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The effect of the antirheumatic gold drug auranofin in *Trichomonas vaginalis*

Bachelor Thesis

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Vienna, June 2020

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1. Introduction

Trichomonas vaginalis is a microaerophilic protist parasite, causing trichomoniasis in humans. Trichomoniasis is the most prevalent non-viral, sexually transmitted infection, that proceeds asymptomatic in most cases. If occurring after all, the symptoms are inflammation, pruritus and odorous vaginal discharge. The protozoon is considered as opportunistic and can increase the risk for HIV-infection, cervix and prostate cancer and may have severe impact on pregnancies (Leitsch 2016). *T. vaginalis* increases the vaginal pH from 4.5 to > 5 (Kissinger 2015) and is often accompanied by bacterial disequilibrium, i.e. mostly alongside with anaerobic bacteria, but also as a host of bacterial or viral endosymbionts, like *Mycoplasma* and Trichomonasvirus, itself (Mercer und Johnson 2018).

Trichomoniasis has so far been treated with metronidazole or tinidazole, both 5-nitromidazol prodrugs. The drug is partially reduced in the cell before it targets the thioredoxin reductase (TrxR) as well as it depleting the thiol pools (Leitsch 2016), both major players in the cell´s protection system against oxidative damage (Hopper et al. 2016). The recommended regimen of metronidazole is a single dose of 2 g peroral (p. o.). Alternatively 500 mg can be applied two times a day for seven days, p. o.. Though being a successful therapy in most patients, about 5 %, have shown to be resistant or responded with allergic reactions to metronidazole. The prevalence of resistance varies across the world. Therefore, further research in this field must be done (Cudmore et al. 2004).

Being microaerophilic, *T. vaginalis* uses an oxidative carbohydrate metabolism to generate energy, partly taking place in the cytoplasm and partly in the hydrogenosome, an organelle similar to the mitochondrion of higher eukaryotes, which is also found in other anaerobic protozoa. In the cytoplasm glucose is converted to pyruvate and further metabolized in the hydrogenosome. There adenosin triphosphate (ATP) is then produced through substrate-level phosphorylation (Petrin et al. 1998). With oxygen having a toxic effect on *T. vaginalis*, it protects itself from reactive oxygen species (ROS) using two different NADPH oxidases and thioredoxin (Trx)-dependent peroxidases. The NADPH-dependent oxidases either reduce oxygen to water or to hydrogen peroxide, the latter being a flavin reductase.

Trx is reduced by TrxR, with NADPH as the reductant, and further activates Trx peroxidase and thiol peroxidase by reduction of cysteines to cysteines in their active sites. The active peroxidases then break down hydrogen peroxide and prevent oxidative damage. Trx is not only important for ROS defence but plays a variety of other roles, some of which are portrayed in Fig. 1 (Leitsch 2016).

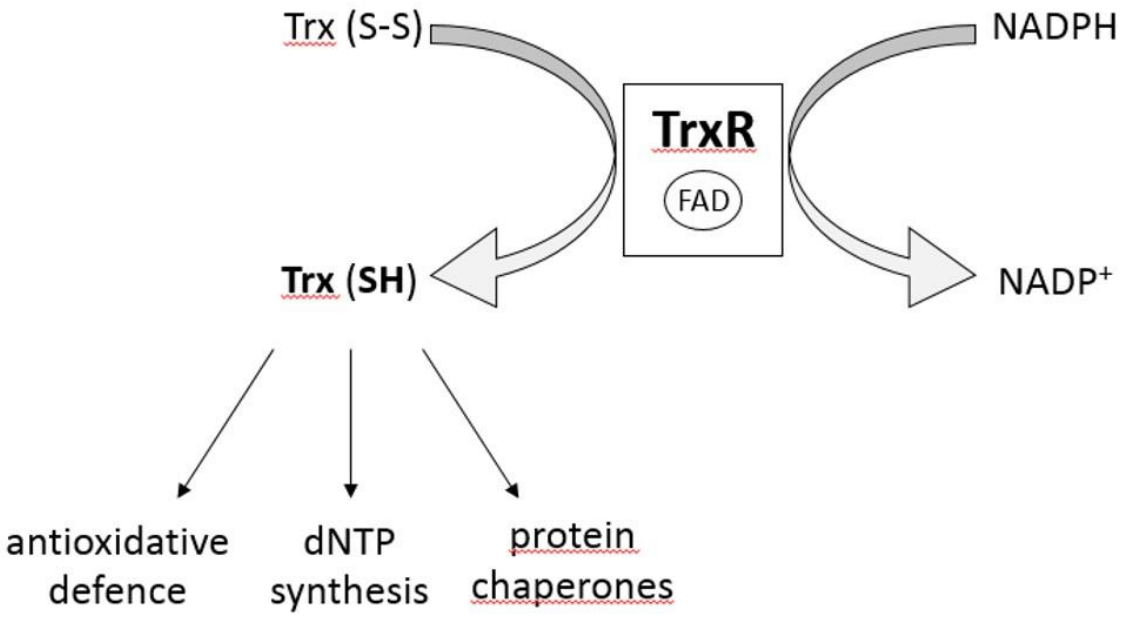

Figure 1. TrxR/Trx dependent processes in *T. vaginalis*. Reduced Thioredoxin (Trx (SH)) has been shown to be participating in a variety of different reactions. Trx reduction is majorly important for the antioxidative defence of the protist. In the process of desoxyribose nucleoside triphosphate (dNTP) synthesis Trx (SH) works as an essential reducer of ribonucleotide reductase and it supposably also aids the protein folding by reduction of chaperons and other mechanisms (Leitsch et al. 2009).

The causes of resistance vary and are not yet fully understood. One of which is based on the necessity of nitromidazole drugs to be activated through reduction at their nitro groups. In this case the activity of NADPH oxidase, which would reduce oxygen to hydrogen peroxide, is decreased or even absent. The remaining oxygen interferes with the reduction of the prodrug by reoxidation and metronidazole can therefore not be converted into its active, toxic state. The remaining oxygen thus inhibits the effect of metronidazole (Leitsch et al. 2014). Resistance can also be traced back to the discovery that TrxR and other flavin reductases acts as electron donors

to 5-nitromidazole drugs and can, when lacking, not activate metronidazole (Leitsch et al. 2010).

Targeting inflammation processes and thiol redox enzymes like TrxR, the gold drug auranofin appears to be a possible therapy for *T. vaginalis* infection. Originally developed and approved against rheumatoid arthritis, but nowadays often replaced by novel antirheumatic drugs, auranofin is still topic of research. Its potential as a therapeutic for microbial and viral infections and different types of cancer is being investigated (Madeira et al. 2012). Previous studies in other protist parasites and helminths have shown effective inhibition of TrxR by auranofin *in vitro* as well as *in vivo* (Hopper et al. 2016). For the antirheumatic purpose auranofin is sold as Ridaura® (Sebela Pharmaceuticals Inc., USA) and is applied orally in form of capsules, each containing 3 mg of auranofin. The daily dosage is usally 6 mg. (https://ridaura.com/wp-content/uploads/2018/04/WestWard_Ridaura_PI_Single-28NOV2017.pdf (accessed 30.07.2020))

The aim of this study was to investigate if auranofin effectively inhibits TrxR-activity in *T. vaginalis*. Moreover, the hypothesis that it depletes the cellular thiol pools was tested. The effect of auranofin (40 µM) was compared to cells treated with metronidazole (50 µM), diphenyleneiodonium chloride (DPI) (10 µM) and to untreated cultures. The US Food and Drug Administration (FDA)-approved agent metronidazole functioned as our positive control, so did DPI, a NAD(P)H oxidase-inhibitor (Li und Trush 1998), in our TrxR-activity assay.

2. Materials & Methods

2.1. Trichomonad cultures

Trichomonas vaginalis strain C1 (ATCC 30001) was used for the experiments. The cells were cultured in trypticase, yeast extract, maltose (TYM) medium (pH 6.2) (DIAMOND 1957) under aerobic conditions in 10 ml Nunc™ Cell Culture Tubes (Thermo Fisher Scientific Inc., USA). The growth medium included 10 % horse serum. It was sterile-filtered and 8 mM cysteine was later added to each individual tube. The cultures were incubated at a temperature of 37 °C. Cysteine, as well as all other drugs were purchased from Sigma-Aldrich (Sigma-Aldrich Inc., USA).

For every assay four culture tubes with each about 10 000 cells/ml were prepared. They were induced with 50 µM metronidazole, 10 µM diphenyleneiodonium (DPI) and 40 µM auranofin. One culture was left untreated as reference. The ideal concentration of each drug was determined in preceding experiments (Leitsch 2017). Stocks of metronidazole were prepared in water, stocks of Auranofin in dimethyl sulfoxide (DMSO). All four were incubated for 3 h at 37 °C.

For all the following assays, *T. vaginalis* cells were taken from the untreated culture tube of the previous assay in advance of cell lysation and protein extraction. 1 ml of cell culture was each transferred into four new 10 ml cell culture tubes with 9 ml of fresh TYM medium, including 8 mM of cysteine, and incubated for about 20 h at 37 °C before inducing the three drugs. This procedure was repeated for the next assay, if on the following day.

2.2. TrxR-activity assay

2.2.1. TrxR extraction

After 3 h of incubation, the cells were centrifuged at 800 RCF for 5 min, the medium was discarded. The cells were washed twice with 1x Phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 2000 x g for five minutes. Further they were resuspended in 100 mM Tris buffer (pH 7.5) and mechanically lysed in a Dounce homogenizer on ice. The homogenate was

centrifuged at 20 000 x g for ten minutes. The supernatant, including TrxR protein, was removed and further used for the activity assay.

2.2.2. TvTrx expression and purification

For the TrxR-activity assay recombinant Thioredoxin was used. TvTrx was expressed in *Escherichia coli*, strain BL21-AI. The TvTrx gene is located on a pET-17b plasmid flanked by a T7-promotor and a 6x histidine tag. The gene for the T7-RNA-polymerase being located on the bacteria genome.

5 ml lysogeny broth (LB) medium containing 50 µg/ml ampicillin were inoculated with one BL21-AI colony. The culture was incubated in the shaker over night at 37 °C. The next day 2 ml of the *E. coli* culture were transferred into 50 ml of fresh LB medium with the same concentration of ampicillin and incubated in the shaker. At optical density $(OD)_{600}$=0.4-0.6, L-arabinose (0.2 %) was added, inducing the expression of TvTrx. All of the measurements were performed under aerobic conditions using Perkin Elmer Lambda 25 UV/VIS spectrophotometer (PerkinElmer, Inc., USA). After three hours the cells were washed in 1x PBS and lysed mechanically with a frozen mortar and pestles. Protein purification was conducted with the Ni-NTA Spin Columns (Qiagen N. V., Netherlands) according to the manufacturer's manual. The concentration of extracted TvTrx was determined by Bradford Assay (Bio-Rad Laboratories, Inc., USA).

To check if the purification of TvTrx was successful, Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. A 5 % stacking gel and 12.5 % separating gel were prepared. Twenty µg protein with 25 % 4x Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., USA) were loaded onto the gel. After electrophoresis at 100 V for about 1.5 h the gel was stained with Coomassie Blue, and thereafter destained in 20 % methanol and 10 % acetic acid.

2.2.3. Enzyme assay

TrxR-activity was determined through an adapted enzyme assay. Auranofin-, DPI- and metronidazole-treated cells were compared to untreated cultures. Metronidazole acted as positive control. Each reaction included 100 mM Tris buffer, 1 mM 5,5′-dithiobis-(2-

nitrobenzoic acid) (DTNB), 0.2 mM NADPH, 20 µg/ml TvTrx and 50 µg/ml extracted TrxR. TrxR-activity was determined through conversion of DTNB to 2-nitro-5-thiobenzoic acid (TNB), detected photometrically at 412 nm (Leitsch et al. 2007). The activity was registered in 30 s intervals, starting at 0 s until 1 min 30 s, which left us with four extinction values.

2.3. Intracellular thiols

To extract the intracellular thiols, the cells were centrifuged at 800 RCF for five minutes, the medium was discarded. The cells were washed twice in 1x PBS (pH 7.4) and centrifuged at 2000 x g for five minutes. They were further resuspended in 20 mM EDTA and mechanically lysed with 20 strokes in a Dounce homogenizer on ice. The homogenate was centrifuged at 20 000 x g for 10 min, the pellet, including big cell particles and organelles, was discarded. To determine the protein concentration of the supernatant a Bradford Assay (Bio-Rad Laboratories, Inc., USA) was conducted. For ensuing protein precipitation 20 % trichloroacetic acid (TCA) was used.

The free intracellular thiols in the supernatant were determined through a colourimetric assay and measured in a photometer at 412 nm. 666 µl of 0.4 M Tris buffer (pH 8.9) and 333 µl of the supernatant were mixed, 1.7 µl 100 mM DTNB was added (Sedlak und Lindsay 1968).

2.4. Data analysis and statistics

For the TrxR-activity assay, two experiments were included into the analysis. For each assay ΔOD/min was calculated from the four detected values, representing the average activity of the enzyme. Unspecific reduction of DTNB in the absence of TrxR was taken into consideration. The TrxR-activity of the treated cultures were then calculated as a percentage of the untreated samples and used for further statistical analysis.

The intracellular thiols were determined in four independent experiments. For the thiols OD_{412}/mg values were calculated and used for significance testing. Further, the mean and the variance were determined for both. Significance was evaluated by a two-sample t-test with the significance level set at 0.05.

5. Results

5.1. Trx expression

To check if the extraction of the recombinant thioredoxin had been successful we ran a SDS-PAGE. Figure 2 shows the gel, which reveals that the extraction of Trx protein had been

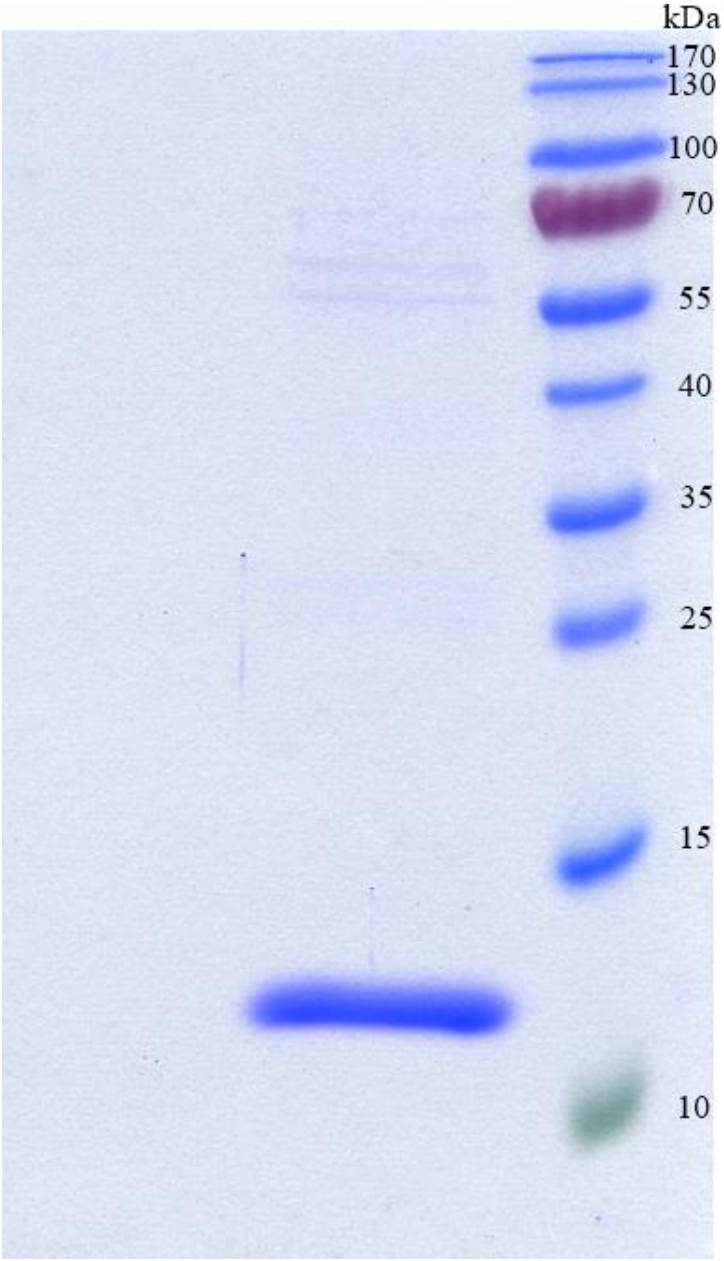

Figure 2. **SDS-PAGE of extracted recombinant Trx.** A strong band is visible at around 12 kDa, corresponding to the size of TvTrx.

successful. A very distinct band at about 12 kDa was visible, matching the actual molecular weight of TvTrx.

5.2. Intracellular thiols

The intracellular thiol concentrations were determined in four independent experiments. Significance testing by t-test showed that auranofin has a highly significant effect in terms of decreasing the intracellular thiol concentration, compared to the untreated samples, as seen in Table 1.

Table 1. Mean and variance s² of the indirect parameter OD412/mg, representing the intracellular thiol concentrations. The mean and variance of the measured OD_{412}/mg values were calculated and used for further statistical analysis by t-test. **p < 0.01

OD_{412}/mg	**0**	**DPI**	**Metronidazole**	**Auranofin****
mean	0,1335	0,1350	0,0540	0,0878
s²	0,0004	0,0003	0,0014	0,0004

5.3. TrxR-activity

As determined by t-test and represented in Table 2 TrxR-activity was significantly lower in Auranofin treated cells in comparison to the untreated cells.

Table 2. Mean and variance s² of the detected TrxR-activity values. TrxR-activity was calculated from the four absorbance values detected in 30 second intervals, the mean and variance were calculated and used for statistical testing. *p < 0.05 **p < 0.01

% activity	**0**	**DPI****	**Metronidazole****	**Auranofin***
mean	100	1,7	17,4	13,1
s²	0	0,5	3,2	18,4

6. Discussion

In previous publications an inhibiting effect of auranofin on the TrxR in *T. vaginalis* has been discovered (Hopper et al. 2016). Confirming these, we also observed a significant inhibition of the TrxR-activity after treatment with the antirheumatic agent. Only two assays could be included in the statistical analysis, because the recombinant thioredoxin did only properly react with TrxR when it was freshly extracted. Storing TvTrx overnight in the refrigerator as well as with added glycerine in the freezer decreased the reactivity of TvTrx as substrate of TrxR. SDS-PAGE of Trx did not reveal degradation through proteases. This observation would have to be further investigated within future research. For now it is recommended to only use freshly extracted TvTrx for the assays.

Additionally, we suspected a depletion of the intracellular thiol pools due to auranofin. This hypothesis too can be confirmed according to the results of the experiment. Intracellular thiol concentrations in *T. vaginalis* were significantly lower in auranofin-treated cells.

Both experiments showed the efficacy of the antirheumatic gold drug in terms of inhibition of the antioxidative defence of *T. vaginalis*. This system is of major importance for the parasite and a lacking protection against reactive oxygen species can cause the cells death. Thus, it presents a promising target for trichomoniasis therapy. If auranofin is as potent *in vivo* as it was shown to be *in vitro* it has potential to become a new agent against *T. vaginalis* infection. *In vivo* experiments in mice have been conducted before where auranofin successfully eradicated *T. vaginalis* (Hopper et al. 2016). Future research should further investigate in the direction of *in vivo* experiments.

The discovery of an alternative agent would be ground-breaking for the therapy of trichomoniasis. In fact, a metronidazole resistance rate of even only 5 % is of relevance, considering the prevalence of about 270 million cases of trichomoniasis worldwide each year (Leitsch 2016). The association between *T. vaginalis* infection and increased prevalence of HIV-infection, poor reproductive outcomes and neoplasia makes it even more important for scientists to focus on (Kissinger 2015).

7. Abstract

Trichomoniasis is the most prevalent non-viral, sexually transmitted disease, caused by the microaerophilic protist parasite *Trichomonas vaginalis*. Though itself not life-threatening, in fact it progresses without any symptoms in most cases, *T. vaginalis* infection can promote urogenital neoplasia, HIV-infection and other severities. Since its discovery in 1960, the 5-nitromidazole drug metronidazole is one of the two only established treatments for trichomoniasis. However, with a significant number of patients resistant or showing major side effects, novel therapies are in need. Targeting the protection system against oxidative damage, several organic gold compounds have been tested as alternative medication. The antirheumatic drug auranofin has shown promising results before by inhibiting the thioredoxin reductase (TrxR), a major player of the antioxidative defence system of the protist.

In the following thesis the activity of TrxR in auranofin (40 µM) and metronidazole (50 µM) treated cell cultures was measured in a photometric assay and compared to the activity in diphenyleneiodonium chloride (DPI) (10 µM) treated and untreated cells. Recombinant *T. vaginalis* Thioredoxin (TvTrx) was therefore expressed in *Escherichia coli*. In addition, intracellular thiol concentrations were determined, based on the hypothesis that auranofin has a negative effect on the thiol pools in *T. vaginalis*, which also have antioxidative properties.

The outcome of our experiments is encouraging. We did observe an inhibiting effect of auranofin in *T. vaginalis* in the assays that were conducted. Both TrxR-activity and intracellular thiol measurements were significantly lower in auranofin treated cells, affirming an inhibiting effect of the gold drug on the reactive oxygen species (ROS) defence mechanisms in *T. vaginalis* and therefore inhibiting the proliferation and eventual death of the parasite. With further research auranofin might be a future alternative agent for *T. vaginalis* infection, in cases where metronidazole is ineffective.

8. Zusammenfassung

Trichomonose ist die häufigste nicht virale, sexuell übertragbare Infektionskrankheit, die von dem anaerobe Protozoon *Trichomonas vaginalis* ausgelöst wird. Eine Infektion ist zwar nicht lebensgefährlich, sie verläuft in den meisten Fällen sogar symptomlos, kann aber die Übertragung von HIV, sowie Neoplasien und Komplikationen während der Schwangerschaft begünstigen. Behandelt wird Trichomonose seit der Entdeckung 1960 fast ausschließlich mit dem Nitroimidazol Metronidazol. Allerdings reagiert ein beträchtlicher Anteil an Patienten mit Resistenzen oder starken Nebenwirkungen, weshalb alternative Wirkstoffe gefragt sind. Verschiedene organische Goldverbindungen, deren Angriffspunkt das System zum Schutz vor Sauerstoffradikalen ist, sind Thema der Forschung für alternative Therapien. Das Antirheumatikum Auranofin zeigte in vorhergegangenen Experimenten vielversprechende Ergebnisse durch Inhibition der Thioredoxinreduktase, ein wichtiges Protein für die Abwehr von Sauerstoffradikalen.

In der folgenden Arbeit wurde die Aktivität der Thioredoxinreduktase in mit Auranofin (40 µM) und Metronidazol (50 µM) behandelten Zellen in einem photometrischen Assay gemessen und mit Diphenyleneiodoniumchlorid (DPI) (10 µM) behandelten und unbehandelten Zellen verglichen. Thioredoxin, das Substrat der Reaktion, wurde rekombinant in *Escherichia coli* hergestellt. Zudem wurde die Hypothese, dass Auranofin auch die intrazellulären Thiol-Speicher depletiert, getestet. Diese wirken ebenfalls als Antioxidantien.

Das Ziel der vorliegenden Arbeit war es den Effekt von Auranofin in *T. vaginalis* zu beobachten. Wie schon in vorherigen Publikationen gezeigt, hat Auranofin auch in unseren Experimenten die TrxR-Aktivität signifikant gehemmt. Die intrazellulären Thiolkonzentrationen waren in den behandelten Zellen ebenfalls weitaus geringer, was den erwarteten Effekt des Antirheumatikums bestätigt. Die Inhibition der Sauerstoffabwehr führt zu einer Intoxikation und kann folglich zum Tod der Zelle führen. Die vorliegenden Ergebnisse bieten demnach eine Grundlage für die weitere Erforschung von Auranofin als alternatives Therapeutikum gegen Trichomonose, in Fällen wo Metronidazol nicht wirksam ist.

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10. List of Figures and Tables

11. Abbreviations

ATP	Adenosin triphosphate
DMSO	Dimethyl sulfoxide
dNTP	Desoxyribose nucleoside triphosphate
DPI	Diphenyleneiodonium chloride
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FDA	U.S. Food and Drug Administration
LB	Lysogeny broth
OD	Optical density
PBS	Phosphate-buffered saline
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TNB	2-nitro-5-thiobenzoic acid
Trx	Thioredoxin
TrxR	Thioredoxin Reductase
TYM	Trypticase-yeast extract-maltose