg/kg) or curcumin (500 mg/kg) combined with piperine (20 mg/kg) - positive control, for 8 days prior to AFB_1 exposure (1 mg/kg). The ELBE group showed a decreased activity of ALP and γ-GT compared to the AFB_1 group. Histopathological examination of the liver demonstrated ameliorative effects of ELBE. Thus, ELBE could have a protective effect against hepatotoxins such as AFB_1.

Key words

Erythrina latissima; Leguminosae; antigenotoxicity; prenylated flavonoids; *in vivo* antihepatotoxic activity

Highlights

- *E. latissima* stem bark extract showed a protective effect against aflatoxin B_1 induced genotoxicity in the Vitotox assay.
- *E. latissima* stem bark extract ameliorated aflatoxin B_1 – induced liver injury in rats.
- An HPLC-DAD method for determination of flavonoids in stem bark extracts of *E. latissima* was developed and validated according to ICH guidelines.

1. Introduction

Mycotoxins comprise a group of chemically diverse compounds originating from secondary metabolism of fungi, mainly belonging to five genera: *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* (Gutleb et al., 2002; Binder et al., 2007). Aflatoxins are well known mycotoxins, and among the most carcinogenic substances (Steyn, 1995). Among the major aflatoxins (B_1, B_2, G_1 and G_2) of *Aspergillus flavus* or *Aspergillus parasiticus*, AFB_1 is the most potent one. It has hepatotoxic, immunosuppressive, carcinogenic, teratogenic and mutagenic activities (Richard, 2007). When ingested, the subsequent toxic effects, mainly in the liver, are due to the biotransformation of AFB_1 to its reactive 8, 9-epoxide by cytochrome P450 enzymes (McLean and Dutton, 1995). After the invasion of the liver by aflatoxins, their metabolites react with different cell proteins involved in carbohydrate, lipid and protein metabolism, leading to necrosis or liver cell death. Nevertheless, the activation of aflatoxins can be counteracted by other constituents present in animal feed, food or herbal preparations. Examples include turmeric and its active ingredient curcumin, ellagic acid and *Calendula officinalis* extracts, which were found to inhibit the *in vitro* mutagenicity of AFB_1 in the Ames test and *in vivo* hepatotoxic effects induced by AFB_1 in rats (Soni et al., 1997; Hamzawy et al., 2013).

The bark of *Erythrina latissima* E. Mey (Leguminosae), a large tree up to 5-8 m tall growing in South-Africa, Zimbabwe and Botswana, contains a range of prenylated flavonoids, such as sigmoidin A and B, 4-*O*-methylsigmoidin B, abyssinin I, II and III, which inhibited the mutagenic effects induced by AFB_1 *in vitro* in the Vitotox assay, a test that is equivalent to the Ames assay (Zarev et al., 2017). Prenylation of flavonoids in nature is relatively rare but still not less interesting. The increased lipophilicity due to the presence of a prenyl side chain may result in a higher affinity for biological membranes and a better interaction with target proteins, resulting in improved bioactivities (Yang et al., 2015; Nikolić and Breemen, 2013). The genus

Erythrina contains a wide range of prenylated flavonoids, compounds with a rather limited distribution, but showing various pharmacological effects: antibacterial, anti-inflammatory, antioxidant, larvicidal and estrogenic activities, but cytotoxicity as well. Results of previous investigations on the bioactivity of prenylated flavonoids are mostly limited to *in vitro* studies (Chen et al., 2014). As the prenylated flavonoids can affect the genotoxic properties of the potent mycotoxin AFB_1 through inhibiting its metabolic activation, a stem bark extract of *E. latissima* could be used as a dietary supplement counteracting harmful effects of these genotoxic and hepatotoxic substances, involuntarily taken, e.g. as food contaminants, or as a feed additive in the livestock industry. Thus, in the present study, firstly an HPLC-DAD method for analysis of antigenotoxic flavonoids in an ethanolic stem bark extract of *E. latissima* was developed and validated according to the ICH guidelines (ICH 1994/1996). Then, the *in vitro* inhibitory activity of the quantified extract against AFB_1 induced genotoxicity was confirmed, followed by an *in vivo* study of the protective effects of the extract against AFB_1-induced hepatotoxicity in rats.

2. Material and methods

2.1. General experimental procedures

Dichloromethane, ethyl acetate, methanol, propan-2-ol and acetonitrile of HPLC-grade were purchased from Fisher Chemicals. Water for HPLC analysis was prepared by a Milli-Q system (Millipore) and passed through a 0.22 µm membrane filter. Standards of rutin (≥95%) and naringenin (≥95%) were purchased from Sigma-Aldrich. Curcumin (≥90%) was obtained from Cayman Chemicals, and piperine (≥97%), sodium carboxymethyl cellulose as well as AFB_1 (≥98%) from Sigma-Aldrich. Formic acid and trifluoroacetic acid were supplied by Acros Organics.

All analyses were performed with an HPLC 1200 series with degasser, quaternary pump, automatic liquid sampler, thermostatic column compartment and diode array detector (DAD) from Agilent. Different HPLC columns were tested: XBridge C_{18} (5 µm, 4.6 x 250 mm) and Kinetex EVO C_{18}, (5 µm, 4.6 x 250 mm). Water with 0.1% formic acid, water with 0.01% trifluoracetic acid, methanol and acetonitrile were evaluated as mobile phases.

2.2. Plant material

Stem bark of *Erythrina latissima* E. Mey (Leguminosae) was collected in 2013 in the Walter Sisulu National Botanical Garden in South Africa. A voucher specimen (No. 1983/141) was deposited at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria, South Africa.

2.3. Extraction and determination of flavonoid content

2.3.1. Method optimization

The extraction procedure, solvents, duration and the HPLC conditions were evaluated and optimized. The extraction efficiency of different solvents (100 ml v/v), i.e. methanol/water 1:4, methanol/propan-2-ol 2:3, methanol/ethyl acetate 1:4, methanol/dichloromethane 1:1, dichloromethane/propan-2-ol 3:7 and ethyl acetate were tested. The extraction procedures were evaluated through heating the samples under reflux or sonicated for one hour. For the determination of the sample/solvent ratio, about 2 g of sample was extracted with 50 ml, 100 ml and 200 ml of methanol/dichloromethane 1:1. Also the number of extractions (1 – 4) and duration of heating needed to exhaust the plant material were evaluated.

As a final step the following was done. To achieve the total extraction about 1 g of powdered stem bark was being extracted for one hour with 150 ml methanol/dichloromethane 1:1 in a round bottom flask and heated under reflux. The solution was filtered through a filter paper and the extraction was repeated twice, adding fresh solvent to the plant material every time. The combined filtrate was evaporated to dryness under reduced pressure. The dried

extract was dissolved in 100% methanol and transferred into a volumetric flask of 25.0 ml and then Nylon syringe filter (0.25 µm) was used to filter the solution.

For the subsequent HPLC analysis 10 µl extracted solution was injected into a Kinetex EVO C_{18} column (5 µm, 250 x 4.6 mm) and for the separation the following gradient program was applied: solvent (A) water + 0.05% trifluoroacetic acid, solvent (B) acetonitrile; 0 min 90% A; 0-10 min 86% A; 10-20 min 82% A; 20-25 min 80% A; 25-30 min 75% A; 30-60 min 60% A; 60-80 min 50% A; 80-110 min 45% A; 110-120 min 40% A. The flow rate during the analyses was 1.0 ml/min. The column eluent was simultaneously monitored at 280 nm, for the none-polar flavonoids (using naringenin as positive control) of the examined plant material and at 350 nm for the polar glycosylated flavonoids (using rutin as positive control). Detection wavelengths corresponded to the absorption maxima of the constituents concerned.

2.3.2. Validation of the analytical method

The developed and optimized method was validated according to the ICH guidelines (ICH 1994/1996). For the statistical analysis Excel 2010 (Microsoft Office) was used. All results were presented as percentages, where *n* means the number of values. A 5% level of significance was selected.

Because of the lack of commonly available primary standards, rutin and naringenin were selected as a secondary external standard for the quantification of the flavonoid glycosides and aglycones, respectively. Both standards are closely related, commercially available analogues of the main compounds of interest, and they show similar chromatographic retention times and UV absorption maximum. For the stock solution of rutin about 50 mg of standard was dissolved in 20 ml methanol, 10 ml water and methanol 50% to reach 50 ml then sonicated for 30 min. The stock solution of naringenin was prepared by dissolving about 25 mg naringenin in 25 ml methanol 50% and sonicating for 30 min. The stock solutions were freshly

prepared prior the analysis. The appropriate dilutions were prepared in 50% methanol each time right before the analysis. The calibration model was determined on nine concentration levels, ranging from 2.02 to 364.32 µg/ml for rutin and 1.02 to 183.36 µg/ml for naringenin, with two injections for each level. The regression line was constructed, the equation generated, and the determination coefficient was calculated. The slope and intercept of the regression line was inspected by using Student's *t*-test. A lack-of-fit (LOF) test was performed to evaluate the lack of fit of the linear model and the residuals were graphically examined.

During the validation procedure precision was investigated at two levels: the repeatability (precision under the same conditions over a short interval of time) and the intermediate precision (investigating the effect of performing the analysis on different days). To investigate this intermediate precision and repeatability six independently prepared samples (100% i.e. 1.0 g) were analyzed on three consecutive days. The standard deviation (SD) and relative standard deviation (RSD %) were calculated for each of the three days. The results from the three different days were analyzed with ANOVA single factor. Within and between days RSD%'s were calculated. The intermediate precision of different concentration levels (linearity of the method over a broad range of concentrations) was examined by analyzing six samples with half of the amount (50% i.e. 0.5 g) and six samples with one and half of the amount (150% i.e. 1.5 g), all within the range of the calibration curve. The extracts for each concentration level were prepared according to the method described above and injected into the HPLC. The SD and RSD% were calculated for each level. Within and between days RSD%'s were calculated and the results were evaluated using Cochran and an ANOVA single factor test.

Accuracy was investigated by calculating the percent recovery of a known, added amount of analyte to the sample. Accuracy was assessed using a minimum of nine measurements over a minimum of three concentration levels. To 50% (0.5 g) of the bark extract

a known amount of rutin and naringenin standard solutions was added, obtaining three different concentration levels e.g. 75%, 100% and 125% in the linear range of the method. For each of the concentration levels three samples were prepared and analyzed according to the above-described method. Recovery (%) for each analyzed sample, mean recovery % and RSD% was calculated. To check whether the observed recovery was significantly different from 100% a Student's *t*-test was performed ($\alpha = 0.05$; n – 1).

2.4. In vitro antigenotoxicity evaluation - Vitotox assay

The antigenotoxic effect of the total methanol/dichloromethane 1:1 extract of *E. latissima* stem bark was evaluated against AFB_1 - induced mutagenicity in the Vitotox assay (Zarev et al., 2017). In the Vitotox assay the total extract (0.016 µg/ml - 500 µg/ml) was added to the cultures of *S. typhimurium* TA104 recN2-4 (Genox strain) and *S. typhimurium* TA104 pr1 (Cytox strain) in the presence of rat liver S9 as well as 1 µg/ml of AFB_1. Antigenotoxic activity was measured as % inhibition of genotoxicity, and IC_{50} values were calculated.

2.5. ADME prediction and drug likeness estimation for major flavonoids from ELBE

Preliminary to the *in vivo* experiment, *in silico* screening of pharmacological properties (ADME) and evaluation of drug-likeness features of the major antigenotoxic flavonoids from the stem bark of *E. latissima* (fig. 1) (Zarev et al., 2017) was performed with the SwissADME tool developed by the Swiss Institute of Bioinformatics, which is freely available at http://www.swissadme.ch. Computational analyses to predict the core pharmacokinetics parameters such as gastrointestinal absorption, P-glycoprotein-mediated efflux and being substrates of essential isoforms of the cytochrome P450 (CYP) family, in particular CYP1A2, CYP2D6, CYP2C9, CYP2C19 and CYP3A4 was performed (Daina and Zoete, 2016). Drug-likeness was assessed with filters such as Lipinski, Ghose, Veber, Egan and Muegge, using

calculations based on parameters like molecular weight, LogP, number of HBA and HBD (Daina et al., 2017).

2.6. In vivo evaluation of antihepatotoxic activity

A total of 48 male Wistar rats with body weight about 140 – 150 g were obtained from the National Breeding Center, Sofia, Bulgaria. The rats were housed in Plexiglas cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20 ± 2°C and humidity 72 ± 4%) with free access to water and standard pelleted rat food 53-3, produced according ISO 9001:2008. Seven days of acclimatization were allowed prior the the study initiation and a veterinary physician monitored the health of the animals regularly. The Vivarium (certificate of registration of farm No. 0072/01.08.2007) was inspected by the Bulgarian Drug Agency to check the husbandry conditions (No. A-11-1081/03.11.2011). All performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (Council of Europe, 1991) were strictly followed throughout the experiment.

For the purpose of the *in vivo* experiment a 90% ethanolic extract from stem bark of *E. latissima* (ELBE) was prepared. The Wistar rats were randomly divided into six test groups of eight rats each (n = 8). The animals were treated via oral gavage. Group A: control rats treated with 0.5% aqueous sodium carboxymethyl cellulose (0.5 ml/100 g bw/day) for 11 days. Group B, C and D: rats treated with respectively 20 mg/kg, 50 mg/kg and 100 mg/kg bw/day ELBE for 8 days. On day 8^{th} the animals were challenged with AFB_1 (1 mg/kg BW), dissolved in 1% solution of DMSO and subsequently treatment with ELBE continued in the respective doses for 3 additional days. Group E: rats treated with the positive control curcumin 500 mg/kg bw/day together with piperine 20 mg/kg bw/day suspended in 0.5% sodium carboxymethyl for

8 days. On day 8th the animals were challenged with AFB_1 (1 mg/kg BW), dissolved in 1% solution of DMSO and subsequently treatment continued for 3 additional days. Group F: animals treated for 8 days with 1% solution of DMSO (0.5 ml/100 g bw/day). On day 8 animals were challenged with AFB_1 (1 mg/kg BW) dissolved in 1% solution of DMSO and subsequently treatment continued with 1% solution of DMSO for 3 additional days.

2.6. Determination of serum biochemical markers

Hepatotoxicity was assessed by determination of the activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transferase (γ-GT) as well as creatinine levels. Serum activities were measured with commercially available standard diagnostic kits (Mindray, Shenzhen, China) using an automatic chemistry analyzer (BS-120, Mindray, Shenzhen, China). On day 11, before collecting blood samples, animals were made comfortable in a restrainer while maintaining the temperature around at 35 to 40°C. Lidocaine cream 2% was applied on the surface of the tails 30 min before the experiment. Blood was collected using a capillary tube containing ethylene glycol tetra-acetic acid. Serum was separated by centrifugation in a bench centrifuge (Eppendorf MiniPlus) at 10 000 rpm for 10 min, 4°C. Serum biochemical markers (8 samples from each group) were analyzed and the data are presented as mean ± S.E.M. Statistical analysis was performed using SPSS. Variances were tested using Levene's test then one-way analysis of variance (ANOVA) or Kruskall Wallis, followed by post-hoc tests (Dunnett's T3 and bonferoni) for multiple comparison was performed. The level of statistical significance was set at $p < 0.05$.

2.7. Histopathologic examination

At the end of the experiment fresh liver tissues samples were obtained from three randomly selected rats of each group. Liver specimens were immediately preserved in 10%

neutral formalin, and then embedded in paraffin then tissue sections (3 µm) were cut. AFB_1-induced necrosis was evaluated by light microscope assessing the morphological changes in the liver sections stained with hematoxylin and eosin (Feldman and Wolfe, 2014).

3. Results

3.1. Method optimization and validation

Since prenylated flavonoids present in the bark of *E. latissima* are not readily commercially available and their large-scale isolation would be very time-consuming, suitable standards such as rutin and naringenin were selected for the analytical quantification method. Concerning the extraction, a methanol/dichloromethane 1:1 mixture was found to be the most appropriate solvent for sample preparation. Heating under reflux was more efficient than sonication. Solvent (600 ml) added in three successive steps and 3 h (3 times × 1 h) extraction time was found to be sufficient for exhausting the plant material.

The use of a Kinetex EVO C_{18} (5 µm, 250 x 4.6 mm) HPLC column together with Milli-Q water containing 0.05% trifluoroacetic acid (A) and acetonitrile (B) (v/v) and a gradient program starting with 10% acetonitrile running for 120 min to 60% acetonitrile allowed separation of the main polar and non-polar constituents of the very complex bark extract. Polar flavonol type constituents were monitored at 350 nm and eluting for 38 min, while flavanone aglycones were observed at 280 nm eluted from 40^{th} min further to 100 min (Fig. 1).

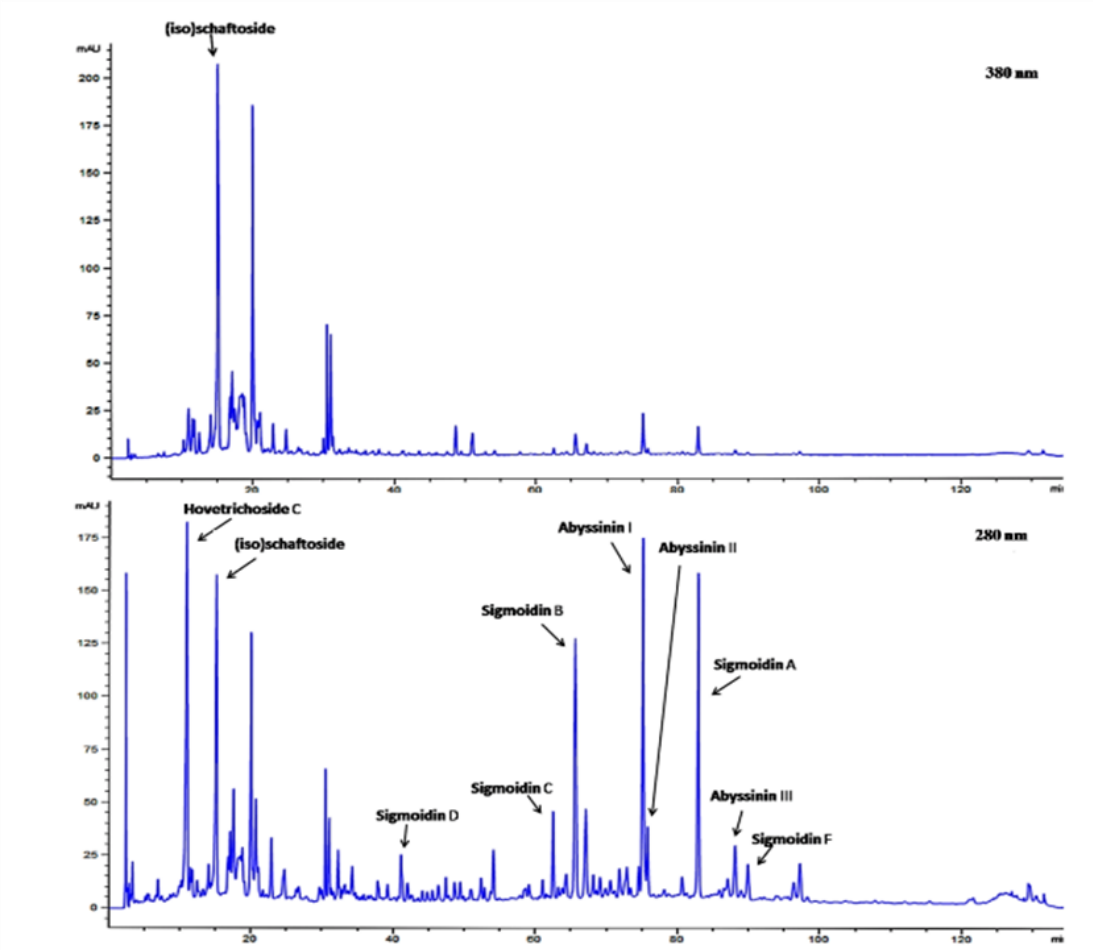

Fig. 1. Chromatographic profile of *E. latissima* bark extract (methanol/dichloromethane 1:1) at 350 nm and 280 nm.

The analytical validation was performed in terms of a calibration model, precision and accuracy concerning total flavonoid glycosides and aglycones. The calibration model of both standards was evaluated by analysing the regression line, the equation and the determination coefficient. A visual evaluation of the linear regression line and residuals plot showed that the method was linear and homoscedastic, while no trend was observed for the residuals. The determination coefficient for both external standards was $R^2 > 0.99$. The slope and intercept of the regression line were investigated with a Student's *t*-test. Results showed that the slope was significant and the 95% confidence interval of the intercept including the point (0, 0). As the t-test confirmed that the origin was included in the regression line a one-point calibration can be

used in further analyses (Table 1). Maximal residual values of 3.45% for naringenin and 1.86% for rutin correspond with accepted system precision of 5% for a UV analytical method.

Table 1. Linear regression data of rutin and naringenin

	Rutin	**Naringenin**
Determination coefficient	0.9998	0.9996
Slope ± standard error	13.93 ± 0.04	27.65 ± 0.14
Intercept ± standard error	-11.02 ± 8.70	-4.89 ± 14.71
Confidence interval (95%) intercept	-29.45 / 7.42	-36.09 / 26.30
tcalc (tcrit) slope	325.56 (2.12)	192.64 (2.12)
tcalc (tcrit) intercept	-1.27 (2.12)	-0.33 (2.12)
Range (µg/mL)	2.02 – 364.32	1.02 – 183.36
Number of standards	9	9
Rt (min)	20.62	41.35

The results used for the evaluation of the precision on different days and at different concentration levels for both the aglycones and the glycosylated flavanoids are presented in Table 2. A Cochran test confirmed the equality of all variances, affirming the correctness of the results obtained by the ANOVA analyses. ANOVA showed that for both the glycosides and the aglycones the calculated F-value was below the critical F-value for the different days and different concentration levels, indicating there was no statistic difference for both parameters. The RSD_{within} and $RSD_{between}$ for both precision on different days and different concentration levels were in the same range and higher than the limit set by Horwitz (Albert and Horwitz, 1997). However, the disadvantage of the limit is that it only includes the concentration level and does not cover any aspects related to the complexity of the sample preparation or analysis.

Table **2**. Validation data: precision on different days (n = 18) and different concentration levels (n = 30) for aglycones and glycosylated flavonoids

		Mean	s	RSD%	RSD_{within}	$RSD_{between}$	RSD_{max}	Cochran	*F*-value(*F*-theor)
Total aglycones	Concentration 50%	1.4480	0.0419	2.89	2.95	2.88	2.52	0.3536	0.70 (2.76)
	Concentration 150%	1.4462	0.0356	2.46					
	Day 1 - 100%	1.4283	0.0524	3.67					
	Day 2 - 100%	1.4190	0.0087	0.62	3.12	3.11	2.53	0.5313	0.93 (3.86)
	Day 3 - 100%	1.4532	0.0565	3.89					
Total glycosides	Concentration 50%	2.1523	0.1871	8.70	10.63	11.97	2.41	0.3438	2.61 (2.76)
	Concentration 150%	1.7913	0.1258	7.02					
	Day 1 - 100%	1.9605	0.2746	14.01					
	Day 2 - 100%	1.8997	0.2097	11.04	12.03	11.62	2.41	0.4475	0.60 (3.68)
	Day 3 - 100%	2.0487	0.2216	10.82					

Still RSD% values for the aglycones are smaller than 5%, confirming the results obtained by ANOVA, and demonstrating the precision of the method. However, RSD_{within} and $RSD_{between}$ for the glycosides exceeded 5%. Based on the results of all samples used for the validation of the precision, the dried bark contained an average amount of 1.44% of aglycones with RSD% of 2.89% and 1.97% of glycosides with RSD% of 11.75% (Table 2). The results of the recovery experiment showed that the method was accurate for the determination of the total amount of aglycones. A mean recovery of 100.31% (RSD% = 6.49%) was observed, which statistically did not differ from 100% (Student's *t*-test). A recovery of 197.77% (RSD% = 55.87) was obtained for the total amount of glycosides.

3.2. In vitro antigenotoxic activity

A methanol/dichloromethane 1:1 extract of *E. latissima* stem bark was evaluated for its antigenotoxic activity in the Vitotox assay. The extract containing 11.82% flavonoid aglycones, calculated as naringenin, and 16.17% of flavonoid glycosides, calculated as rutin,

was found to inhibit genotoxicity induced by AFB_1 after metabolic activation with an IC_{50} value of 44.32 µg/ml (data not shown).

3.3. In silico ADME and drug-likeness evaluation

The main ADME parameters concerning pharmacokinetic behavior of the tested flavonoids are presented in Table 3. All compounds are estimated to have high gastrointestinal absorption, except as expected the two glycosides hovetrichoside C and isoschaftoside. High gastrointestinal absorption can be considered as a favorable advantage in case of oral administration. The diversity in chemical structures and the large variation in possible active regions of the molecules could be possible reason for the observed inhibition of different CYP isoforms, related to inhibition of the metabolic activation of AFB_1. Except for the glycosylated compounds, all evaluated flavonoids proved to comply with Lipinski's rule-of-five, considered to be the source of all drug-likeness tools. The tested flavonoids also did not violate the Veber, Ghose and Egan filters. In line with the gastrointestinal absorption, the tested compounds showed a good bioavailability score (55%). The data about the tested flavonoids being substrates or non-substrates of the permeability glycoprotein (P-gp), considered to be the most important member among ATP-binding transporters or ABC-transporters, allow to assess the possible active efflux through biological membranes. Interestingly, all molecules which are P-gp substrates, except 5, 7, 4′-trihydroxy-3′-methoxyflavanone and isoschaftoside, have a B-ring fused with a 2,2-dimethyldihydropyran ring, such as erylatissin G, dihydroabyssinin I, sigmoidine C, sigmoidine D, 3′, 4′-dihydro-3′-hydroxy-8′-methylether of sigmoidin C and abyssinoflavone V. In addition, in the Vitotox assay those molecules exhibited the weaker activity with IC_{50} values ranging from 134.3 µM (abyssinoflavone V) to 271.1 µM (erylatissin), when compared to sigmoidine A and sigmoidine B with IC_{50} values of 44.1 µM and 52.5 µM, respectively (Zarev et al., 2017).

Table. 3 Calculated ADME, pharmacokinetic parameters and drug likeness parameters for major flavonoids isolated from ELBE.

Compounds	GI absorption	Pgp substrate	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4	Lipinski	Ghose	Veber	Egan	Muegge	Bioavailability Score
					inhibitor			# violations					
3'4'-Dihydro-3'-hydroxy-8'-methylether of sigmoidin C	High	Yes	No	No	No	Yes	Yes	0	0	0	0	0	0.55
4-O-Methylsigmoidine B	High	No	Yes	No	Yes	No	Yes	0	0	0	0	0	0.55
5'-(3-Methylbut-2-enyl)pratensin A	High	No	No	No	Yes	No	No	0	0	0	0	1	0.55
5,7,4'-Trihydroxy-3'-methoxyflavanone	High	Yes	Yes	No	No	No	Yes	0	0	0	0	0	0.55
5,7-Dihydroxy-2',4',5'-trimethoxyisoflavanone	High	No	Yes	No	Yes	No	Yes	0	0	0	0	0	0.55
7-Demethylrobistigenin	High	No	Yes	No	Yes	Yes	Yes	0	0	0	0	0	0.55
Abyssinin I	High	Yes	Yes	Yes	Yes	Yes	Yes	0	0	0	0	0	0.55
Abyssinin II	High	No	Yes	No	Yes	No	Yes	0	0	0	0	0	0.55
Abyssinin III	High	No	No	No	Yes	No	Yes	0	0	0	0	1	0.55
Abyssinoflavone IV	High	No	Yes	Yes	Yes	No	Yes	0	0	0	0	0	0.55
Abyssinoflavone V	High	Yes	No	No	Yes	No	Yes	0	0	0	0	0	0.55
Dihydroabyssinin I	High	Yes	Yes	Yes	Yes	Yes	Yes	0	0	0	0	0	0.55
Erylatissin D	High	No	Yes	No	Yes	Yes	Yes	0	0	0	0	0	0.55
Erylatissin E	High	No	Yes	Yes	Yes	Yes	Yes	0	0	0	0	0	0.55
Erylatissin F	High	No	Yes	No	Yes	No	Yes	0	0	0	0	0	0.55
Erylatissin G	High	Yes	Yes	No	Yes	Yes	Yes	0	0	0	0	0	0.55
Glycyrrhisoflavone	High	No	Yes	No	Yes	Yes	No	0	0	0	0	0	0.55
Hovetrichoside C	Low	No	No	No	No	No	No	2	1	1	1	3	0.17
Isoschaftoside	Low	Yes	No	No	No	No	No	3	3	1	1	4	0.17
Olibergin A	High	No	Yes	No	Yes	Yes	Yes	0	0	0	0	0	0.55
Sigmoidine A	High	No	No	No	Yes	No	Yes	0	0	0	0	1	0.55
Sigmoidine B	High	No	Yes	No	Yes	Yes	Yes	0	0	0	0	0	0.55
Sigmoidine C	High	Yes	Yes	No	Yes	Yes	Yes	0	0	0	0	0	0.55
Sigmoidine D	High	Yes	No	No	No	No	Yes	0	0	0	0	0	0.55
Sigmoidine F	High	No	Yes	No	Yes	No	Yes	0	0	0	0	1	0.55

3.4. In vivo antihepatotoxic activity

The quantitative analysis of a 90% ethanolic extract of *E. latissima* bark used in the *in vivo* experiment showed that ELBE contained 7.5% non-glycosylated flavonoids and 12.7% glycosylated flavonoids. Administration of ELBE for 11 days did not affect the food consumption of the animals nor their weight, and no adverse behavior changes, toxicity symptoms or mortality were observed in rats at doses up to 100 mg/kg. Based on these findings, ELBE could be considered safe in rats. The effect on the level of the selected biological markers of the different concentrations of ELBE in a model of AFB_1-induced liver damage is shown in Table 4.

Table **4**. Effects of ELBE on the levels of hepatic ALP (A), *γ*-GT (B), AST (C), ALT (D) and creatinine (E) after AFB_1 treatment in rats.

Animal Groups	**Creatinine mmol/l**	**AST U/l**	**ALT U/l**	**ALP U/l**	***γ*-GT U/l**
Control (A)	42.31 ± 0.94	192.96 ± 10.12	62.31 ± 3.03	491.00 ± 30.68	2.59 ± 0.19^{b}
AFB_1 + 20 mg/kg ELBE (B)	37.46 ± 2.54	252.50 ± 4.02^{a}	84.69 ± 4.26^{a}	477.63 ± 14.05^{b}	2.93 ± 0.30^{b}
AFB_1 + 50 mg/kg ELBE (C)	42.73 ± 1.22	287.96 ± 12.92^{a}	88.08 ± 5.04^{a}	418.00 ± 16.33^{b}	3.39 ± 0.13
AFB_1 + 100 mg/kg ELBE (D)	44.86 ± 1.47	270.89 ± 16.54^{a}	83.63 ± 8.12	491.13 ± 16.15^{b}	2.86 ± 0.19^{b}
AFB_1 + Curcumin 500 mg/kg and Piperine 20 mg/kg (E)	46.23 ± 1.74	$151.94 \pm 9.78^{b,\sim}$	88.01 ± 2.23^{a}	657.00 ± 32.55^{a}	4.06 ± 0.14^{a}
AFB_1 (F)	47.34 ± 0.85^{a}	291.90 ± 16.79^{a}	90.59 ± 2.20^{a}	588.63 ± 20.98	4.67 ± 0.36^{a}

Values are expressed as means of ± S.E.M. of 8 animals in each group, ~ means of ± S.E.M. of 7 animals in each group

[a] significantly different from untreated control group without aflatoxin-B_1 treatment $p < 0.05$ vs control

[b] significantly different from AFB_1 model group $p < 0.05$ vs control

A significant increase ($p < 0.05$) of these serum biomarkers of liver damage as well as creatinine was observed in AFB_1-intoxicated rats when compared to the control group. ALP activity in the rats of the AFB_1 treated group, although elevated compared to the control group, appeared to be below the level of significance ($p = 0.28$). In the animals in which ELBE (20, 50 and 100 mg/kg) was administered the activity of ALP and γ-GT was decreased compared to AFB_1 group. However, no differences could be observed in the level of ALP between the different administered doses. The activity of γ-GT was decreased when animals were treated with ELBE 20 mg/kg bw/day ($p = 0.041$) and 100 mg/kg bw/day ($p= 0.018$), but appeared not to be statistically significant compared to animals treated with ELBE 50 mg/kg bw/day ($p = 0.122$). The administration of ELBE did not change the activity of ALT and AST as well as the creatinine level, when compared to the AFB_1 group. The administration of curcumin 500 mg/kg bw/day together with piperine 20 mg/kg bw/day only led to a decrease in AST activity, when compared to the AFB_1 group.

Histopathological examination of a liver section of a rat of control group A displayed normal liver architecture. Group B showed no significant portal dilatation with slight cellular infiltration, which did not disturb portal spaces (without "piecemeal necrosis") or without interface hepatitis. Vacuoles were observed only in single lobules localized next to the bigger branches of the portal vein. In group C, the architecture was comparable with group B but portal spaces have slight to moderate dilatation with moderate cellular infiltration, which forms focal necrosis with incipient interface hepatitis. More severe changes of hepatic lobules in the centrilobular zone were observed. Portal spaces were closer to the bigger branches of portal vein, with more significant dilatation and with further developed interface hepatitis. In group D portal spaces were moderately infiltrated with advance interface hepatitis where local necrosis links were observed. Rats treated

only with AFB_1 (Group F) showed a clear dilatation of portal spaces with interface hepatitis. The necrosis links and fibrosis proliferation formed single pseudo-lobules. Severe bile ducts proliferation in all portal spaces were observed. Fatty liver dystrophy of hepatocytes was found around the central vein, only in the liver lobule next to the branches of the portal vein. The positive control treated with curcumin together with piperine (Group E) had the tendency of reverse development of the described changes. The infiltration and dilation of portal spaces were decreased, while the proliferation of bile ducts remained the same. Bile ducts were significantly dilated. The central vein was almost normal (Fig. 2).

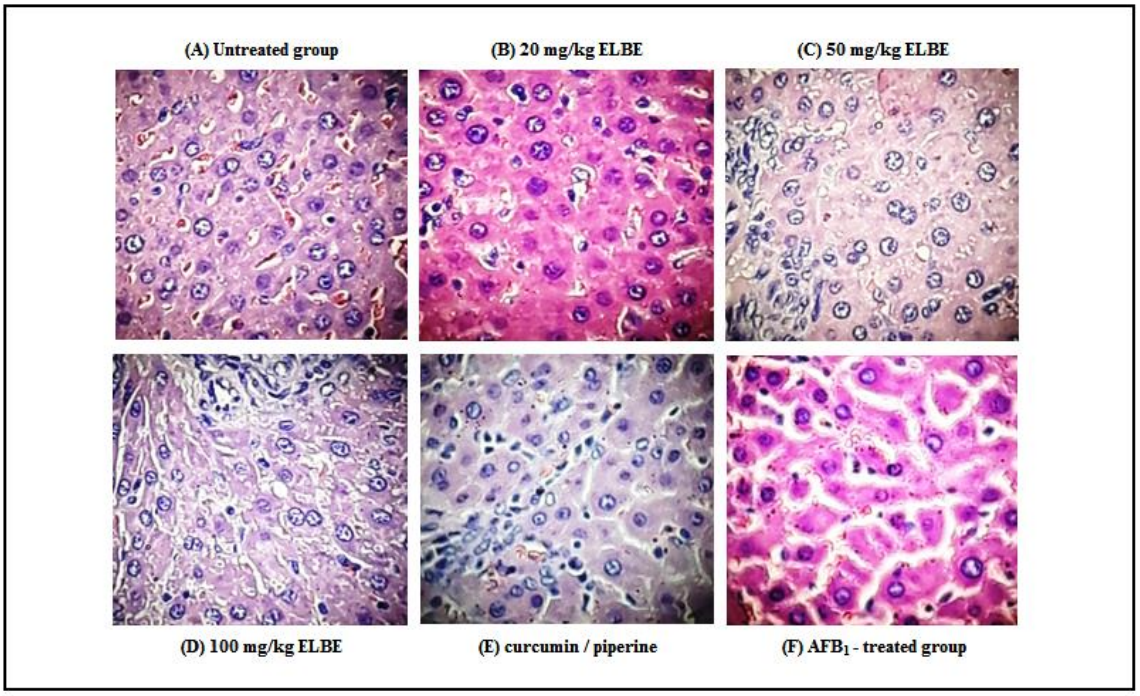

Fig. 2 H & E stained sections of the liver Group **A** (untreated control group); Group **B** (AFB_1 + 20 mg/kg ELBE), Group **C** (AFB_1 + 50 mg/kg ELBE) and Group **D** (AFB_1 + 100 mg/kg ELBE) - portal spaces are moderate infiltrated. Group **F** (AFB_1-treated group) showed clear dilatation of portal spaces with interface hepatitis. Group **E** (AFB_1 + curcumin 500 mg/kg together with

piperine 20 mg/kg) have the tendency of reverse development of the described changes in group F.

4. Discussion

Both results from the precision and recovery experiments confirmed that, although the method is fully applicable for the quantification of non-glycosylated flavonoids of the stem bark of *E. latissima,* it has some drawbacks for the quantification of the glycosylated flavonoids, i.e. the long chromatographic gradient, needed for the separation of the complex mixture of glycosides, and the rather long sample preparation. Therefore, the development of a separate method for the quantification of the glycosides may be envisaged, but then 2 different assays have to be carried out. Previously, the antigenotoxic flavonoid aglycones such as sigmoidin A and B (IC_{50} = 18.7 µg/ml) were isolated from 90% methanol fraction, whereas the flavonoid glycosides were present in the inactive aqueous fraction (Zarev et al., 2017). Nevertheless, it is considered that the flavonoid glycosides might also contribute to *in vivo* effects, since the glycosidic moieties can be removed in the gastro-intestinal tract after oral administration, liberating potentially active flavonoid aglycones. Therefore, when designing *in vivo* experiments, it is important to know not only the level of flavonoid aglycones, but also level of flavonoid glycosides. The results from *in vitro* test of the methanol/dichloromethane 1:1 extract (IC_{50} = 44.32 µg/ml) revealed it as a promising antigenotoxic agent against aflatoxin induced mutagenicity, even without the flavonoid glycosides contributing to this activity. The *in silico* data of the antigenotoxic flavonoids present in the ELBE extract revealed those flavonoids to possess acceptable pharmacokinetic properties. Therefore, a more detailed pharmacological and toxicological evaluation was carried out *in vivo.*

In the *in vivo* study, it was observed that AFB_1 intoxication significantly increased AST, ALT, γ-GT as well as creatinine levels compared to the control rats. In general, chronic or acute

liver injury eventually results in increased serum levels of aminotransferases. ALT is considered as the gold standard biomarker for hepatic injury. It is the most reliable indicator for hepatic histomorphological changes and shows infrequent false negative signals as well as limited false positive signals. AST is a less specific biomarker of liver function compared to ALT activity. AST is localized in heart, brain, muscles and liver tissue, while ALT is primary localized in the liver, and an increase of ALT serum level is therefore more specific for liver damage. γ-GT and ALP are additional biomarkers of liver function. In humans with primary biliary cirrhosis or chronic liver disease with unknown etiology, levels of ALP and γ-GT are increased while serum ALT levels could be normal. In rats, γ-GT could be considered as more reliable marker for cholestasis compared to ALP (Ozer et al., 2008). In the present study, curcumin was used as a positive control drug due to its known hepatoprotective activity against aflatoxin-induced toxicity (Mathuria and Verma, 2008). Due to poor absorption, rapid metabolism, and rapid systemic elimination, oral administration of curcumin leads to very low curcumin plasma and tissue levels. To improve the bioavailability of curcumin, piperine was used as an adjuvant, since it interferes with glucuronidation of curcumin and increases its blood levels (Anand et al., 2007). However, the addition of piperine can also interfere with the toxicity of aflatoxins, both *in vitro* as *in vivo*, decreasing hepatic injuries when given to intoxicated animals (Gadini et al., 2010).

The effect of the administration of AFB_1 clearly could be observed in the altered serum levels of the liver enzymes and creatinine. The protective effect of the treatment with curcumin / piperine, however, could only be observed by the level of AST; the levels of the other enzymes and creatinine were not significantly different. On the contrary, treatment with the extract of *E. latissima* induced a reduction of both ALT and γ-GT levels. No clear dose response effect could be observed when investigating the different amounts of extract administered. However,

histopathological examination of the liver demonstrated ameliorative effects of ELBE after acute ABF_1 induced hepatotoxicity. At doses of 100 mg/kg bw/day histopathological changes were moderate, while the AFB_1 treated group clearly showed signs of necrosis as well as dilatation of portal spaces with interface hepatitis.

Hepatoprotective properties of *Erythrina* extracts have been reported before. A methanolic leave extract from *Erythrina indica* showed protective effects against liver injury (Mujahid et al., 2017). Furthermore, the administration of various extracts of stem bark of *E. indica* showed improvement in the biochemical parameters profile in CCl_4 induced toxicity in albino rats, which confirmed its hepatoprotective potential (Balachandar and Indumathy, 2017). The ethanolic extract of whole plant of *Erythrina variegata* displayed significant protection against CCl_4 induced hepatocellular injury (Venkateswarlu and Karunambigai, 2013). However, these Erythrina extracts were not phytochemically characterized or quantified. Till now no study on the hepatoprotective activity of *E. latissima* bark extract was reported.

In conclusion, administration of *E. latissima* bark extract ameliorated aflatoxin B_1 – induced liver injury in rats. Improvement of histopathological architecture precedes retrieval of serum biochemical markers, which could be overcome in future studies with administration of ELBE during a longer time.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Albert R, Horwitz W. A heuristic derivation of the Horwitz curve. Anal Chem 1997; 69: 789-790. doi: 10.1021/ac9608376

Anand P, Kunnumakkara A, Newman RA, Aggarwal BB. Bioavailability of curcumin: Problems and Promises. Mol Pharm 2007; 4: 807-818. doi: 10.1021/mp700113r

Balachandar C, Indumathy R. Hepatoprotective activity of stem bark of *Erythrina indica* on Wistar Albino rats. WJPPS 2017; 6: 1324-1333 DOI: 10.20959/wjpps20176-9345

Binder EM, Tan LM, Chin LJ, Handl J, Richard J. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Anim Feed Sci Technol 2007; 137: 265-282. https://doi.org/10.1016/j.anifeedsci.2007.06.005

Chen X, Mukwaya E, Wong M-S, Zhang Y. A systematic review on biological activities of prenylated flavonoids. Pharm Biol 2014; 52: 655-660. https://doi.org/10.3109/13880209.2013.853809

Daina A, Zoete V. A BOILED-Egg to predict gastrointestinal absorption and brain penetration of small molecules. Chem Med Chem 2016 11(11): 1117-1121.

Daina A, Michielin O, Zoete, V. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci Rep 2017; 7: 42717.

Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. Methods Mol Biol 2014; 1180: 31-43. doi: 10.1007/978-1-4939-1050-2_3

Gadini T, Silva R, Castro I, Soares B, Lima M, Brito M, Mazur C, Direito G, Danelli M. Oral administration of piperine for the control of aflatoxin intoxication in rats. Braz J Microbiol 2010; 41: 345-348. doi: 10.1590/S1517-838220100002000013

Gutleb AC, Morrison E, Murk AJ. Cytotoxicity assays for mycotoxins produced by *Fusarium* strains: a review. Environ Toxicol Pharmacol 2002; 11: 309-320. https://doi.org/10.1016/S1382-6689(02)00020-0

Hamzawy MA, El-Denshary ESM, Hassan NS, Mannaa FA, Abdel-Wahhab MA. Dietary supplementation of *Calendula officinalis* counteracts the oxidative stress and liver damage resulted from aflatoxin. ISRN Nutr 2013; 12: 538427. doi: 10.5402/2013/538427

Mathuria N, Verma RJ. Ameliorative effect of curcumin on aflatoxin induced toxicity in serum of mice. Acta Pol Pharm 2008; 65: 339-343.

McLean M, Dutton MF. Cellular interactions and metabolism of aflatoxin: an update. Pharmacol Ther 1995; 65: 163-192. https://doi.org/10.1016/0163-7258(94)00054-7

Mujahid M, Hussain T, Siddiqui HH, Hussain A. Evaluation of hepatoprotective potential of *Erythrina indica* leaves against antitubercular drugs induced hepatotoxicity in experimental rats. J Ayurveda Integr Med 2017; 8: 7-12. doi: 10.1016/j.jaim.2016.10.005

Nikolić D, Breemen RB. Analytical methods for quantitation of prenylated flavonoids from hops. Curr Anal Chem 2013; 9: 71-85.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of biomarkers in hepatotoxicity. Toxicology 2008; 245: 194-205. doi: 10.1016/j.tox.2007.11.021

Richard JL. Some major mycotoxins and their mycotoxicoses-an overview. Int J Food Microbiol 2007; 119: 3-10. doi:10.1016/j.ijfoodmicro.2007.07.019

Soni KB, Lahiri M, Chackradeo P, Bhide SV, Kuttan R. Protective effect of food additives on aflatoxin-induced mutagenicity and hepatocarcinogenicity. Cancer Lett 1997; 115: 129-133. https://doi.org/10.1016/S0304-3835(97)04710-1

Steyn PS. Mycotoxins, general view, chemistry and structure. Toxicol Lett 1995; 82/83: 843-851. https://doi.org/10.1016/0378-4274(95)03525-7

Text on Validation of Analytical Procedures–ICH Harmonised Tripartite Guideline. ICH 1994.

Validation of Analytical Procedures: Methodology–ICH Harmonised Tripartite Guideline, ICH. 1996.

Venkateswarlu B, Karunambigai M. Hepatoprotective effect of *Erythrina variegata* against carbon tetrachloride induced hepatotoxicity in Wistar albino rats. IJPPR 2013; 4: 75-80

Yang X, Jiang Y, Yang J, He J, Sun J, Chen F, Zhang M, Yang B. Prenylated flavonoids, promising nutraceutical with impressive biological activities. Trends Food Sci Technol 2015; 44: 93-104. https://doi.org/10.1016/j.tifs.2015.03.007

Zarev Y, Foubert K, de Almeida VL, Anthonissen R, Elgorashi E, Apers S, Ionkova I, Verschaeve L, Pieters L. Antigenotoxic prenylated flavonoids from stem bark of *Erythrina latissima*. Phytochemistry 2017; 141: 140-146. https://doi.org/10.1016/j.phytochem.2017.06.003