RESEARCH ARTICLE

Toxin Neutralization Efficacy of Catechin and Silymarin against Anthrax Toxins

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Abstract

The present study investigated the *in-vitro* toxin neutralization potential of FDA-approved polyphenols; catechin and silymarin against anthrax lethal and edema toxin. The titration of protective antigen (PA) against a fixed concentration of LF, and EF induced a linear dose-dependent cytotoxic effect with maximum attainable cytotoxicity at 10 µg/mL PA, and 1 µg/mL LF/EF in RAW264.7 and CHO.K1 cells. The LT and ET-induced cytotoxicity were assessed using MTT assay, and cAMP ELISA, respectively. All the tested concentrations (100, 10, 1, 0.1 µM) of catechin and silymarin exhibited a highly significant and dose-dependent reduction in LT-induced cytotoxicity ($p<0.01$). Further, a highly significant reduction in cAMP levels in ET-intoxicated cells was observed for the higher concentrations of both compounds ($p<0.01$), except at 0.1 µM concentration, while the mean cAMP levels at 100, and 10 µM of silymarin were less as compared to that at similar concentrations of catechin, the difference was insignificant ($p>0.05$). The study emphasized the anti-toxin potential of catechin and silymarin, which could augment the antimicrobials in anthrax infection. Further, future studies focused on their pharmacodynamics, kinetics, and determination of clinically safe therapeutic doses *in vivo* are warranted.

Key words: Anthrax, cAMP, Edema toxin, Lethal toxin, MTT.

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Introduction

Bacillus anthracis, a Gram-positive, rod-shaped bacterium causes a serious disease called anthrax (D'Amelio *et al.,* 2015). The virulence of the bacterium is attributed to two large plasmids, *viz.,* pX01, and pX02, encoding for proteinaceous factors, *viz.,* lethal factor (LF), edema factor (EF), protective antigen (PA), and poly-d-glutamic acid capsule, respectively (Bower *et al.,* 2022). While the factors are non-toxic independently, the combination of lethal factor (LF)/edema factor (EF) with protective antigen (PA) forms a bipartite toxin, *viz.,* lethal toxin (LT), and edema toxin (ET) responsible for the pathophysiology of the disease (Liu *et al.,* 2014; Bower *et al.,* 2022). PA acts as a binding moiety that translocates LF inside the cell, which is a Zn-dependent *metalloproteinase* that disrupts the host cell-signaling pathway by cleaving *mitogen-activated protein kinase kinases* (MAPKKs) and *Nlrp1* (Liu *et al.,* 2014). The pathogenesis of EF is attributed to the altered cell transcription resulting from elevated cyclic adenosine monophosphate (cAMP) levels via calcium and calmodulin-dependent *adenylate cyclase* activity of EF (Leppla, 1982).

The treatment for anthrax includes antimicrobials including but not limited to penicillins, and fluoroquinolones like doxycycline, and ciprofloxacin (Moayeri *et al.,* 2015), however, they are no longer effective once the intoxication sets in (Abrami *et al.,* 2013). Further, vaccines help prevent and stop the chain of transmission from infected animals to humans or healthy animals (Baillie and Read, 2001). PA is used as an immunogen in clinically used vaccines, *i.e.,* the US BioThrax vaccine (formerly called Anthrax Vaccine Adsorbed) and AVP vaccine (Moayeri *et al.,* 2015). Albeit the benefits that AVA offers, it has certain limitations including high production costs, lack of standardization, the need for repeated dosing, and transient side effects (Baillie and Read, 2001). Therefore an alternate therapeutic approach augmenting the antimicrobials against anthrax toxins is the need of the hour.

Flavonoids, a class of natural polyphenolic compounds produced by plants possesses anti-inflammatory, antioxidant, chemoprotective, anticarcinogenic, and antimutagenic properties (Panche *et al.,* 2016). Additionally, they are capable of modulating cellular enzymes like xanthine oxidase (XO), cyclo-oxygenase (COX), lipoxygenase, phosphoinositide 3-kinase, and matrix metalloproteinases

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(Hou and Kumamoto, 2010; Ribeiro *et al.,* 2015; Lin *et al.,* 2015). Catechins are an important subset of flavonoids accounting for more than 75% of total polyphenols in tea leaves (Bae *et al.,* 2020) with similar therapeutic properties as other flavonoids including inhibitory activity against the activity of toxins (Wu and Brown, 2021). Primarily, there are eight catechins distinguished by distinct chemical structures, and catechin is one among them (Singh *et al.,* 2011). Previously the anti-toxin potential of epigallocatechin gallate (EGCg) against different bacterial toxins (Cherubin et al., 2016; Miyamoto *et al.,* 2014; Chang *et al.,* 2019) and anthrax lethal toxin *in vitro* and *in-vivo* models has been explored (Dell'Aica *et al.,* 2004), however there are still no reports on catechin being investigated as an anti-anthrax toxin. Silymarin is another popularly known hepatoprotective flavonoid with cardioprotective, neuroprotective, cytoprotective, anti-inflammatory, and anti-carcinogenic properties (Škottová *et al.,* 2003). Furthermore, the protective effects of silymarin against some mycotoxins, snake venoms, jellyfish venom (Asirvatham *et al.,* 2023), and bacterial and chemical toxins have been studied in depth previously (Fraschini *et al.,* 2002; Fanoudi *et al.,* 2020). Keeping in view the previously established anti-toxin potential of a few flavonoids, the present study was planned to investigate the toxin neutralization potential of catechin and silymarin against anthrax lethal and edema toxin in an *in-vitro* model.

Materials and Methods

Cell Culture and Reagents

The cell lines employed in the study (RAW 264.7 and CHO. K1) were procured from the National Center for Cell Sciences (NCCS), Pune. The FDA-approved polyphenols; catechin and silymarin, were procured from Sigma Aldrich, India. LT-induced cytotoxicity assay was performed in RAW 264.7 cells (murine macrophage-like cell line) maintained in Dulbecco's modified Eagle media (DMEM) and supplemented with 4.5 g/L D-glucose, 110 mg/L sodium pyruvate, 5% heat-inactivated bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin, and 10 mM HEPES. Furthermore, ET-induced cytotoxicity assays were performed in Chinese hamster ovary cells (CHO.K1) maintained in Ham's F12 media, supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin, and 10% heat-inactivated bovine serum. The cell monolayer was trypsinized using 0.25% trypsin-EDTA and transferred to a 96-well plate for experimentation.

Affinity Purification of Recombinant Lethal Factor (LF), Edema Factor (EF) and Protective Antigen (PA) using Ni-NTA Column Chromatography

The nickel-nitrilotriacetic acid (Ni-NTA) affinity purification of LF, EF and PA was performed using the previously described protocol (Kumar *et al.,* 2001; Manish *et al.,* 2013).

Western Blot Assay

SDS-PAGE was performed using a discontinuous gel system with 12% resolving and 5% stacking gels containing 0.1% SDS. A 180 kDa protein marker (PUREGENE) was included. After electrophoresis, proteins were transferred to a nitrocellulose membrane overnight. The membrane was incubated with anti-His primary antibody (1:10,000), washed with PBST, and then incubated with anti-mouse IgG-Alkaline Phosphatase (AP) secondary antibody (1:10,000). After further washes, target proteins were visualized using NBT/BCIP in AP buffer

In Vitro Studies

Optimization of cytotoxic dose of lethal toxin (LT) and edema toxin (ET): The *in vitro* cytotoxicity dose for LT and ET was respectively optimized in RAW 264.7 cells and CHO.K1 cells using MTT assay and cAMP ELISA (Dell'Aica *et al.,* 2004; Taskeen *et al.,* 2023). The percent cytotoxicity was calculated using the formula (Dell'Aica *et al.,* 2004)

$$\% \text{ Cytotoxicity} = 100 \times (O.D_{Control} - O.D_{Sample}) / (O.D_{Control})$$

In-vitro LT and ET neutralization by catechin and silymarin: The *in-vitro* LT neutralization, using different concentrations of catechin and silymarin (100 µM, 10 µM, 1 µM, and 0.1µM) was optimized using our previously optimized protocol (Taskeen *et al.,* 2023). Briefly, both catechin and silymarin were separately prepared and pre-incubated with 1 µg/mL of LF and EF in incomplete DMEM (LF) and Ham's F12 media (EF) for 15 min at 37°C. Later, PA (optimized concentration) was added to each well, followed by incubation of the plate at 37°C and 5% CO_2 for 4 h. Following incubation, an MTT assay (LT) and a cAMP ELISA (ET) were performed, and the percent cytotoxicity was calculated.

Statistical Analysis

The data for dose optimization (LT and ET) is illustrated as a means plot to represent the linearity in cytotoxicity and cAMP elevation, and as mean ± standard deviation for toxin-neutralization assays using IBM SPSS Statistics v.27.0.1 (IBM Corporation, Armonk, NY). A one-way analysis of variance was performed to compare the homogeneity of variance between different cytotoxic doses of LT and ET for dose optimization and to assess the toxin-neutralization potential of the compounds against both toxins. Further, *post-hoc* analysis was performed using Tukey's test if the Levene statistic $p>0.05$, however, for Levene statistic $p\leq0.05$, the Welch test with Games-Howell *post hoc* test was used. The effect size, *i.e.,* partial eta square (n^2_p) was calculated using a general linear model and univariate analysis. For the analysis, $p\leq0.05$ was considered statistically significant, while $p\leq0.01$ was considered highly significant.

Results and Discussion

Confirmation of Recombinant Lethal Factor (rLF), Edema Factor (rEF), and Protective Antigen (rPA)

The eluted purified fractions of recombinant LF, EF, and PA proteins using Ni-NTA column chromatography were collected and electrophoresed on 12% SDS-PAGE. On SDS-PAGE resolution a 90 kDa, 89 kDa, and 83 kDa protein band

was evident for rLF, rEF, and rPA, respectively, which was further confirmed on western blot (Fig. 1).

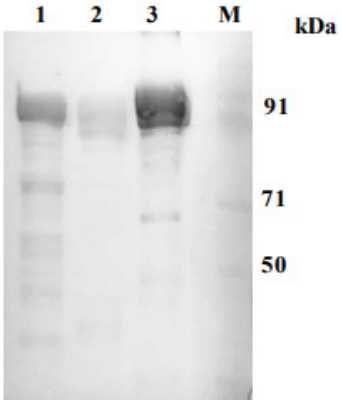

Fig. 1: Confirmation of rPA, rLF, and rEF using Western blot assay: Lane M: Protein marker (BIO-RAD Precision Plus Protein TM Standards, prestained ladder), Lane1: rPA (83 kDa), Lane 2: rLF (90 kDa), Lane 3: rEF (89 kDa)

Optimization of Cytotoxic Dose of Lethal Toxin (LT)

The study observed a concentration-dependent highly significant increase in cytotoxicity (Welch statistic, $p< 0.001$, $n^2_p= 0.986$) when variable concentrations of PA were tested against a constant concentration of LF (1 µg/mL) (Fig. 2), with maximum attainable cytotoxicity (74.44%) at PA 10 µg/mL of PA and 1 µg/mL of LF. Therefore, PA 10 µg/mL of PA and 1 µg/mL of LF were optimized as a cytotoxic dose and used for further experimentation. Further, on the comparison, a significant difference in cytotoxicity was observed between the optimized cytotoxic dose and the lower concentrations of LT (0.625, 0.3125, 0.15, 0.07 µg/mL) ($p<0.05$).

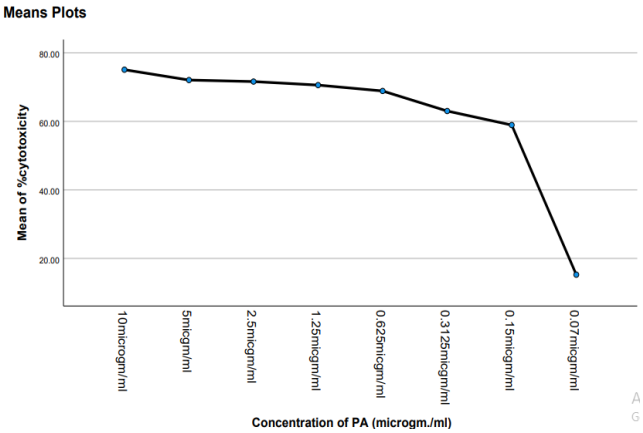

Fig. 2: Optimization of cytotoxic dose of LT in RAW 264.7 cells: RAW264.7 cells (2×10^4/well) in a 96-well plate were pre-incubated with LT (varying PA, constant LF at 1 µg/mL) for 4 h at 37 °C, 5% CO_2. DMEM-only wells served as negative controls. Cytotoxicity was measured via MTT assay, and dose-dependent effects were shown as mean plots from three independent experiments.

In Vitro LT Neutralization by Catechin and Silymarin

Different concentrations of catechin and silymarin (100, 10, 1, and 0.1 µM) were tested against a cytotoxic dose of LT (Fig. 3). The study observed that all the tested concentrations of both compounds produced a dose-dependent highly significant reduction in LT-induced cytotoxicity when compared to the toxin control (Welch statistic, $p<0.001$, $n^2_p=0.967$). Further, Tukey's *post-hoc* comparison revealed that the difference in cytotoxicity between different concentrations of catechin was insignificant ($p>0.05$), while in the case of silymarin, the difference was highly significant between 10 µM and 0.1 µM (Games Howell statistic, $p<0.005$); and 1 µM and 0.1 µM (Games Howell statistic, $p<0.046$). LF is a Zn-dependent *metalloprotease* with m*itogen-activated protein kinase kinase* 1 and 2 (MAPPK1 and 2, or MEK1 and 2) as the original substrates (Duesbery *et al.,* 1998; Vitale *et al.,* 1998). Previous studies have established the role of EGCg (Hwang *et al.,* 2020) and silymarin (Asirvatham *et al.,* 2023) as potent inhibitors of jellyfish *metalloproteinase*. Likewise, EGCg and catechin gallate (CG) have been reported as inhibitors of the proteolytic activity of LF, responsible for reduced LT-mediated macrophage cytotoxicity, and survival of rats from LF-induced death (Dell'Aica *et al.,* 2004). Given that the inhibitory role of catechins and silymarin on *metalloproteinases* has been previously established, the dose-dependent reduction in LT-induced cytotoxicity observed in the present study could be a result of inhibition of LT *metalloprotease*.

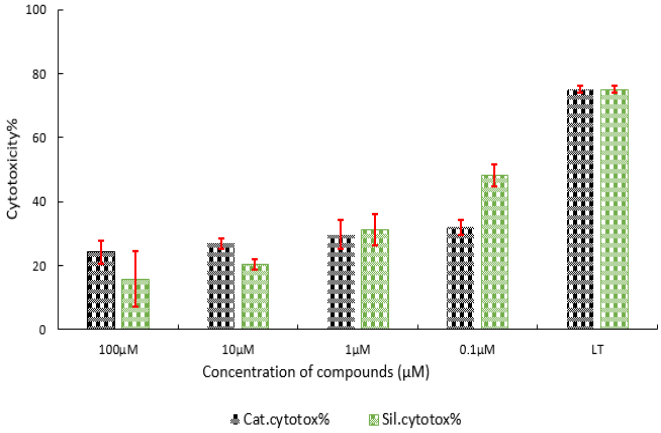

Fig. 3: RAW264.7 cells (2×10^4/well) were pre-incubated with varying concentrations of catechin or silymarin and 1 µg/mL LF for 15 min at 37 °C. PA (10 µg/mL) was then added, followed by 4 h incubation. Cytotoxicity was measured via MTT assay and expressed as mean ± SD from three independent experiments.

Optimization of Cytotoxic Dose of Edema Toxin (ET)

Edema toxin is responsible for elevating the intracellular cAMP in CHO.K1 cells. The study revealed a linear dose-dependent highly significant increase in cAMP concentration when variable concentrations of PA (20, 10, 5, 2.5, 1.25, 0.625 µg/mL) were tested against a constant concentration of EF (1 µg/mL) ($p<0.001$) (Fig. 4). On analysis, the observed difference in cAMP levels at PA 20 µg/mL, and 10 µg/mL was insignificant ($p>0.05$), while all other concentrations depicted a significant difference in cAMP levels. Therefore, 10 µg/mL PA and 1 µg/mL EF were optimized as the cytotoxic dose of ET. Previously,

PGE2-imidazole, the first identified inhibitor of mammalian *adenylate cyclase*, has been reported to effectively inhibit EF at micromolar concentrations.

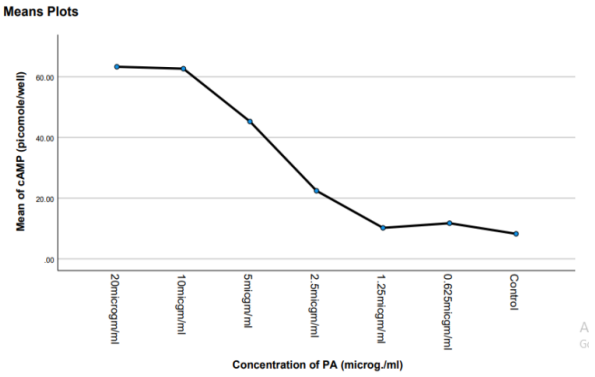

Fig. 4: CHO.K1 cells (1×10^5/well) were pre-incubated with ET in Ham's F12 medium for 4 h at 37 °C, 5% CO_2. Medium-only wells served as controls. Post-incubation, cells were lysed and cAMP levels measured via ELISA. Dose-dependent cAMP increase is shown as mean plots from three independent experiments.

In Vitro ET Neutralization by Catechin and Silymarin

The study observed that the higher concentrations of catechin ($F_{4, 10}$= 483.585, p<0.001, n^2_p= 0.995) and silymarin ($F_{4, 10}$=600.467, p<0.001, n^2_p= 0.996), except 0.1 µM elicited a dose-dependent reduction in cAMP levels when compared to the cytotoxic dose of ET (Fig. 5). Further, the mean cAMP levels at 100, and 10 µM of silymarin were less than that at similar doses of catechin, however, this difference was found to be statistically insignificant (p>0.05). EF is an *adenylate cyclase* which is responsible for catalyzing the intracellular conversion of ATP to cAMP (Shen *et al.,* 2005) which disrupts the cellular water homeostasis thus leading to altered intracellular signaling pathways and activation of

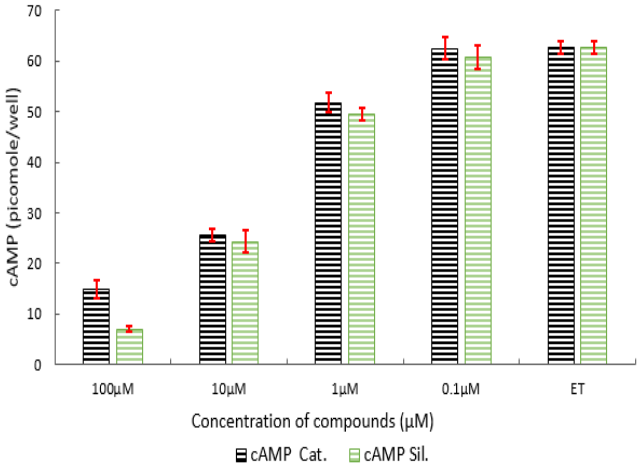

Fig. 5: CHO.K1 cells (1×10^5/well) were pre-incubated with varying concentrations of catechin or silymarin and 1 µg/mL EF for 15 min at 37 °C. PA (10 µg/mL) was then added, followed by 4 h incubation. After lysis, cAMP levels were measured via ELISA and presented as mean ± SD from three independent experiments.

chloride channels (Ahuja *et al.,* 2004). The inhibition of the *adenylate cyclase* activity of EF can therefore prevent further pathogenesis of anthrax.

Conclusion

The study concluded with the anti-toxin potential of catechin and silymarin against anthrax toxins at micromolar concentration *in vitro*. These findings prove to be supportive of the usage of both compounds as alternate therapeutics in the advent of anthrax infection. However, studies encompassing the accumulation, distribution, metabolism, and excretion (ADME) of these compounds *in vivo* and their formulations for therapeutic use are warranted in the future.

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