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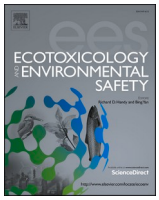

Mechanistic insight into human androgen receptor-mediated endocrine disrupting potential of cyclic depsipeptide mycotoxin, beauvericin, and influencing environmental factors for its biosynthesis in *Fusarium oxysporum* KFCC 11363P on rice cereal

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ABSTRACT

In current study, *Fusarium* mycotoxin, beauvericin (BEA), has endocrine disrupting potential through suppressing the exogenous androgen receptor (AR)-mediated transcriptional activation. BEA was classified as an AR antagonist, with IC_{30} and IC_{50} values indicating that it suppressed AR dimerization in the cytosol. BEA suppress the translocation of cytosolic activated ARs to the nucleus via exogenous androgens. Furthermore, we investigated the impact of environmental conditions for BEA production on rice cereal using response surface methodology. The environmental factors affecting the production of BEA, namely temperature, initial moisture content, and growth time were optimized at 20.28 °C, 42.79 % (w/w), and 17.31 days, respectively. To the best of our knowledge, this is the first report showing that BEA has endocrine disrupting potential through suppressing translocation of cytosolic ARs to nucleus, and temperature, initial moisture content, and growth time are important influencing environmental factors for its biosynthesis in *Fusarium* strains on cereal.

1. Introduction

The concern of secondary metabolites, which are biosynthesized in contaminated *Fusarium* strains on food materials including cereal because of their occurrence in various food types (Placinta et al., 1999; Sidhu, 2002; Jestoi, 2008; Garcia et al., 2010; Neme and Mohammed, 2017; Bryla et al., 2018; Moretti et al., 2019). Secondary metabolite residues in food and the environment may cause critical affect human health (IARC, 2002). BEA is biosynthesized in *Fusarium* strains (Zocher and Keller, 1997), and this structural characteristic imparts ionophoric properties to BEA (Lee et al., 1992). BEA is a modulator of cholesteryl ester formation in the adrenal gland, acyl-CoA: cholesterol acyltransferase (ACAT) (Liscum and Dahl, 1992; Tomoda et al., 1992). This indicates that BEA may have physiological effects on the endocrine system because steroidogenic factor-1 dependent up-regulation of ACAT is important for maintaining readily available cholesterol esters, which in turn are needed during steroidogenesis (Ferraz-de-Souza et al., 2011). From this possibility for endocrine disruptor, previous studies tried to investigate critical potential on human endocrine system using cell-based assays (Kalayou et al., 2015; Fernández-Blanco et al., 2016). However, the major mechanism of endocrine disrupting potential induced by BEA is not well-know, yet.

Therefore, we assessed antagonistic effects of BEA on the human androgen receptor (AR), using the OECD Test Guideline (TG) No.458 AR transcriptional activation (TA) assay (OECD, 2021). Additionally, we confirmed that BEA has endocrine disrupting potential through suppressing the cytosolic ligand-bound ARs to nucleus using Bioluminescence Resonance Energy Transfer (BRET)-based assay and western blotting. Furthermore, the environmental conditions for BEA biosynthesis in *Fusarium* strain on rice cereal were investigated using response surface methodology (RSM) to reduce the contamination by BEA in food matarials.

Abbreviations: BEA, beauvericin; AR, androgen receptor-alpha; TA, transcriptional activation; BA, binding affinity; RSM, response surface methodology.

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2. Materials and methods

2.1. Chemicals and reagents

BEA used in the current study was purified from a culture of *F. oxysporum* KFCC11363P. Other chemicals used as reference substrates for cell-based assays and as reagents for the liquid chromatographic analysis of BEA were obtained commercially. The details of the protocol for the purification and analysis of BEA are presented in Supplemental Materials and Methods 1. All chemicals, including reference substances for assays, were of analytical grade. For the agonist assay, reference standards (positive; 5α-dihydrotestosterone (DHT) and bisphenol A (BPA), negative; bis(2-ethylhexyl) phthalate (DEHP)) were commercially obtained (Sigma-Aldrich, St. Louis, MO, USA). Additionally, AR (abcam, Cambridge, MA, USA) and β-actin (Cell Signaling, Danvers, MA, USA), and HRP (horseradish peroxidase)-conjugated secondary antibody were purchased by Cell Signaling Technology (Danvers, MA, USA).

2.2. Reporter gene assay

The inhibition of BEA on the exogenous AR agonistic activity was determined according to the OECD TG No. 458 protocol. All quantitative data are shown as mean ± standard deviation. The assay procedure is described in Supplemental Materials and Methods 2.

2.3. Confirmation of ligand-induced cytosolic AR dimerization

The interference effect of BEA on cytosolic AR dimerization was investigated using a BRET-based *in vitro* assay, according to the protocol described in our previous study (Lee et al., 2021). All quantitative data are shown as mean ± standard deviation. The assay procedure is described in Supplemental Materials and Methods 3.

2.4. Immunoblotting

Proteins harvested from the cells were transferred onto polyvinylidene fluoride membranes via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The transferred proteins were then allowed to react sequentially with primary and secondary antibodies. All quantitative data are shown as mean ± standard deviation. Further details of the process are reported in Supplemental Materials and Methods 4.

2.5. Design of statistical model

The effect on environmental factors, temperature (Temp), initial moisture content (IMC), and growth time (GT), for biosynthesis of BEA in contaminated *Fusarium* strain on rice were investigated based on the central composition design (CCD) in response surface methodology using Minitab 18 software (Minitab Inc., State College, PA, USA). To predict the response variables (Y), the following fitting equation was used:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \beta_{12} X_1 X_2 \quad (1)$$

The details of the experimental design for model are presented in Supplemental Materials and Methods 5.

3. Results

3.1. Inhibitory effect on androgen-induced transcriptional activation by BEA

BEA was classified to be an AR antagonist with an IC_{50} value of 1.67 $\times 10^{-6}$ M which when expressed as a percentage compared to the IC_{50} value of bicalutamide (an AR antagonist positive substance) was 23.35 (Table 1 and Fig. 1).

3.2. Effect on cytosolic AR dimerization of BEA

To confirm whether the AR antagonistic activity of BEA was due to its interference of cytosolic AR dimerization, a BRET-based *in vitro* assay was performed. Cytosolic AR dimerization was not observed in the presence of BEA (Fig. 2). The ranges of BRET signal values and the relative binding affinity (%) to the positive control (10 nM DHT) of by serially diluted BEAs were similar to those of the vehicle control.

3.3. Localization of cytosolic AR proteins to nucleus

The localization of AR proteins from cytoplasm to nucleus was investigated by western blotting to characterize the mode of mechanism underlying BEA-induced antagonistic effect of AR (Figs. 3 and 4). Western blotting showed that AR proteins were highly expressed in the nucleus after 3 h when cells were treated with DHT. Conversely, the AR levels co-treated by BEA and DHT in the nucleus after 3 h were similar to vehicle control (Fig. 3). In terms of quantitative expression level, treatment of only DHT induced increasing expression level of nucleus ARs, whereas expression level of nucleus ARs was suppressed in the presence of BEA (Fig. 4). As a result, we confirmed that BEA acts suppressing translocation of cytosolic AR to the nucleus.

3.4. Model fitting to optimize the production conditions of BEA by F. oxysporum

The principle of RSM applied to model the BEA biosynthesis by *F. oxysporum* KFCC 11363P as functions of the influencing environmental factors, Temp., IMC, and GT on rice cereal. Table 2 lists the amounts of BEA produced (1.71–522.07 mg/kg) in each of the 20 experimental runs suggested by model. The coefficients of the polynomial differential equation denoting the BEA production, established using the experimental results, were expressed along with their statistical significance in Table 3. The fitting of the quadratic polynomial model was evaluated in terms of not only R^2 and adjusted R^2, which indicated the fraction of variation in the response explained by the model, but also the lack of fit from ANOVA (Table 3). The linear and quadratic terms of influencing environmental factors (Temp., IMC, and GT) had significant ($p < 0.05$) effects on the biosynthesis of BEA. In case of interaction terms between investigated environmental factors, although it was discovered that interaction between Temp. X IMC and Temp. X GT had significant ($p < 0.05$) effects on the biosynthesis of BEA, interaction terms between Temp. X GT had no significant effects with a p-value of 0.792. From the ANOVA analysis with respect to association between independent environmental factor and BEA biosynthesis, the importance of the independent variables on the BEA biosynthesis in *F. oxysporum* KFCC11363P on rice cereal could be ranked in the following order with p-values of <0.0001, 0.005, and 0.045, respectively: Temp.> GT > IMC. The fits of the quadratic polynomial models were evaluated by determination coefficients of R^2 and adjusted R^2 and a test for lack of fit from the ANOVA. R^2 and adjusted R^2 for BEA biosynthesis were 0.972 and 0.946, respectively; the model conveyed no

Table 1
Quantitative level of suppressing exogenous AR-mediated transcriptional activation by BEA.

Substances	Inhibitory of DHT-induced AR agonistic effect		
	IC_{30} (M)	IC_{50} (M)	RTI[a]
Bicalutamide	2.43×10^{-7}	3.90×10^{-7} A	100
Beauvericin	1.41×10^{-6}	1.67×10^{-6} B	23.40

[a] Relative transcriptional inactivity (RTI) = (A/B) × 100.

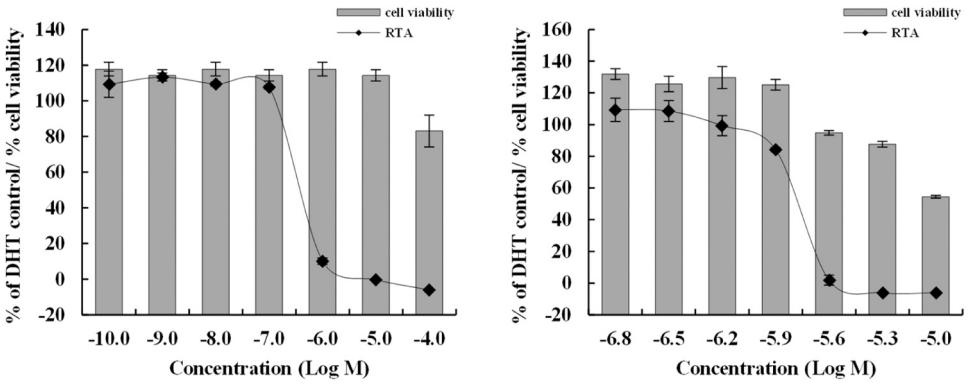

Fig. 1. Concentration-response curves of inhibition of DHT-induced AR agonistic effect by bicalutamide (A) and BEA (B).

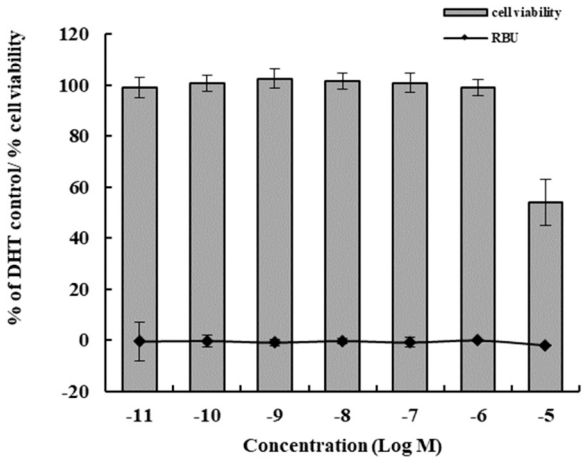

Fig. 2. Cytosolic AR homodimerization induced by BEA.

lack of fit at the 95 % significance level with p = 0.134. In addition, the predicted R^2, which indicates the fraction of the variation in the response predicted by the model, was 0.807. Additionally, the probability for the regression observed < 0.0001, indicating that all the proposed models were significant. Therefore, well-fitting models of the associations between the influencing environmental factors and the amounts of BEA biosynthesis by *F. oxysporum* KFCC 11363P were successfully established.

The optimal conditions (Temp., IMC, and GT) for BEA production by *F. oxysporum* KFCC11363P were established using a model equation. The response surface plot of the quantity of BEA produced as a function of the reaction conditions is shown in Fig. 5. The optimal Temp., IMC, and GT were 20.28°C, 42.79 % (w/w), and 17.31 days, respectively.

4. Discussion

In current study, the condition of influencing environmental factors for biosynthesis in *Fusarium* strain was investigated on rice because it was selected as the optimal cereal for BEA biosynthesis in *Fusarium* strain from our previous report (Song et al., 2009). The optimal temperature conditions for the production of mycotoxins, such as fusaproliferin, enniatins, moniliformin, and BEA generated from *Fusarium* strains are between 20 and 25 °C (Gupta et al., 1991; Moretti et al., 1994; Song et al., 2008). *F. subglutinans* ITEM-1434 produces the highest level of BEA at 25 °C in rice (Kostecki et al., 1999). Song et al. (2008)

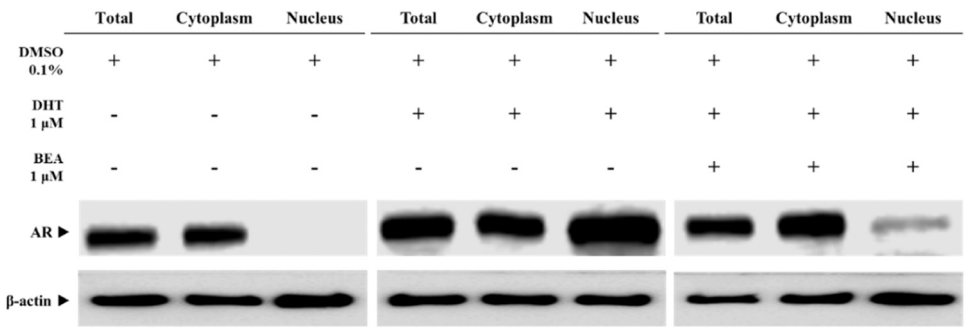

Fig. 3. DHT-induced translocation of AR protein in the presence of BEA in western blot.

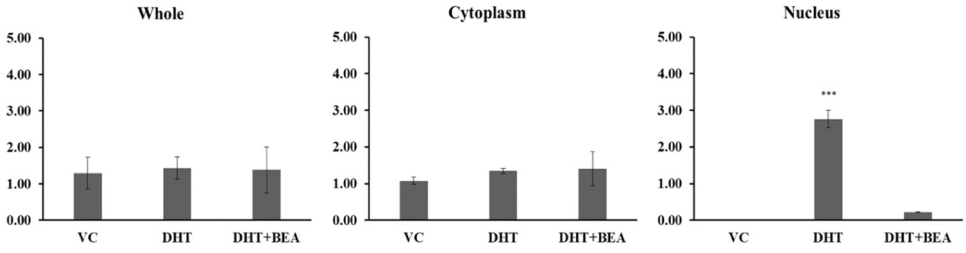

Fig. 4. Quantitative expression levels of AR protein by 0.1 % DMSO (vehicle control), DHT (AR agonistic positive control), and BEA, *** $p < 0.001$.

Table 2
Quantity of BEA produced under each experimental run.

Serial No.	Independent variables			
	Temp. (°C) X_1	Initial moisture content (%) X_2	Growth Time (days) X_3	BEA Y_1
1	15	20	20	219.13 ± 1.46
2	35	40	15	325.55 ± 0.02
3	35	20	20	50.55 ± 0.01
4	35	20	10	1.72 ± 1.25
5	25	40	20	386.43 ± 0.62
6	15	60	10	131.75 ± 0.20
7	25	40	15	491.22 ± 64.47
8	25	40	15	514.85 ± 14.56
9	25	60	15	316.58 ± 0.30
10	25	40	15	522.07 ± 16.74
11	25	40	15	433.42 ± 0.06
12	25	40	15	502.39 ± 1.34
13	35	60	10	1.71 ± 0.07
14	15	20	10	19.41 ± 0.16
15	25	40	15	507.44 ± 8.91
16	25	20	15	251.31 ± 1.06
17	35	60	20	19.82 ± 3.5
18	25	40	10	402.16 ± 2.7
19	15	40	15	402.57 ± 0.36
20	15	60	20	396.49 ± 0.15

Table 3
Statistical analysis table.

Variables			Coefficients	*p* values
Intercept			487.5	
Temp.			-77.0	< 0.0001
IMC			32.4	0.045
GT			51.6	0.005
Temp.×Temp.			-111.8	0.002
IMC×IMC			-191.9	< 0.0001
GT×GT			-81.6	0.013
Temp.×IMC			-40.1	0.030
Temp.×GT			-49.7	0.011
IMC×GT			4.3	0.792
Variables	**Sum of squares**	**Degrees of freedom**	**Mean square**	***p* values**
Model	686,530	9	76,281	< 0.0001
Residual	20,059	10	2006	
Lack of fit	14,917	5	2983	0.134
Pure error	5142	5	1028	
R^2	0.972			
Adjusted R^2	0.946			
Predicted R^2	0.807			

obtained the maximum production of cyclic hexadepsipeptides at 40 % IMC in rice. Song et al. (2009) showed that the maximum amount of cyclic hexadepsipeptides from *F. oxysporum* could be obtained in the

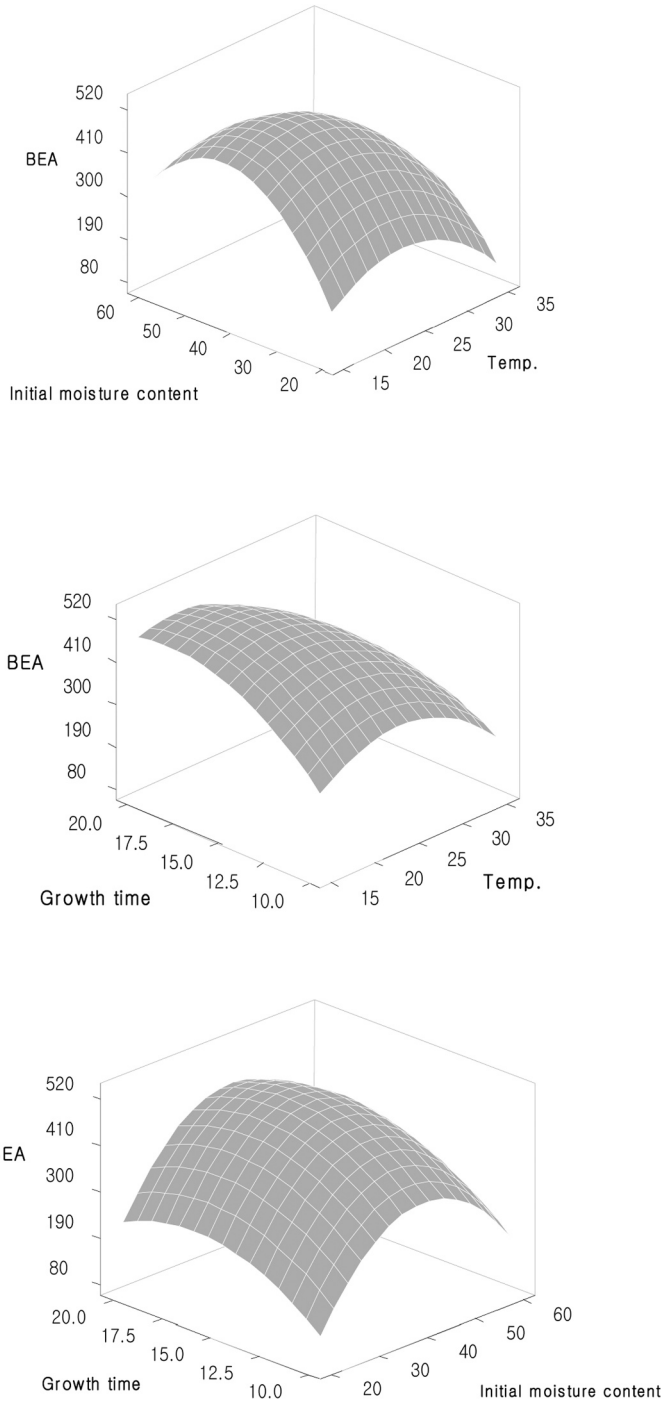

Fig. 5. Response surface plots for the quantity of BEA produced.

second week after inoculation on a solid rice medium (Song et al., 2009). Overall, the model estimated that the highest amount of BEA was 519.89 mg/kg when *F. oxysporum* KFCC11363P was grown under selected conditions by prediction model, and approximately 550 mg/kg of BEA was produced at the selected condition using the model equation.

In a previous study on the AR antagonistic effect of BEA, AR-mediated transcriptional activation induced by BEA was not detected using an RGA reporter gene assay (Fernández-Blanco et al., 2016). BEA was not classified as an AR antagonist in RGA reporter gene assays because BEA showed cytotoxicity to test cell lines (Fernández-Blanco et al., 2016). The intrinsic toxicity of test substances to cell lines is critical because cell death caused by test substances may interfere with the detection of antagonistic responses (OECD, 2021, 2016). If the cell

viability is reduced by 20 % or more by the test substance at any concentration, OECD TGs recommend the concentrations at or above the cytotoxic concentration to be excluded from the data analysis (OECD, 2021). In contrast, in this study, BEA suppressed the exogenous AR-mediated transcriptional activation in the OECD TG No.458 *in vitro* assay at non-toxic concentrations.

We focused on proving the AR-mediated endocrine disrupting potential of BEA by interference of AR genomic pathway using OECD TG No.458 because exogenous androgens regulate the growth, differentiation, and function of various target tissues by activating AR. In the genomic pathway of AR-mediated endocrine-disrupting chemicals, ligands act as endogenous androgens by directly binding to ARs, thereby triggering downstream transactivation events. When ligands bind to AR, the ligand-AR complex dimerizes in the cytoplasm. It translocates to the nucleus, where it binds to androgen response elements; subsequently, together with coactivators and corepressors, AR controls gene transcription (Heinlein and Chang, 2004). In the current study, BEA acts as a suppressor of androgen-induced AR dimerization in the cytosol through competition reaction with DHT for binding to ARs (Supplemental data 1). BEA had a direct binding affinity to ARs with an IC_{25} value of 5.70×10^{-6} M in a fluorescence polarization competitive binding assay. This inhibitory acting caused blocking the translocation of activating ARs to the nucleus.

Previous publications indicated that the phenyl ring of AR antagonist containing BPA is very important structural characteristic because phenyl ring linked to docking site of the AR DNA binding domain through hydrogen bonding (Li et al., 2014). Therefore, based on these previous reports, we considered that the Inhibition of exogenous androgen-induced AR transcriptional activation induced by BEA might involve the phenyl ring in structure (Fig. 6).

Regarding the prevalence of BEA in food products, EFSA provided a scientific opinion on the concentration of beauvericin in food. The study found that 80 % of results for beauvericin in food and 46 % for beauvericin in unprocessed grains were below the detection limit or quantification limit. The highest average concentrations of 131.8 ug/kg beauvericin were found in dried fruits, followed by oilseeds and cereal-based food (21.3 ug/kg) for infants and young children (European Food Safety Authority, 2014). Additionally, considering the overall critical effect data from *in vivo* model by EFSA(European Food Safety Authority), the point of departure (POD) value of critical effect is 0.1 mg/kg b.w./day based on reducing colloid and altered T4 serum levels in male mice (European Food Safety Authority, 2018). The maternal POD value is 0.1 mg/kg b.w./day due to the significant effect on thymus weight and follicle degeneration (European Food Safety Authority, 2018). In terms of the risk of ingesting contaminated food based on the activity strength of BEA, and POD value and detection levels in food stuff, the quantitative values of AR-mediated endocrine disrupting effect by OECD in vitro TGs on BEA were lower than its POD value (0.1 mg/kg b.w./day) and detection levels of BEA in food. However, the interpretation in association with AR-mediated disrupting value from *in vitro* model and its POD value from animal model has a critical limitation. Furthermore, to prove the association between end point of critical effects induced by BEA and endocrine disrupting potential, the EFSA expert committee recommended that even if BEA didn't show any critical effects on the offspring, further studies should be considered to clarify mechanism of endocrine disrupting potential (European Food Safety Authority, 2018). Due to that, further studies containing animal model should be conducted to clarify in association with AR-mediated endocrine disrupting potential and POD value. Additionally, mechanistic insight into AR-mediated endocrine disrupting evidence in current study will be a suitable reference for evaluating the human health-based guidance value of BEA.

5. Conclusion

This study, for the first, provides mechanistic evidence for the AR-mediated endocrine-disrupting potential of BEA, and influencing environmental factors for BEA biosynthesis in contaminated *Fusarium* strains on cereal. Although the current study was conducted in cellular level, our data found that BEA exhibited inhibitory effect on androgen-induced transcriptional activation through suppressing the nuclear translocation of cytoplasmic AR proteins. Furthermore, this study might help regulate the environmental conditions to reduce the production of BEA by contaminated *Fusarium* strains in food materials.

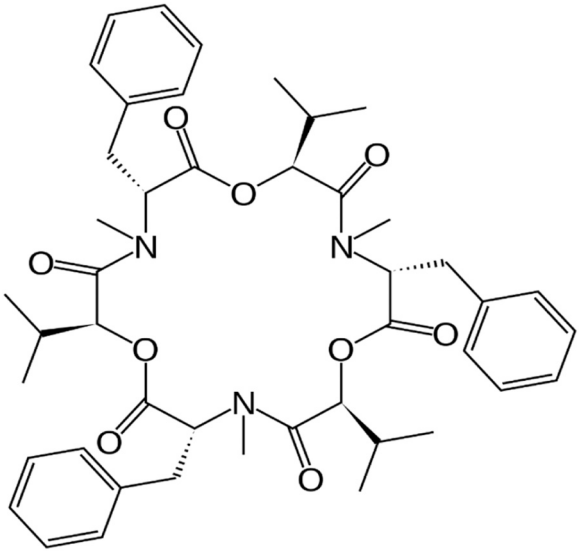

Fig. 6. Chemical structure of BEA.

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CRediT authorship contribution statement

Hee-Seok Lee: Writing – review & editing, Writing – original draft, Validation, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Chaemin You:** Validation, Writing – review & editing. **Da-Woon Jung:** Writing – review & editing, Validation. **Da-Hyun Jeong:** Writing – original draft, Investigation.

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.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116227.

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