RESEARCH ARTICLE

In-Vitro Neutralization Efficacy of Taxifolin against Anthrax Toxins

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Abstract

The present study was undertaken to evaluate the protective efficacy of Taxifolin against anthrax toxins, *viz.,* lethal toxin (LT) and edema toxin (ET) in *in-vitro* model. The MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay and cAMP ELISA was deployed to estimate the LT induced cytoxicity and ET induced intracellular increase in cyclic AMP, respectively, wherein 10 µg/mL PA + 1 µg/mL LF/EF was deduced as the cytotoxic dose. The assays revealed an anticipated dose dependent decrease in cytotoxicity at varying concentrations (100 µM to 0.1 µM) of taxifolin when challenged against optimized cytotoxic dose of LT (lethal toxin). Similar dose dependent decrease in intracellular cAMP levels was observed at all the evaluated concentrations of taxifolin (100 µM to 0.1 µM) against cytotoxic dose of ET (edema toxin). The neutralization potential of taxifolin against both the anthrax toxins pave a way for future prospects of experimentation using combination of taxifolin with other such inhibitors and/or antibiotics in *in vitro* and *in vivo* model, which could therefore be helpful in formulating therapeutics against anthrax.

Key words: Anthrax, Edema toxin, *In vitro* efficacy, Lethal toxin, Protective antigen, Taxifolin.

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Introduction

Anthrax is a fatal zoonotic disease caused by Gram-positive, rod-shaped, spore-forming bacterium *Bacillus anthracis*. The pathophysiology of the disease is attributed to the production of three plasmid encoded proteinaceous factors namely, protective antigen (PA), lethal factor (LF) and edema factor (EF) (Bower *et al.,* 2022). These factors in isolation are however incapable of producing toxicity and need to combine to form a tripartite toxin in order to cause disease. The anthrax toxin so formed is therefore a binary combination (A/B) of the three protein moieties: PA, LF and EF. Lethal Factor (LF), a Zn dependent metallo-protease is transported inside the cell by PA, which acts as a binding moiety for LF to form lethal toxin (LT) (Cote and Welkos, 2015). Lethal factor (LF) is responsible for disrupting the host cell signaling pathway by cleaving the mitogen-activated protein kinase kinases (MAPKKs) and Nlrp1 (Levinsohn *et al.,* 2012). However, edema factor (EF) is a Ca^{2+} and calmodulin-dependent adenylyl cyclase that intracellularly converts adenosine triphosphate (ATP) to cyclic-adenosine monophosphate (cAMP) leading to elevated cAMP levels and dissemination of the bacterium in the host (Dumetz *et al*., 2011).

The treatment guidelines laid by CDC recommend the use of an anti-toxin agent in combination with antibiotics for patients suffering with systemic illness. However, despite the continuous use of appropriate antimicrobial agents and advancement in supportive care, individuals with systemic anthrax still remain at high risk of death due to the detrimental effects of the toxins formed inside the body after the infection. Therefore, a multifaceted approach is required to augment the use of antimicrobials either by inhibiting toxin formation or its neutralization using inhibitor molecules.

Taxifolin is a naturally available flavonoid abundantly found in olive oil, grapes, citrus fruits and onions with hepato-protective (Tapas *et al*., 2008), cardioprotective, anti-cancer (Polyak *et al*., 2010), anti-inflammatory, anti-fibrotic and anti-angiogenic properties. The anti-microbial, anti-tuberculosis (Kozhikkadan Davis *et al*., 2018) and anti-toxoplasmosis (Abugri *et al*., 2018) properties of taxifolin have been previously documented in the *in-vitro* model. Apart from that, multiple studies have previously reported the efficacy of taxifolin as a potential antioxidant, hepato-protectant, anti-inflammatory, antiviral and anti-bacterial agents. However, to the best of our knowledge, none of the studies have ever reported the efficacy of taxifolin against anthrax toxins. Therefore, present study was undertaken to

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evaluate the toxin neutralization potential of taxifolin in the *in-vitro* model against LT and ET.

Materials and Methods

Cell Lines and Reagents

The cell lines employed in the study (RAW 264.7 & CHO.K1) were procured from National Center for Cell Sciences (NCCS), Pune. Taxifolin was procured from Sigma Aldrich, India. The purified fractions of LF, EF and PA were obtained through recombinant constructs and were kindly provided by School of Biotechnology (SBT), Jawaharlal Nehru University, New Delhi, India.

In Vitro Studies

Cell Culture

The murine macrophage like cells RAW 264.7 were employed for toxin neutralization assay with lethal toxin (LT). The cells were cultured and maintained in Dulbecco's modified Eagle media (DMEM) supplemented with 4.5 g/liter D-glucose, 110 mg/liter sodium pyruvate, 5% heat inactivated bovine serum, 2 mM L-glutamine, 1% penicillin-streptomycin and buffered with 10 mM HEPES.

Chinese hamster ovary cells (CHO.K1) were employed for experimentation with edema toxin (ET). The cells were maintained in Ham's F12 media supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin and 10% heat-inactivated bovine serum until the formation of a monolayer. The adherent cells were trypsinised with 0.25% trypsin-EDTA and transferred to a 96 well plate for further experimentation.

Optimization of *In Vitro* Cytotoxicity Dose for Lethal Toxin (LT) and Edema Toxin (ET)

The cytotoxic dose for LT was optimized by MTT [3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay in RAW 264.7 cells as per the protocol suggested by Dell'Aica *et al*. (2004) and % LT induced cytotoxicity was calculated using the formula:

(%) Cytotoxicity = 100 x (Control – Sample) / (Control)

Where, sample - OD of cells treated with toxin, and control - OD of the negative control.

The cytotoxic dose of edema toxin (ET) was optimized in Chinese hamster ovary (CHO.K1) cell line by employing commercial c-AMP ELISA kit (R&D Systems, USA) using the previously described protocol by Maddugoda *et al*. (2011). The supernatant was further subjected to competitive cAMP ELISA. The observations (OD at 450_{nm} and 540_{nm}) for each concentrations of ET tested was interpolated with the standard curve plot.

Evaluating Neutralization Ability of Taxifolin against LT and ET

The neutralization ability of varying concentrations of Taxifolin (100 µM, 10 µM, 1 µM and 0.1 µM) were tested against LT using the protocol described by Dell'Aica *et al*. (2004). Further, the MTT assay was performed and the percent cytotoxicity was calculated as stated previously.

The previously described concentrations of taxifolin were further employed to test their neutralization efficacy against ET using the protocol described by Maddugoda *et al*. (2011) and concentrations of cAMP was interpolated using standard curve.

Statistical Analysis

The results of the *in vitro* cytotoxicity trials with toxins and taxifolin were analyzed using SPSS software and plotted using Graphpad prism. The data was expressed as mean ± S.D. The experiments were conducted in triplicate and subjected to one way analysis of variance (ANOVA) to analyze the *p* value. The *p* value <0.05 was considered as statistically significant.

Results and Discussion

All the experiments were conducted in triplicate in order to improve the reproducibility of the results. When variable concentrations of PA were tested against constant concentration of LF (1 µg/mL), a concentration dependent increase in cytotoxicity in RAW 264.7 cells was observed (Fig. 1). This pattern of dose-dependent increase in LT induced cytotoxicity was consistent with the findings of Pohl *et al*. (2013) and Artenstein *et al*. (2004). After the assessment of cytotoxicity induced at various combinations of PA and LF, 10 µg/mL PA and 1 µg/mL LF was fixed as the final cytotoxic dose, which was capable of killing 74.4% of RAW264.7 cells (Table 1). This dose combination however differs from that used by Pohl *et al*. (2013), wherein 1 µg/ mL each of PA and LF was determined as a cytotoxic dose to evaluate the protective effect of monoclonal antibody against LT. Besides, even lower concentrations of LT (each of PA and LF in equal proportion ranging from 100 to 600 ng/mL) have been found to be effective in inducing maximum cytotoxicity in RAW 264.7 cell line (Artenstein *et al*., 2004). The difference observed in cytotoxic doses of LT might be a result of differences in procedures employed to generate recombinant clones (rPA, rLF), purification protocols and subsequently functionality of the proteins.

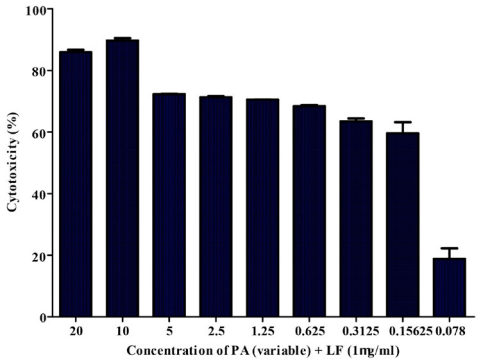

Fig.1: Optimization of cytotoxic dose of LT in RAW264.7 cells

Table 1: Optimization of cytotoxic dose of LT using MTT assay in RAW264.7 cells

PA (µg/mL)	LF (µg/mL)	Cytotoxicity %
10	1	74.44694
5	1	72.27222
2.5	1	71.29734
1.25	1	70.45369
0.625	1	68.35396
0.3125	1	63.47957
0.15625	1	59.58005

While evaluating different concentrations of taxifolin against predetermined toxic dose of LT, it was observed that all the tested concentrations of taxifolin (1 µM to 100 µM) produced a significant dose dependent reduction in cytotoxicity when compared with the toxin control (Fig. 2, Table 2), however, no significant difference in cytotoxicity was observed in between the two concentrations of taxifolin, *viz*., 10 µM and 1 µM. These findings coincided with those of Dell'Aica *et al*. (2004), where the green tea polyphenols exhibited anti-toxin potential against LF in RAW 264.7 cells even at concentration of 0.01 µM (Dell'Aica *et al*., 2004). Our findings ran parallel with the findings of Antonelli *et al*. (2014), where curcumin and its related compounds inhibited the proteolytic activity of LF in a dose dependent fashion in *in-vitro* model.

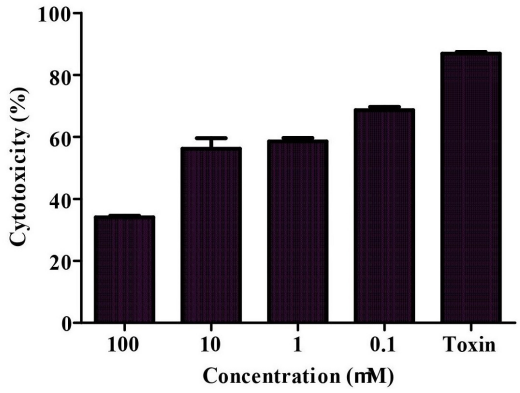

Fig. 2: Neutralization efficacy of Taxifolin against LT in RAW264.7 cells

Table 2: Neutralization efficacy of taxifolin against cytotoxic dose of LT in RAW264.7 cells

Taxifolin (µM)	Average OD	Survival %
100	2.319	91.56
10	1.269	50.10
1	1.461	57.69
0.1	1.258	49.67
Toxin control	0.681	25.55

Similarly, following same titration protocol for optimizing cytotoxic dose of ET, the intracellular cAMP concentration increased in a dose dependent manner and 10 µg/mL of PA and 1 µg/ mL of EF combination was fixed as the cytotoxic dose which elevated the intracellular cAMP levels to 66 picomole compared to that in media control (8.24 picomole) (Fig. 3, Table 3). Consequently, the neutralization efficacy of taxifolin at the above mentioned concentrations (0.1 µM to 100 µM) against ET was evaluated in CHO.K1 cells using cAMP ELISA. It was observed that all the tested concentrations of taxifolin produced a significant dose-dependent decline in cAMP levels when compared to the toxin control (Fig.4, Table 4).

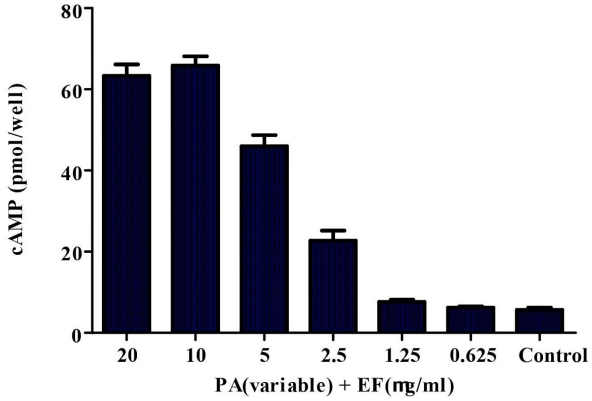

Fig. 3: Optimization of cytotoxic dose of ET in CHO.K1 cells

Table 3: Optimization of cytotoxic dose of ET using cAMP ELISA in CHO.K1 cells

PA (µg/mL)	EF (µg/mL)	cAMP (pmole/well)
20	1	63.3
10	1	66.0
5	1	45.26
2.5	1	22.42
1.25	1	10.20
0.625	1	8.40
Media control	-	8.24

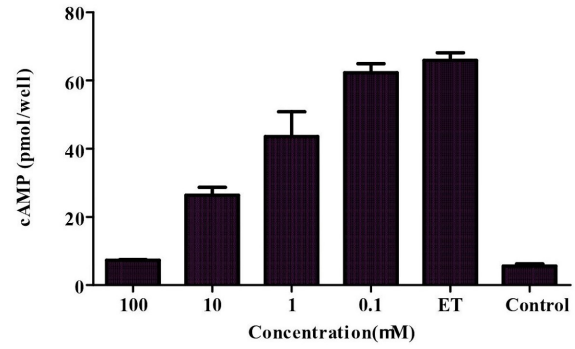

Fig. 4: Neutralization efficacy of taxifolin against ET in CHO.K1 cells

The neutralization potential of taxifolin could be attributed to its pre-established role as an immune-modulator in suppressing the production of pro-inflammatory cytokines (IL-1 beta, IL-6, GM-CSF and TNF-α) (Rhee *et al*., 2008) against LPS activated RAW 264.7 cells. Since, the macrophages infected with LT of *B. anthracis* are known to produce increased levels of tumor necrosis factor alpha (TNF-α) which eventually leads to shock-like symptoms (Hanna *et al*., 1993).

Concurrently we could hypothesize that the decreased cytotoxicity observed after treatment with each inhibitor(s) might be due to down regulation of the inflammatory cytokines (mainly TNF-α), ROS scavenging and subsequent cyto-protective action, however the exact mechanism still needs to be explored in further studies.

Table 4: Neutralization efficacy of taxifolin against cytotoxic dose of ET in CHO.K1 cells

Taxifolin (µM)	cAMP (pmole/well)
100	12.2
10	25.44
1	45.24
0.1	63.46
Toxin control	66.0
Media control	8.24

Conclusion

The present study highlights the potential of taxifolin as an effective tool in preventing the LT and ET induced cytoxicity in *in vitro* model, however, there is a need for extensive research on the cumulative effect of taxifolin when used in conjunction with antibiotics and/or other anti-toxin compounds. The findings of the present study could be used as a footprint to trace the role of taxifolin in treating intoxication also in *in vivo* model, in order to extrapolate the results in human settings. Additionally, further experimentation on studying the ADME (accumulation, distribution, metabolism and excretion) profile of taxifolin in *in vivo* model is needed to estimate the therapeutic dose with significant bioavailability in the blood on administration.

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References

Abugri, D.A., Witola, W.H., Russell, A.E., & Troy, R.M. (2018). *In vitro* activity of the interaction between taxifolin (dihydroquercetin) and pyrimethamine against *Toxoplasma gondii*. *Chemical Biology & Drug Design*, *91*(1), 194-201.

Antonelli, A.C., Zhang, Y., Golub, L.M., Johnson, F., & Simon, S.R. (2014). Inhibition of anthrax lethal factor by curcumin and chemically modified curcumin derivatives. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *29*(5), 663-669.

Artenstein, A.W., Opal, S.M., Cristofaro, P., Palardy, J.E., Parejo, N.A., Green, M.D., & Jhung, J.W. (2004). Chloroquine enhances survival in *Bacillus anthracis* intoxication. *Journal of Infectious Diseases*, *190*(9), 1655-1660.

Bower, W.A., Hendricks, K.A., Vieira, A.R., Traxler, R.M., Weiner, Z., Lynfield, R., & Hoffmaster, A. (2022). What is anthrax? *Pathogens*, *11*(6), 690.

Cote, C.K., & Welkos, S.L. (2015). Anthrax toxins in context of *Bacillus anthracis* spores and spore germination. *Toxins*, *7*(8), 3167-3178.

Dell›Aica, I., Dona, M., Tonello, F., Piris, A., Mock, M., Montecucco, C., & Garbisa, S. (2004). Potent inhibitors of anthrax lethal factor from green tea. *EMBO reports*, *5*(4), 418-422.

Dumetz, F., Jouvion, G., Khun, H., Glomski, I.J., Corre, J.P., Rougeaux, C., ... & Goossens, P.L. (2011). Non-invasive imaging technologies reveal edema toxin as a key virulence factor in anthrax. *The American journal of pathology*, *178*(6), 2523-2535.

Hanna, P.C., Acosta, D., & Collier, R.J. (1993). On the role of macrophages in anthrax. *Proceedings of the National Academy of Sciences*, *90*(21), 10198-10201.

Kozhikkadan Davis, C., Nasla, K., Anjana, A.K., & Rajanikant, G.K. (2018). Taxifolin as dual inhibitor of Mtb DNA gyrase and isoleucyl-tRNA synthetase: Molecular docking, dynamics simulation and *in vitro* assays. *In Silico Pharmacology*, *6*, 1-11.

Levinsohn, J.L., Newman, Z.L., Hellmich, K.A., Fattah, R., Getz, M.A., Liu, S., ... & Moayeri, M. (2012). Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLoS pathogens*, *8*(3), e1002638.

Maddugoda, M.P., Stefani, C., Gonzalez-Rodriguez, D., Saarikangas, J., Torrino, S., Janel, S., ... & Lemichez, E. (2011). cAMP signaling by anthrax edema toxin induces transendothelial cell tunnels, which are resealed by MIM via Arp2/3-driven actin polymerization. *Cell Host & Microbe*, *10*(5), 464-474.

Pohl, M.A., Rivera, J., Nakouzi, A., Chow, S.K., & Casadevall, A. (2013). Combinations of monoclonal antibodies to anthrax toxin manifest new properties in neutralization assays. *Infection and Immunity*, *81*(6), 1880-1888.

Polyak, S.J., Morishima, C., Lohmann, V., Pal, S., Lee, D.Y., Liu, Y., ... & Oberlies, N.H. (2010). Identification of hepatoprotective flavonolignans from silymarin. *Proceedings of the National Academy of Sciences*, *107*(13), 5995-5999.

Rhee, M.H., Endale, M., Kamruzzaman, S.M., Lee, W.M., Park, H.J., Yoo, M.J., & Cho, J.Y. (2008). Taxifolin inhibited the nitric oxide production and expression of pro-inflammatory cytokine mRNA in lipopolysaccharide-stimulated RAW264.7 cells. *Biomedical Science Letters*, *14*(3), 147-155.

Tapas, A.R., Sakarkar, D.M., & Kakde, R.B. (2008). Flavonoids as nutraceuticals: A review. *Tropical Journal of Pharmaceutical Research*, *7*(3), 1089-1099.