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Cytogenotoxic evaluation of the acetonitrile extract, citrinin and dicitrinin-A from *Penicillium citrinum*

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ABSTRACT

Endophytic fungi are promising sources of bioactive substances; however, their secondary metabolites are toxic to plants, animals, and humans. This study aimed to evaluate the toxic, cytotoxic, mutagenic and oxidant/antioxidant activities of acetonitrile extract (AEPc), citrinin (CIT) and dicitrinin-A (DIC-A) of *Penicillium citrinum*. For this, the test substances at 0.5; 1.0; 1.5 and 2 Ig/mL were exposed for 24 and 48 h in *Artemia salina*, and 48 h in *Allium cepa* test systems. The oxidant/antioxidant test was evaluated in pre-, co- and post-treatment with the stressor hydrogen peroxide (H₂O₂) in *Saccharomyces cerevisiae*. The results suggest that the AEPc, CIT and DIC-A at 0.5; 1.0; 1.5 and 2 Ig/mL showed toxicity in *A. salina*, with LC₅₀ (24 h) of 2.03 Ig/mL, 1.71 Ig/mL and 2.29 Ig/mL, and LC₅₀ (48 h) of 0.51 Ig/mL, 0.54 Ig/mL and 0.54 Ig/mL, respectively. In *A. cepa*, the test substances also exerted cytotoxic and mutagenic effects. The AEPc, CIT and DIC-A at lower concentrations modulated the damage induced by H₂O₂ in the proficient and mutant strains of *S. cerevisiae* for cytoplasmic and mitochondrial superoxide dismutase. Moreover, the AEPc at 2 Ig/mL and CIT at the two highest concentrations did not affect the H₂O₂-induced DNA damage in the test strains. In conclusion, AEPc, CIT and DIC-A of *P. citrinum* may exert their toxic, cytotoxic and mutagenic effects in the test systems possibly through oxidative stress induction pathway.

KEYWORDS

Endophytic fungi; *Penicillium citrinum*; mycotoxins; cytotoxicity; genotoxicity; mutagenicity

1. Introduction

Secondary metabolites, also known as natural products, are an abundant source of compound with promising biological activities (Blunt et al. 2008, 2009). Fungi, derived from marine algae represent a potential source for obtaining secondary metabolites (Wali et al. 2019) that can be widely used in human medicines (Gerwick and Fenner 2013). Several studies suggest that endophytic fungi have a variety of biotechnological potential, such as enzyme production, bioremediation, biodegradation, biotransformation, biocontrol, and treatment of various diseases, including cancer (Arora and Chandra 2011, Yao et al. 2011, Wen et al. 2014, Blunt et al. 2015, Zheng et al. 2016).

Among the various secondary metabolites derived from fungi, citrinin (CIT), a polyketide, was first isolated by Hetherington and Raistrick from *Penicillium citrinum* (Thacker et al. 1997). Species belonging to the genus *Penicillium* have several metabolic routes, commanded by specific genes, allowing the biosynthesis of potent secondary metabolites, being widely used as antibiotics and antifungals, in addition to other metabolites applied as immunosuppressants and antitumor agents (Visagie et al. 2014). The species *P. citrinum* corresponds to a filamentous fungus with extensive territorial expansion, and well known for its potential to produce secondary metabolites (Bennett and Klich 2003). Studies show that the species *P. citrinum* produces an eccentric diversity of secondary metabolites, including the natural metabolite CIT

(El-Neketi et al. 2013, Hu et al. 2017). Since CIT possesses good antibiotic properties, studies have endeavored to reduce its toxicity (Xu et al. 2006), rather than evaluating its antitumor effect (Du et al. 2010, Wang et al. 2013). However, CIT, as well as its co-product, a polyketide known as dicitri-nin-A (DIC-A) has been explored for their cytotoxicity (Yao et al. 2011), antimicrobial effect, genotoxic and mutagenic properties (Kumar et al. 2014). CIT has been identified as an important antitumor agent, due to its antagonistic properties, such as antioxidant and cytotoxic properties (Lesgards et al. 2014). Moreover, CIT has been reported as a neuroprotectant, as it can prevent glutamate excitotoxicity and neuronal death (Nakajima et al. 2016). Fungi belonging to the genus *Penicillium* have several metabolic routes, managed by specific genes, which allow the biosynthesis of potent biomolecules used as antibiotics (Penicillin and some of its derivatives), immunosuppressants (Cyclosporin), cholesterol inhibitors (Levostatin) and antitumor (Paclitaxel) (Keller 2019, Williams and Andersen 2020).

In order to understand the effects of natural substances obtained from endophytic fungi, regarding nuclear DNA damage, it is necessary to apply several non-clinical tests in eukaryotic systems, involving in vitro and in vivo studies, such as *Artemia salina* and *Allium cepa* for the evaluation of toxicity, cytotoxicity and mutagenicity. *A. salina* is a minicrustacean that provides an important information on toxicity (Gajardo and Beardmore 2012), it is easy to culture, has low costs and is readily commercially available (Nunes et al. 2006, Shaala et al. 2015). On the other hand, *A. cepa* test is able to evaluate different DNA damages such as toxic, cytotoxic, genotoxic and mutagenic effects, which are analyzed by the number of mitotic

cells (mitotic index); formation of micronuclei and chromosomal changes (Leme & Marin-Morales 2009). Moreover, such tests can evaluate DNA repair activities (Santos et al. 2015), and more importantly, shows similar results in other test systems constituted with prokaryotic and eukaryotic test models (Fiskesjö 1985).

The cellular response to oxidative damage to fungal DNA is similar to the response of mammalian cells, and 30% of genes related to human diseases have functional homologous genes in yeasts (De La Torre-Ruiz et al. 2015). The tests with *Saccharomyces cerevisiae* are important for measuring the mechanisms of changes in replication fidelity, sensitivity to DNA damage and the cellular responses (Skoneczna et al. 2015), including oxidative stress and antioxidant capacity (De Oliveira et al. 2014). Therefore, the aim of the present study was to isolate and characterize compounds (CIT and DIC-A), from acetonitrile extract (AEPc) of the *P. citrinum* of endophytic of the marine macroalgae *Dichotomaria marginata* and to evaluate their toxic/cytotoxic, mutagenic and oxidant/anti-oxidant effects in *A. salina*, *A. cepa* and *S. cerevisiae* test systems.

2. Materials and methods

2.1. Experimental design and isolation of substances

Analytical grade solvents such as methanol (CH₃OH), ethyl, n-hexane and acetonitrile (CH₃CN) were purchased from the

Synth, Dynamics, Merck. These solvents were used for extraction and fractionation. Sephadex LH-20 Sigma-Aldrich stationary phase was used for the open-column chromatographic separation and solid media were used for the growth and isolation of microorganisms: Parboiled rice (Marcon^{VR}) and PDA (Potato Dextrose Agar-Sigma^{VR}). ¹H NMR (500 MHz), ¹³C NMR (126 MHz), HMBC, HMQC, were obtained on a Bruker Avance DRX-500 spectrometer with the solvents CDCl₃, DMSO-d₆ (CIL and Isotec-INC), and the not-deuterated residual solvent as internal reference. The electrospray ionization mass spectrometry was obtained in the positive mode using Orbitrap XL Hybrid with Fourier Transform (Thermo Scientific Instruments) coupled to a Thermo Instruments CLAE system (Accela PDA Detector, Accela automatic sampler and Accela pump, Thermo Instruments Scientific). The following conditions were used: capillary voltage of 4.5 kV; capillary temperature of 260 C; auxiliary gas flow rate of 10–20 arbitrary units; carrier gas flow rate of 40–50 arbitrary units; spray voltage of 4.5 kV; mass range of 100–1000 u.m.a (maximum resolution 30 000).

2.2. Collection of *Dichotomaria marginata*

The red macroalgae *Dichotomaria marginata* was collected in December 2009 in the northern region of São Paulo state, on the beach of Fortaleza, in the city of Ubatuba, Brazil (23°24'03" S and 45°03'41" W) during low tide. Dr. Nair Yokoga (Institute of Botany, São Paulo, Brazil) identified *D. marginata*, and a voucher specimen was deposited in the Herbarium of the Botany Institute of São Paulo, Brazil (Voucher no. SP 400960).

2.3. Isolation and identification of endophytic fungus

The endophyte fungus *P. citrinum* was isolated as previously described (Gubiani et al. 2014), from the inner tissue of the marine red macroalga *D. marginata*. After isolation, pure *P. citrinum* culture was identified by Dr. Anil Sazak (Ondokuz Mayıs Universities Fen Edebiyatları Fakültesi Biyoloji Bölümü Kurupelit/Samsun Turkey).

2.4. Acetonitrile extract of *P. citrinum* (AEPc) and isolation of CIT and DIC-A

The endophytic fungus *P. citrinum* was grown in five Erlenmeyer flasks, each containing 90 g of rice. The medium was autoclaved four times (on four consecutive days) at 121 C for 40 min. After sterilization, the medium was inoculated and incubated at 25 C for 21 days. At the end of the incubation period, the cultures were combined, ground and extracted with CH₃OH (5 250 mL). The solvent was evaporated, producing a raw extract of CH₃OH, which was dissolved in CH₃CN and hexane partitioned to obtain the AEPc.

A portion (900 mg) of the AEPc was fractionated by Sephadex LH-20 column chromatography (70 cm × 3.0 cm) and eluted with CH₃OH: DCM (1: 1), producing 45 fractions of approximately 80 mL each. The analysis by charge-coupled device (CCD) and mass spectrophotometry (CLAE-DAD-IES-

MS) [Column C18; CH₃OH: H₂O (5–100% CH₃OH), 254 nm], allowed their clustering in 16 new fraction groups through the similarity of the chromatograms (FDm1Se-FDm16Se). The FDm9Se fraction (79 mg), after methanol washing, provided the CIT (30 mg) and FDm11Se (198 mg) was rechromatographed on Sephadex LH-20 column (70 cm x 3 cm) and eluted isocratically with CHCl₃/CH₃OH (1/1). From the fractions, 22 subfractions were collected, which resulted in the DIC-A isolation (27 mg, in subfraction 3).

2.5. Characterization of AEPc, CIT and DIC-A

The analysis of RMN¹H and ¹³C revealed the substance CIT, HRESIMS m/z [M⁺ Na]^p 251.0917 (calculated for C₁₃H₁₄O₅) and m/z 273.0735 [M⁺ Na]^p. ¹H RMN (CDCl₃, 500 MHz) 8.23 (s, H-1), 4.76 (qd, J ¼ 6.8, H-3), 2.97 (qd, H-4), 1.23 (d, J ¼ 6.8, H-9), 1.35 (d, J ¼ 7.2, H-10), 2.01 (s, H-11), and DIC, HRESIMS m/z [M⁺ H]^p 381.1701 [M⁺ H]^p, (calculated for C₂₃H₂₄O₅), including m/z 403.1521 [M⁺ Na]^p and m/z 783.3252 [2M⁺ H]^p. ¹H RMN (CDCl₃, 500 MHz) 6.39 (s, H-7), 4.98 (qd, J ¼ 8.5, H-3), 4.62 (m, H-2⁰), 3.16 (m, H-3⁰), 3.12 (m, H-4), 2.11 (s, H-11), 1.33 (d, J ¼ 9.0, H-9⁰), 1.41 (d, J ¼ 8.0, H-8⁰), 1.43 (d, J ¼ 8.5, H-9), 1.31 (d, J ¼ 7.0, H-10), 2.20 (s, H-10⁰). The substances were identified according to the method developed by Wakana et al. (2006) and Yao et al. (2011). The ¹H NMR spectrum of the AEPc extract shows a variety of methyl, methylenic, methylenic and olefinic hydrogen signals (Supplementary materials: Figure 1S–7S).

2.6. Brine shrimp lethality bioassay (BSLB)

The microcrustacean toxicity assay was conducted according to the method described by Meyer et al. (1982). Cysts of *A. salina* were incubated in artificial salted water (23 g of NaCl, 11 g of MgCl₂·6H₂O, 4 g of Na₂SO₄, 1.3 g of CaCl₂·2H₂O or 1.3 g of CaCl₂·6H₂O and 0.7 g KCl in 1000 mL of distilled water) at 25–30 C. Sodium bicarbonate (Na₂CO₃) was used as a buffering agent for the adjustment of pH 9.0. After 48 h, ten live *A. salina* nauplii were collected and transferred to the test tube. The final volume of each sample was 5 mL of saline and tap water (1:1). Solutions of AEPc, CIT and DIC-A were prepared in quadruplicate at concentrations of 0.5, 1.0, 1.5 and 2.0 Ig/mL. After 24 and 48 h, the number of live nauplii was counted. Artificial saline and potassium dichromate (K₂Cr₂O₇) were used as negative control (NC) and positive control (PC), respectively.

2.7. Evaluation of toxicity, cytotoxicity and mutagenicity in *A. cepa*

Medium size bulbs of *A. cepa* were obtained from the Center Supply company- CEAPI in Teresina- Piauí, 2017. The *A. cepa* test was carried out according to Fiskesjö (1985). Briefly, onions were kept in direct contact with AEPc, CIT and DIC-A for 48 h, at a temperature of 25 ± 1 C, protected from light and maintenance of the solution every 24 h. The concentrations tested were same as BSLB assay. The positive (PC) and negative control (NC), were composed of copper sulfate

(CuSO₄·5H₂O) at 6 Ig/mL and dechlorinated water, respectively. After treatment, the roots were removed and fixed for 24 h in Carnoy solution. The roots were washed with distilled water (3 baths of 5 min each), root hydrolysis was performed with 1 N HCl at 60 C for 11 min and the bath was repeated. The basophilic structures were stained with Schiff reagent for 2 h and then rinsed with the aid of running tap water. For assembling the slides, the root meristematic region was removed with a scalpel and a drop of 2% acetic carmine was added to stain the nuclear region and covered with the cover slip. A slight pressure was exerted on the coverslip in order to scatter the meristematic cells throughout the slide.

The toxicity of the test samples and controls was evaluated by determining the root growth inhibition in millimeters of each onion. Cytotoxicity (mitotic index, MI) and mutagenicity (chromosomal changes, CA) were evaluated by counting 5000 meristematic cells (experimental unit: 1000 cells/slide, total of 5 slides per treatment) by using a light microscope DM 500 (400x). Genotoxicity includes aneugenic effects (C-metaphases, metaphases with chromosomal adhesions, chromosomal losses, multipolar anaphases and poly-ploid metaphases) or clastogenic effects (chromosomal fragments, chromosomal bridges and other changes). In addition, the presence or absence of MN, which may result from aneugenic or clastogenic effects, was also evaluated along with the other chromosomal alterations (CA) (Mazzeo et al. 2011).

2.8. Oxidant/antioxidant evaluation in *S. cerevisiae*

The *S. cerevisiae* strains used for oxidant and/or antioxidant test included the proficient cytoplasmic superoxide dismutase (SodWT), three simple deficient strains (Sod1D, Sod2D and Cat1D) and two double deficient strains (Sod1D/Sod2D and Sod1D/Cat1D) (Table 1). These strains were proficient and deficient in Superoxide dismutase (Sod) and catalase (Cat) and were kindly provided by the Toxicological Genetics Research Group from the Federal University of Rio Grande do Sul (UFRGS). For the assay, *S. cerevisiae* strains were replicated in solid YEPD medium (1% yeast extract, 2% glucose, 2% peptone and 2% agar) and stored under appropriate conditions according to Andrade et al. (2011). Cells were seeded from the center to the edge of a petri dish in a continuous cycle, to both sides of the plate containing, in the center, a sterile filter paper disk, in which was added 10 IL of the test substances.

The *S. cerevisiae* strains (Sodwt, Sod1D, Sod2D, Sod1DSod2D, Cat1D, Sod1DCat1D) were seeded in petri dishes from the center to the margin, where a disk of sterile filter paper was placed into the center of the plate and 10 IL of AEPc, CIT and DIC-A (at concentrations of 0.5–2.0 Ig/mL) were added to three different treatment conditions (pre-, co- and post-treatment). In pretreatment, the concentrations of the tested samples were first added to a filter paper disk in the center of the YEPD plate and 2 h later the oxidizing agent (30% hydrogen peroxide, H₂O₂) was added. In co-treatment, the samples and H₂O₂ were simultaneously added. In post-treatment, the H₂O₂ was first added and the sample

Table 1. Strains of *S. cerevisiae*.

Description	Genotype	Origin
EG103 (SOD WT)	MATa leu2-3,112 trp1-289 ura3-52 GALp	Edith Gralla, L Angeles
EG118 (Sod1D)	Sod1:URA3 all other markers as EG103	
EG110 (Sod2D)	Sod2:TRP1 all other markers as EG103	
EG133 (Sod1DSod2D)	Sod1:URA3 sod2::TRP1 double mutant all other markers as EG103	
EG223 (Cat1D)	EG103, except cat1: TRP1	
EG (Sod1DCat1D)	EG103, except sod1: URA3 and cat1:: TRP1	

concentrations were added 2 h later. After 48 h of incubation, in an oven at 30 C, the growth inhibition halo was measured in millimeters (mm) from the margin of the filter paper disk to the cell growth initiation. All assays were performed in triplicate. 20 IL of 30% H₂O₂ was used as a stressor.

2.9. Statistical analyzes

A. salina data were normalized, logarithmically transformed and subjected to non-linear regression analysis, in order to determine the concentration that causes death of 50% of the microcrustaceans (LC₅₀). The results for A. cepa test were expressed as mean ± standard deviation (SD) and analyzes performed using two-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons. *S. cerevisiae* data were expressed as mean ± SD, with ANOVA analysis, followed by the Bonferroni test for multiple comparisons. All analyzes were performed using the GraphPad Prism software (version 6.0), considering $p < .05$, with a 95% confidence level.

3. Results and discussion

3.1. Effects AEPc, CIT and DIC-A on BSLB

The AEPc and the metabolites CIT and DIC-A showed toxicity in A. salina with LC₅₀ for 24 h 2.03 Ig/mL (with 95% CI: 1.59–2.60 Ig/mL); 1.71 Ig/mL (95% CI: 1.41–2.06 Ig/mL) and 2.29 Ig/mL (95% CI: 1.69–3.12 Ig/mL), respectively. At 48 h, the LC₅₀ values of AEPc, CIT and DIC-A were 0.51 Ig/mL (95% CI: 0.39–0.69 Ig/mL); 0.54 Ig/mL (95% CI: 0.39–0.73 Ig/mL) and 0.54 Ig/mL (95% CI: 0.34–0.85 Ig/mL), respectively. The LC₅₀ values for the PC at 24 h was 1.05 Ig/mL (95% CI: 0.93–1.18 Ig/mL) and, at 48 h, it was 0.7 Ig/mL (95% CI: 0.59–0.85 Ig/mL). The evaluation of lethality in A. salina has been suggested as a toxicological test for a wide variety of substances, including crude extracts, isolated and synthetic compounds (Sangian et al. 2013, Islam et al. 2017). Compounds with a low LC₅₀ may have potential antitumor activity (Nunes et al. 2008, Arcanjo et al. 2012). CIT is evident to induce dermal toxicity and apoptosis in the skin of rats, possibly due to its oxidative stress induction capacity (Kumar et al. 2011).

DIC-A (also named as penicitrinone-A) is a co-product of CIT (dimer CIT) (Clark et al. 2006, Wakana et al. 2006). DIC-A with an IC₅₀ value 58.4 ± 4.0 (Nong et al. 2013) is known to inhibit the protein tyrosine phosphatase 2 (Shp2), which is a protein associated with breast cancer and other types of neo-plasms such as leukemia, lung, liver and gastric carcinoma. In addition, the Shp2 is involved in several cancer-related

processes, including metastasis, apoptosis, DNA damage, cell proliferation and anti-cancer drug resistance (Zhang et al. 2015).

In this study, AEPc, CIT and DIC-A induced toxicity in A. salina at 24 and 48 h at all concentrations (0.5–2.0 Ig/mL) as observed by the reduction of survival rate of the nauplii when compared to the NC group. No statistical differences were observed between the substances and concentrations, as well as in relation to the PC group. The PC group significantly reduced survival rate in comparison to the test substances, especially at higher concentrations (Figure 1(A)). Similar data were observed at 48 h exposure, except for CIT and DIC-A at higher concentrations, where the nauplii survival rate was statistically different from the PC group (Figure 1(B)).

3.2. Cytotoxic and mutagenic effects of AEPc, CIT and DIC-A in A. cepa

To date, CIT has been extensively studied for its toxic properties (Blasko et al. 2013), including nephrotoxicity, hepatotoxicity (Bennett and Klich 2003, Flajs and Peracia 2009, EFSA, 2012, Shi and Pan 2012), cytotoxicity and genotoxicity (Chang et al. 2009). In our study, AEPc, CIT and DIC-A induced cytotoxicity at all concentrations, where these substances increased the number of cells in interphases and reduced the number of dividing cells in A. cepa meristematic cells (Table 2).

Toxic and cytotoxic alterations are related to the effects of anti-neoplastic drugs, since they induce apoptosis (Xiao et al. 2003, Jordan and Wilson 2004). In this sense, CIT, at 20–100 IM concentrations, have been reported to induce apoptosis in several human cell lines, including leukemic cells (Yu et al. 2006, Chan 2007). However, at 50 IM, CIT was not cytotoxic but affected microtubules organization, which is a risk factor for carcinogenesis (Gayathri et al. 2015, Yu et al. 2015). CIT cytotoxicity may be associated with the mitochondrial dysfunction and the influx of calcium ions, leading to increase in membrane permeability, as observed in renal and hepatic cell membranes (Chagas et al. 1995, Da Lozzo et al. 1998). The mitotic index (MI) from A. cepa meristematic cells reliably identifies cytotoxic effects, when the MI shows a reduction of 50% in comparison to the NC group, it is considered a sublethal effect (Mesi and Kopicu 2013).

The mutagenic effects of the isolates of P. citrinum was evaluated by cytogenetic damage, including MN formation and increased in c-metaphase CA, bridges, loose chromo-somes and anaphasic delays, leading to a significant increase in CA. AEPc, CIT and DIC-A, at all tested concentrations induced significant CA parameters. AEPc and DIC-A also

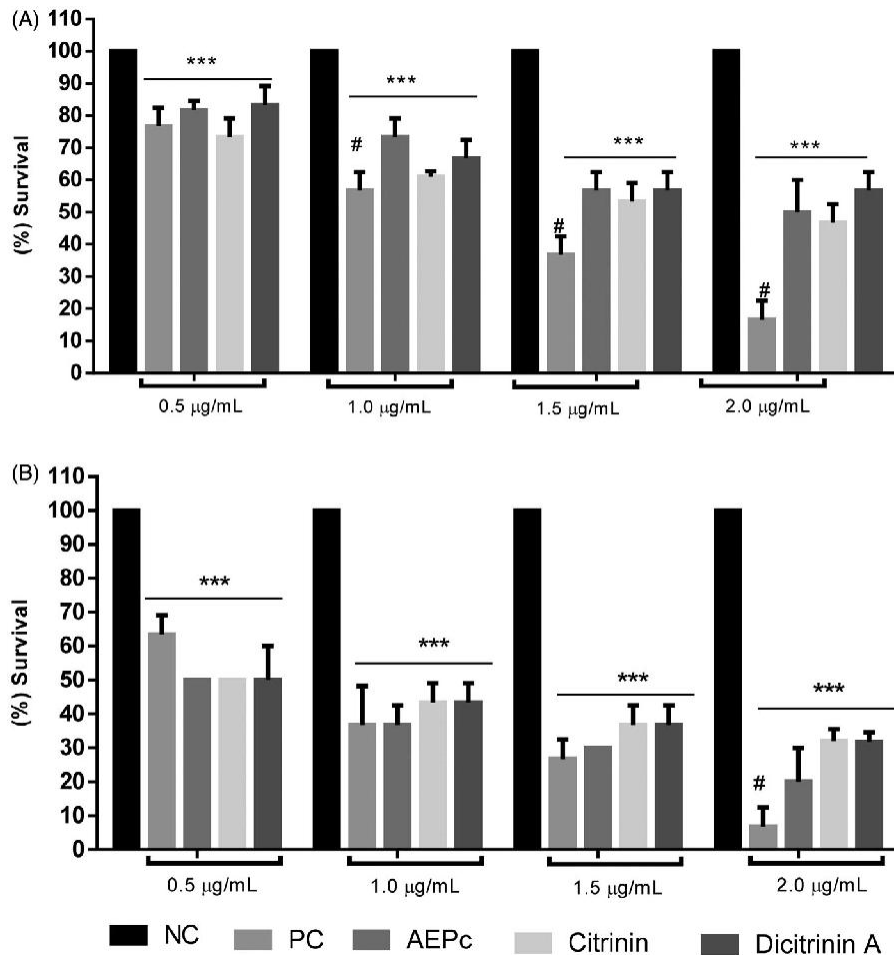


Figure 1. A. salina survival (%) to *P. citrinum* isolates: AEPc, CIT and DIC-A.

Table 2. Cytotoxicity of AEPc, CIT and DIC-A from *P. citrinum* in *A. cepa* meristematic cells after 48 h of exposure.

Treatments	Conc. (mg/mL)	Interphase	Prophase (P)	Metaphase (M)	Anaphase (A)	Telophase (T)	Mitotic index (%)
NC	0	439.4 ± 9.1	458.8 ± 18.9	43.4 ± 7.1	29.8 ± 3.1	28.6 ± 3.0	56.0 ± 0.9
PC	6.0	876.8 ± 15.6	62.6 ± 14.6	29.0 ± 5.4	18.8 ± 3.3	13.6 ± 2.3	12.4 ± 1.5
AEPc	0.5	585.2 ± 28.5	357.6 ± 22.8	24.4 ± 12.0	15.6 ± 7.1	16.8 ± 3.3	41.3 ± 2.1
	1.0	545.2 ± 23.1	399.0 ± 18.2	25.0 ± 7.5	17.0 ± 3.7	13.4 ± 1.9	45.4 ± 1.8
	1.5	561.2 ± 11.9	361.4 ± 6.8	29.0 ± 6.4	23.4 ± 3.1	25.0 ± 1.2	43.8 ± 1.2
	2.0	597.6 ± 12.2	329.2 ± 8.8	24.2 ± 2.9	25.2 ± 2.1	23.8 ± 2.1	40.2 ± 1.2
	CIT	0.5	559.2 ± 28.0	367.0 ± 24.8	22.8 ± 5.9	23.4 ± 8.5	27.2 ± 4.4
CIT	1.0	613.2 ± 35.9	332.2 ± 38.7	27.5 ± 4.0	14.8 ± 6.4	13.2 ± 1.78	38.7 ± 3.6
	1.5	589.2 ± 56.6	367.2 ± 38.3	16.0 ± 5.5	11.2 ± 1.8	17.2 ± 7.1	41.2 ± 4.1
	2.0	623.0 ± 13.5	326.4 ± 17.5	22.6 ± 5.7	10.6 ± 2.5	17.4 ± 1.6	37.7 ± 1.3
DIC-A	0.5	569.8 ± 96.6	359.2 ± 57.0	21.4 ± 2.8	23.6 ± 2.8	26.0 ± 2.1	43 ± 5.9
	1.0	591.8 ± 19.6	344.4 ± 21.8	18.6 ± 1.7	21.6 ± 1.3	23.6 ± 1.5	40.8 ± 2.0
	1.5	603.2 ± 14.3	327.2 ± 13.9	23.4 ± 2.0	24.4 ± 2.3	21.8 ± 1.6	39.7 ± 1.4
	2.0	626.4 ± 19.3	305.4 ± 19.0	20.0 ± 2.5	23.0 ± 3.1	25.2 ± 2.2	37.4 ± 1.9

Varules are mean ± SD of 5000 cells (n ¼ 5 bulbs / group). p < .05; p < .01; p < .001 compared to the NC group; ANOVA with Tukey Post-test, by multiple comparisons.

increased the number of MN formation by the two highest concentrations (Table 3).

Micronucleus (MN) formation is an irreversible nuclear alteration process, and it is the result of the final process of DNA changes. This parameter is commonly used to detect DNA damage after exposure to mutagenic agents (Fernandes et al. 2007). There are reports of CIT cytotoxicity and geno-toxicity in embryonic cells, as observed by the induction of different mechanisms, including reactive oxygen species (ROS) formation, nitric oxide (NO) production, Bax and Bcl-2 overexpression, mitochondrial membrane loss, cytochrome C

release, caspase-9/3 activation, p21, kinase 2 and c-JUN-terminal activation. In another study, CIT induced an increase in the frequency of MN, in concentrations 30 mM, from in vitro tests performed on V79 cells (Follmann et al. 2014). Moreover, CIT also causes apoptosis due to HSP90 inactivation and Ras and Rad 1 degradation (Chan 2008).

Polyketides are evident to induce apoptosis and MN formation (Yu et al. 2006, Chan 2007, Donmez et al. 2007). CIT induces DNA damage via ROS formation through mitogen-activated protein kinase (MAPK) activation (Chan et al. 2007, Farrugia and Balzan 2012). In rats, CIT at high

Table 3. Mutagenic effects of AEPc, CIT, DIC-A on meristematic cells of *A. cepa*, after 48 h exposure by micronucleus formation and chromosomal aberrations.

Treatment	Conc. (mg/mL)	Types of chromosomal alterations (CA)					Total
		Micronucleus	c-metaphase	Bridges	Loose chromosomes	delays	
NC	0	0.6 ± 0.4	0.8 ± 0.2	0.2 ± 0.0	0.8 ± 0.1	1.0 ± 0.4	3.4 ± 0.5
PC	6.0	3.4 ± 1.1	4.8 ± 0.8	5.0 ± 2.3	10.2 ± 0.8	10.2 ± 0.8	33.6 ± 3.7
AEPc	0.5	0.8 ± 0.0	3.4 ± 0.5	2.8 ± 1.3	1.8 ± 1.0	3.0 ± 2.2	15.4 ± 2.9
	1.0	2.2 ± 1.0	3.0 ± 0.7	2.6 ± 1.0	3.0 ± 1.4	3.4 ± 0.7	15.0 ± 1.3
	1.5	3.0 ± 0.8	4.6 ± 1.5	3.6 ± 0.8	3.6 ± 1.0	3.8 ± 1.0	17.8 ± 4.6
	2.0	4.4 ± 1.1	4.0 ± 1.0	4.4 ± 2.6	4.6 ± 1.5	3.0 ± 0.8	16.8 ± 1.8
CIT	0.5	1.8 ± 1.1	2.6 ± 1.9	1.8 ± 1.4	1.2 ± 0.7	4.2 ± 2.4	12.6 ± 2.9
	1.0	1.9 ± 1.1	4.2 ± 1.1	3.2 ± 2.1	0.6 ± 0.4	7.6 ± 1.6	19.2 ± 1.3
	1.5	2.2 ± 1.2	3.6 ± 1.5	2.0 ± 1.0	4.6 ± 1.6	7.2 ± 2.3	19.6 ± 4.6
	2.0	3.6 ± 1.5	4.2 ± 0.8	4.2 ± 1.3	5.6 ± 1.1	5.8 ± 0.8	21.6 ± 1.8
DIC-A	0.5	0.6 ± 0.3	3.2 ± 0.8	3.8 ± 0.8	3.2 ± 1.1	2.2 ± 1.3	13.8 ± 1.9
	1.0	0.2 ± 0.4	2.8 ± 1.3	3.8 ± 1.3	4.0 ± 1.5	1.2 ± 0.4	12.0 ± 3.2
	1.5	0.2 ± 0.4	4.2 ± 1.3	3.8 ± 0.8	4.8 ± 1.3	2.0 ± 0.7	15.0 ± 1.4
	2.0	2.4 ± 0.5	4.4 ± 1.1	4.8 ± 1.4	6.2 ± 1.6	2.0 ± 1.0	18.0 ± 2.3

AEPc: Acetonitrile extract of *Penicillium citrinum*; CIT: Citrinin; DIC-A: Dicitrinin A; NC: Dechlorinated water; PC: Copper sulfate. Values are mean ± SD of 5000 cells (n ¼ 5 bulbs/group), ANOVA with Tukey Post-test, by multiple comparisons; p < .05; p < .01; p < .001 compared to the NC group.

doses was seen to increase mRNA expression for *Ccna2*, *Ccnb1* and *E2f1* transcription factors, leading to cell cycle modifications, CA and genotoxicity (Liu et al. 2003, Knasmüller et al. 2004, Bouslimi et al. 2008, Folkmann et al. 2009, Chang et al. 2011, Kuroda et al. 2013). In addition, the induction of MN, mediated by CIT, and several other damages caused to DNA were observed in HepG2 cells (Knasmüller et al. 2004). A good candidate for an antitumor agent should have the ability to induce cytotoxic, genotoxic and mutagenic effects in neoplastic cells, generating blocking effects of the neoplastic process. CIT is capable of causing clastogenic effects in in vivo and in vitro test systems (Liu et al. 2017).

Chromosomal aberrations (CA) during the cell cycle induced by chemical compounds are indicative of mutagenicity and cytotoxicity (Tacar et al. 2013). However, an increase in CA, such as c-metaphases and loose chromosomes, by any test substance is not necessarily due to direct clastogenic effects, rather to an effect resulting from the breakdown of the cell division machinery during mitosis, which is controlled by the cellular cytoskeleton and microtubules (Eleftheriou et al. 2012). Chromosomal abnormalities can lead to apoptosis. In a study, an administration of 0.9 mg/kg (i.p.) or 0.1 mg/kg of CIT in rats was found to reduce CA and double strand breaks as well as aneuploidy due to cell cycle arrest (Jeswal 1996, Yu et al. 2006). On the other hand, a genotoxic effect of CIT on Vero cells was also reported by Yu et al. (2006). In this study, we have seen that numerous cells in interphase as well as CA such as chromosomal fragments, anaphase bridges, delayed chromosomes and MN formation by the treatment of AEPc, CIT and DIC-A in *A. cepa* meristematic cells (Figure 2).

There are reports that CIT exerted nephropathic and hepatotoxic effects on cultured cell lines and animal models (Arai and Hibino 1983, Aleo et al. 1991, Kogika et al. 1993). CIT induced nephrotoxicity by mechanisms associated with cell cycle arrest and microtubule formation, as evidenced in HEK293 cells, and also altered the expression of p53 and p21 proteins during the cell cycle, interfering in the cell division process (Chang et al. 2009).

3.3. Oxidant/antioxidant and DNA repair capacities of AEPc, CIT and DIC-A in *S. cerevisiae*

AEPc, CIT and DIC-A modulated the oxidative effects of H₂O₂-induced oxidative stress in proficient and mutated *S. cerevisiae* at all concentrations (Table 4).

AEPc, CIT and DIC-A, in most of the concentrations, modulated the effects of H₂O₂, but did not reduce its oxidative damages when compared to the NC group. Antioxidant effects were observed in the two lowest concentrations of AEPc against all the test strains of *S. cerevisiae*, and at all concentrations for the double mutant *Sod1Dca11D* when compared to the PC group. Conversely, CIT and DIC-A exerted no antioxidant effects at 2 µg/mL (Table 5). It may be due to their oxidative effects at this concentration (data not shown). According to Iwahashi et al. (2007), CIT inhibits *S. cerevisiae* growth only at concentrations higher than 100 µg/mL, but in our study, CIT was found to show an oxidative stress at 2 µg/mL.

According to Arora and Chandra (2011) isolated compounds of *P. citrinum* demonstrated potent antioxidant activities (in vitro). However, another study indicates that CIT induces oxidative damage and lipid peroxidation at concentrations above 15 mg/kg in rats (Kumar et al. 2014). In addition, genotoxicity was also observed by DNA fragmentation and apoptosis (Kumar et al. 2014). Previous studies have reported that ROS mediate DNA damage in rat skin with an increased expression of p53, p21/waf1 and Bax and cell cycle arrest in G0/G1 and G2/M (Kumar et al. 2011).

The results demonstrate that the compounds tested have similar characteristics of antineoplastic agents, however, at low concentrations, the tested compounds are antioxidant. Several studies have reported that antioxidants such as natural phenolic compounds can act as cytotoxic agents (Kashif et al. 2015, Perveen & Al-Taweel 2017, Csepregi et al. 2020) and that capacity is linked to anti-proliferative and cytotoxic mechanisms in some cases (Yanez et al. 2004). CIT inhibits yeast growth at high concentrations (100 ppm), activating stress response genes such as AADs, FLR1, OYE3, GRE2 and MET17 that are responsible for the glutathione synthesis.

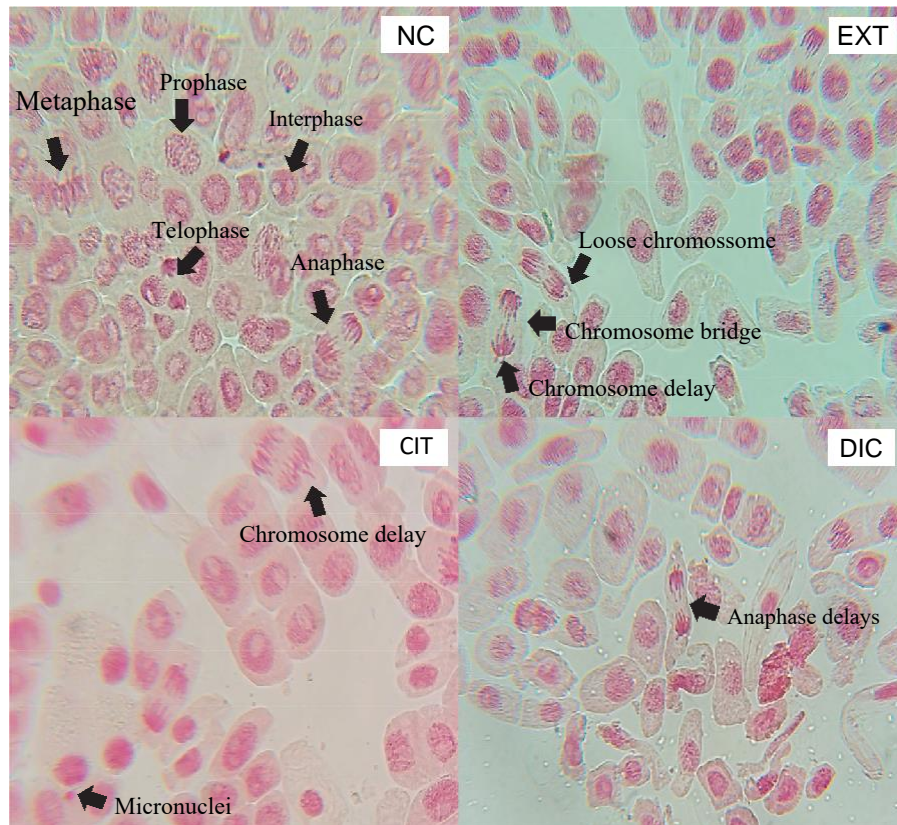


Figure 2. Cell division and chromosomal aberration in *A. cepa* meristematic cells.

Table 4. Pretreatment of *S. cerevisiae* strains with AEPc, CIT and DIC-A.

Groups	SODWT	Sod1D	Sod2D	Sod1DSod2D	Cat1D	Sod1DCat1D
NC	0.50 ± 0.58	0.50 ± 0.61	0.37 ± 0.39	0.41 ± 0.45	0.52 ± 0.49	0.50 ± 0.55
PC	11.25 ± 0.9	15.00 ± 2.16	15.00 ± 0.81	20.25 ± 1.70	20.00 ± 2.06	22.00 ± 1.82
AEPc (mg/mL)						
0.5	3.25 ± 0.50	4.50 ± 0.58	4.25 ± 0.96	3.5 ± 0.58	4.25 ± 0.50	6.75 ± 0.96
1.0	2.00 ± 0.00	7.25 ± 0.96	7.75 ± 0.96	7.25 ± 1.50	7.00 ± 0.82	3.00 ± 0.81
1.5	5.50 ± 0.58	6.25 ± 0.96	7.75 ± 0.50	6.5 ± 0.58	8.25 ± 0.50	6.00 ± 0.58
2.0	6.75 ± 0.50	8.0 ± 1.15	7.25 ± 0.96	7.25 ± 0.96	5.25 ± 0.50	6.00 ± 0.82
CIT (mg/mL)						
0.5	8.75 ± 0.50	7.50 ± 0.58	8.75 ± 0.58	6.00 ± 0.82	6.25 ± 0.96	3.80 ± 1.00
1.0	6.75 ± 0.50	7.25 ± 0.96	9.00 ± 0.82	6.00 ± 0.00	5.75 ± 0.96	6.50 ± 0.57
1.5	10.25 ± 0.96	8.50 ± 0.58	8.75 ± 0.50	7.25 ± 0.96	7.75 ± 0.96	7.00 ± 0.82
2.0	9.75 ± 1.25	7.00 ± 1.15	9.75 ± 0.96	8.25 ± 0.96	8.75 ± 0.96	9.50 ± 0.58
DIC-A (mg/mL)						
0.5	2.00 ± 0.00	4.50 ± 0.58	7.00 ± 0.82	7.25 ± 0.96	4.50 ± 0.58	3.50 ± 0.50
1.0	8.00 ± 0.82	6.00 ± 0.82	6.25 ± 0.50	7.75 ± 0.96	7.25 ± 1.00	8.25 ± 0.96
1.5	6.50 ± 0.58	8.50 ± 0.58	8.25 ± 0.50	8.00 ± 0.82	6.75 ± 0.96	7.25 ± 0.50
2.0	6.70 ± 0.50	7.25 ± 0.50	8.0 ± 1.15	8.25 ± 0.50	9.25 ± 0.96	7.50 ± 0.50

AEPc: Acetonitrile extract of *Penicillium citrinum*; CIT: Citrinin; DIC-A: Dicitrinin A; NC: (vehicle); PC: Hydrogen peroxide (stressor). Values are mean ± SD of inhibition halos measured in mm; ANOVA, two-way, Bonferroni post-test. $p < .01$ compared to the NC; $p < .01$ compared to the PC.

Conversely, CIT was not found to induce DNA repair gene expression (Iwahashi et al. 2007), although it may allow activation of certain defensive genes, promoting adaptation and survival (Santos et al. 2012). In another study, using yeast cells, CIT (1600 IM) induced strong gene expression of 68 genes related to oxidative stress, suggesting a confirmation of the toxicity triggered by CIT is fundamentally based on its ability to generate ROS (Vanaclouig-Pedros et al. 2016). In this context, CIT is capable of triggering oxidative stress responses through induction of natural genes and transcription factors (e.g., Skn7 and Yap1) (Pascual-Ahuir et al. 2014).

AEPc, CIT and DIC-A, in the three lowest concentrations and all strains, participated in the modulation of H₂O₂-

induced damage, but did not eliminate oxidative damage when compared to the NC group. However, CIT at the concentration of 2.0 µg/mL did not modulate H₂O₂-induced damage, as observed in all tested strains (Table 6).

In this study, AEPc, CIT and DIC-A exerted a concentration dependent toxic and cytotoxic effects on *A. salina* and *A. cepa* test systems. In *A. salina* the test substances showed toxic effects by increasing the percentage mortality with the increasing of test concentration. In *A. cepa* test system, the substances reduced the MI value more than 50% in comparison to NC group, which indicates the cytotoxic characteristics of them. Moreover, AEPc, CIT and DIC-A, at all tested concentrations significantly increased the CA parameters, where

Groups	SODWT	Sod1D	Sod2D	Sod1DSod2D	Cat1D	Sod1DCat1D
NC	0.50±0.57	0.50 ± 0.57	0.50 ± 0.57	0.50± 0.57	0.50 ± 0.57	0.50 ± 0.57
PC	11.25±0.95	15.00 ± 2.16	15.00 ± 0.81	17.25± 1.7	15.00 ± 2.06	16.00 ± 1.82
AEPc (mg/mL)						
0.5	0.75±0.95	1.75 ± 0.50	1.00 ± 1.15	1.75±0.50	2.25 ± 1.70	0.25 ± 0.50
1.0	1.25±0.95	3.00 ± 0.82	2.25 ± 0.50	6.25±0.50	4.75 ± 0.95	0.25 ± 0.50
1.5	2.50±1.29	5.75 ± 0.96	2.25 ± 0.96	7.25±0.96	6.25 ± 0.96	0.25 ± 0.50
2.0	2.75±0.96	4.5 ± 1.29	1.25 ± 0.96	8.50±0.58	4.25 ± 0.96	1.75 ± 1.26
CIT (mg/mL)						
0.5	3.75±1.26	8.75 ± 1.50	6.25 ± 1.26	6.00±1.15	6.75 ± 1.50	5.25 ± 0.96
1.0	5.75±0.96	9.00 ± 0.82	8.00 ± 0.82	7.75±1.26	6.00 ± 0.00	6.75 ± 0.50
1.5	6.00±1.15	8.75 ± 0.96	7.25 ± 0.96	6.75±0.96	7.25 ± 0.96	7.00 ± 0.82
2.0	8.25±1.26	9.25 ± 1.26	8.00 ± 0.82	8.00±1.29	9.00 ± 0.58	8.00 ± 1.41
DIC-A (mg/mL)						
0.5	5.50±0.58	5.75 ± 0.96	5.00 ± 1.41	7.25±0.96	5.50 ± 0.58	3.75 ± 0.96
1.0	6.75±0.96	9.00 ± 0.82	6.00 ± 0.82	8.29±0.96	8.00 ± 0.82	4.25 ± 0.50
1.5	6.25±1.26	9.25 ± 0.96	7.75 ± 0.96	7.00±0.82	7.50 ± 1.29	5.75 ± 0.96
2.0	9.00±1.29	9.00 ± 1.41	9.25 ± 0.96	8.25±1.26	8.00 ± 1.41	9.00 ± 1.70

AEPc: Acetonitrile extract of *Penicillium citrinum*; CIT: Citrinin; DIC-A: Dicitrinin A; NC: (vehicle); PC: Hydrogen peroxide (stressor). Values are mean ± SD of inhibition halos measured in mm; ANOVA, two-way, Bonferroni post-test. $p < .0001$ compared to the NC; $p < .0001$ compared to the PC.

Table 6. Effects of AEPc, CIT, DIC-A and control groups on damages induced by H₂O₂ in *S. cerevisiae* strains.

Groups	SODWT	Sod1D	Sod2D	Sod1DSod2D	Cat1D	Sod1DCat1D
NC	0.50±0.58	0.50±0.58	0.50±0.58	0.50±0.58	0.50±0.58	0.50±0.58
PC	11.25±0.95	15.00±2.16	15.00±0.81	16.25± 1.7	17.00±2.06	15.00±1.82
AEPc (mg/mL)						
0.5	5.75±0.96	6.00±0.82	6.0±1.15	5.50±0.58	4.00±0.82	4.50±0.58
1.0	6.25±0.5	8.00±1.15	7.25±0.96	6.00±1.15	5.00±0.0	8.25±0.96
1.5	8.50±0.58	8.75±1.5	7.75±0.96	8.00±1.63	6.75±0.58	5.75±0.5
2.0	9.50±0.58	12.50±1.29	11.50±1.29	9.00±0.82	9.00±1.29	8.50±0.5
CIT (mg/mL)						
0.5	6.50±1.0	7.00±0.82	7.50±1.29	6.50±1.0	7.00±0.0	4.50±0.58
1.0	5.00±0.82	7.75±0.96	7.25±0.96	6.75±0.96	7.50±0.58	7.75±0.50
1.5	7.00±1.63	9.50±2.38	7.75±1.89	6.50±1.0	7.25±2.22	5.50±0.58
2.0	10.25±0.96	13.25±0.96	12.00±0.58	10.00±0.5	11.00±0.96	8.00±0.82
DIC-A (mg/mL)						
0.5	4.25±0.5	7.00±0.82	5.0±0.82	5.50±1.0	4.75±0.96	2.25±0.96
1.0	6.00±1.63	7.75±0.5	5.50±0.58	5.50±0.58	5.75±0.96	5.00±0.82
1.5	6.50±1.29	6.50±0.58	7.75±0.5	6.75±0.5	8.00±0.82	4.75±0.96
2.0	8.00±0.82	10.00±0.82	11.00±0.5	9.00±0.82	9.25±0.96	9.00±0.58

AEPc: Acetonitrile extract of *Penicillium citrinum*; CIT: Citrinin; DIC-A: Dicitrinin A; NC: (vehicle); PC: Hydrogen peroxide (stressor). Values are mean ± SD of inhibition halos measured in mm; ANOVA, two-way, Bonferroni post-test. $p < .0001$ compared to the NC; $p < .0001$ compared to the PC.

AEPc and DIC-A were seen to increase the number of MN formation at the two highest test concentrations. In mutagenic and non-mutagenic *S. cerevisiae* strains, AEPc, CIT and DIC-A modulated the H₂O₂-induced oxidative damage in comparison to the NC group. AEPc at 2 Ig/mL and CIT at the two highest concentrations did not affect the H₂O₂-induced DNA damage in the test strains.

4. Conclusion

AEPc, CIT and DIC-A showed toxicity in *A. salina*. In *A. cephalopoda* system, the compounds also showed an inhibitory effect on cell division phases. The test substances also induced mutagenicity, especially at higher concentrations. Pre-, co- and post-treatments of AEPc, CIT and DIC-A significantly modulated H₂O₂-induced oxidative damage in *S. cerevisiae* strains. The substances also showed a DNA damage repair capacity in *S. cerevisiae* test strains. Further investigations are necessary to understand the exact mechanisms regarding the toxic, cytotoxic and mutagenic effects of these substances.

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