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Original article

Study of Role of piper betle on Ferroptosis

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Abstract

Ferroptosis is a recently discovered programmed cell death triggered by accumulation of iron-induced toxic lipid peroxides. Ferroptosis was first recognized in year 2012 during screening of anticancer drugs that selectively killed RAS-mutated cancer cells. Since than, a large number of studies have implicated ferroptosis in many degenerative disease especially in neurological diseases, renal diseases, ischemic reperfusion injury and respiratory diseases. Genetic or pharmacological inducers of ferroptosis have shown to augment disease process whereas chemical inhibitors of ferroptosis have shown to be protective against the same diseases. Therefore, there is unmet need for discovery of novel drugs targeting ferroptosis for treatment of various degenerative diseases. This formed the rationale for our project. Erastin is widely used as chemical inducer of ferroptosis and many studies have used erastin induced ferroptosis model system for screening inhibitors of ferroptosis. Erastin is a chemical inhibitor of system Xc^- that depletes cellular glutathione (GSH) and facilitates iron induced lipid peroxidation leading cell death. Plants are richest source of medicinal drugs and currently majority of FDA-approved drugs are derived from plant source. This prompted us to screen medicinal plants for discovery of novel ferroptosis inhibitor. we identified a solvent extract of beetle leaves (BT^{SX}) as best lead candidate for characterization. In pre and co-treatment protocols, BT^{SX} showed a dose dependent inhibition of erastin-induced ferroptosis. Mechanistic studies revealed that BT^{SX} treatment increased levels of antioxidant GSH and NQO1, which are regulated by transcription factor Nrf2. We also found that BT^{SX} increases mRNA expression of GPX4, a major antioxidant enzyme that protects from ferroptosis by reducing toxic hydroperoxides. In summary, our study has identified solvent extract of beetle leaves as a novel ferroptosis inhibitor. Further studies are undergoing to identify the bioactive compound in the beetle leave extracts.

Keywords: Nrf2. GPX4, GSH , NQO1, piper betel soxhlet extract

Introduction

Herbal medicine is widely used to promote good health and treat diseases. However due to lack of standardized formulations and systematic studies such as pharmacological target and mechanism of action, it has not been developed as mainstream pharmacotherapy. [1] Medicinal plants are commonly used for the treatment of various diseases, as they are considered to have advantages over the conventionally used drugs that are much expensive and known to have harmful side effects.[2] In India, Betel leaf (BL) plays an important role since ancient culture. Its use in India dates back to 400 BC. As per ancient books of Ayurveda, Charaka, Sushruta Samhitas, and Kashyapa Bhojanakalpa, the practice of chewing BL after meals became common between 75 AD and 300 AD.[3] Toward the 13th century, European traveler Marco Polo recorded betel chewing among kings and nobles in India. Importance of BL has been described in ancient books of Ayurveda. Use of BL was known for centuries for its curative properties. In Chinese folk medicine betel leaves are used for the treatment of various disorders and claimed to have detoxification, antioxidation, and antimutation properties.[4] There are number of research experiments on BL, where the leaf extract, fractions, and purified compounds are found to play a role in oral hygiene, and to have various properties including anti-diabetic, cardiovascular, anti-inflammatory/immunomodulatory, anti-ulcer, hepato-protective, anti-infective, etc., Patents were also awarded for some of the biological activities like anti-inflammatory, anti-cancer, and immunomodulatory associated with the leaf extracts and purified compounds.[5] Oxidative stress and inflammation have been mainly implicated in many of the disease

pathology. Therefore considerable attention has been focused among phytotherapy researchers in identifying dietary and medicinal phytochemicals with antioxidant and anti-inflammatory activity that can inhibit, retard or reverse the multi-stage pathophysiological events underlying the disease pathology. Recently non-apoptotic cell death pathways have gained special attention particularly due to their role in inflammation and as potential therapeutic target in cancer. [6] Ferroptosis, a newly discovered regulated form of cell death leading to glutathione depletion resulting from inhibition of cysteine uptake or inactivation of the lipid repair enzyme glutathione peroxidase (GPX4) causes iron-dependent accumulation of ROS and lipid based ROS, particularly lipid hydroperoxides. [7] GPX4, a selenoprotein, is the only reduced glutathione peroxidases which handles lipid hydroperoxides and uses GSH to convert potentially toxic lipid hydroperoxides to non-toxic lipid alcohols. [8] GSH, a tripeptide of glutamate cysteine and glycine, is a well-known intracellular antioxidant protecting cells from oxidative or other forms of stress and serves as a cofactor for glutathione peroxidase and S-trasnferase enzyme families. [9] Tight regulation of cell death is vital during development, normal homeostasis as well as pathogenesis of diseases. Several acute and chronic Diseases are associated with excessive cell death resulting tissue damage and organ failure. [10] Caspase dependent apoptosis is well studied programmed cell death which is implicated in normal and disease pathogenesis. Ferroptosis is representing a pathogenic programmed cell death characterized by iron mediated production of toxic lipid hydroperoxides. [11] Ferroptosis is not inhibited by caspase inhibitors and therefore represent a unique form cells death which is widely implicated in several acute and chronic diseases such as asthma, COPD, Ischemic heart diseases, brain trauma, neurological diseases and renal failure.[12] Since Ferroptosis involves accumulation of toxic lipid peroxides, enzymes involved in generation and or detoxification of lipid peroxides modulate ferroptosis. [13]Oxidative stress and activation of lipoxygenase (oxygenate polyunsaturated fatty acid (PUFA)-phospholipids) are key inducers of Ferroptosis.[14] On the other hand Glutathione and Glutathione peroxidase 4 plays critical role in detoxification of phospholipid hydroperoxides and protect from ferroptosis.[15] Pharmacological depletion of GSH or inhibition of GPX4 activity is shown to induce ferroptosis. In contrast, agents that increase GSH or GPX4 activity are shown to inhibit ferroptosis. [16] Recently non-apoptotic cell death pathways have gained special attention particularly due to their role in inflammation and as potential therapeutic target in cancer. [17] Ferroptosis, a newly discovered regulated form of cell death leading to glutathione depletion resulting from inhibition of cysteine uptake or inactivation of the lipid repair enzyme glutathione peroxidase (GPX4) causes iron-dependent accumulation of ROS and lipid based ROS, particularly lipid hydroperoxides. [18] GPX4, a selenoprotein, is the only reduced glutathione peroxidases which handles lipid hydroperoxides and uses GSH to convert potentially toxic lipid hydroperoxides to non-toxic lipid alcohols. [19] GSH, a tripeptide of glutamate cysteine and glycine, is a well-known intracellular antioxidant protecting cells from oxidative or other forms of stress and serves as a cofactor for glutathione peroxidase and S-trasnferase enzyme families. [20] Medicinal plants are commonly used for the treatment of various diseases, as they are considered to have advantages over the conventionally used drugs that are much expensive and known to have harmful side effects. Oxidative stress and inflammation have been mainly implicated in many of the disease pathology.[21] Therefore, considerable attention has been focused among phytotherapy researchers in identifying dietary and medicinal phytochemicals with antioxidant and anti-inflammatory activity that can inhibit, retard or reverse the multi-stage pathophysiological events underlying the disease pathology.

The current study focuses on investigating the protective effects of piper beetle on the lung cell line Beas2B against erastin-induced ferroptosis at the cellular level. Understanding these protective effects is crucial for gaining insight into potential therapeutic interventions. This study provides a theoretical basis for exploring non-apoptotic treatments for lung cancer. As a result, it opens up new avenues for research and potential clinical applications.

Methodology

1 Extraction Procedure:

The below schema illustrates leaves collection and processing.

Cell culture: We used normal human bronchial epithelial cells (Beas2B) for the entire cell based assay. Beas2B cells were cultured in DMEM:HAMF12(Dulbecco modified eagles media) and supplemented with 10% v/v foetal bovine serum (FBS), 1% w/v penicillin and 1% w/v L-glutamine (200 mM). All these reagents were obtained from Sigma–Aldrich, UK.

Standardization of betel leaf extract against erastin induced ferroptosis:

Assessment of Cytotoxicity by MTT assay:

To screen and identify standardized BT solvent extracts for inhibiting erastin-induced ferroptosis in normal Beas2b cell line and to know toxic doses of plant extract we performed MTT assay.

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase[NADPH dependent].The dye enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The formazan product is solubilised with 100% DMSO[10% SDS and 0.1N Hcl] and absorbance was measured at 570nm. Only live cells will reduce MTT and form formazan product.

Bioassay for ferroptosis inhibitors using beetle soxhlet extract:

For screening for ferroptosis inhibitors, we used erastin-induced ferroptosis in vitro model system. Normal human lung epithelial cells (Beas2B) cells were treated with increasing concentrations (2.5, 5.0 and 10 μM) of erastin. After 24h, the cell death was assessed by MTT assay. The data was expressed percentage cell death as compared to vehicle control. The cells were treated with test agent 24h prior to erastin treatment to identify identified ferroptosis inhibitor.

Bioassay: Beas2B cells were first treated with 20 or 100 μg/mL concentrations of Beetle soxhlet extract for 24h and subsequently, the cells were incubated for additional 24h with erastin (5 or 10μM). At the end of erastin treatment, the cell death was assessed by MTT assay. Test agents that inhibited erastin-induced cell death by at least 25% were selected as lead studies.

Clarification of the ferroptosis inhibitors' mode of action:

The mode of action by which BTSX betel extract inhibits ferroptosis was assessed. This assessment involved analyzing both enzymatic and non-enzymatic content in a ferroptosis-induced Beas2b cell line.

Preparation of cell lysates: After indicated treatment periods, cells were washed with phosphate buffered saline (PBS) and then lysed with lysis buffer (25 mM Tris, pH 7.5/1 mM EDTA/ % NP40) for 20 min with constant shaking. After centrifugation, an aliquot of cell lysates was used for protein measurement by Bicinchoninic acid (BCA) method (Thermo Scientific) and remaining cell lysate was used for measuring NQO1 enzyme activity and total GSH.

GSH Assay :-To investigate whether selected plant extracts inhibited erastin-induced ferroptosis by increasing GSH. GSH is an important antioxidant tripeptide, existing to different form the reduced sulfhydryl form (GSH) and glutathione disulphide (GSSG) oxidised form. Its intracellular concentration is an indicator of oxidative stress. GSH neutralizes the free radicals by the detoxification mechanism involving glutathione peroxidase enzyme.

NQo1 Assay: To screen the BT extracts for Nrf2 activation by measuring NQo1 enzyme activity using Beas2B cell line. NQO1 is transcriptional target of Nrf2 and prior studies have shown that NQO1 enzymes activity is good surrogate marker for Nrf2 activity. Therefore, we used induction of NQO1 enzyme activity as indicator of Nrf2 activity.

Results

Cytotoxicity of Beetle extract: To determine the non-lethal dose of Beetle extract for screening studies, Beas2B cells were exposed to aqueous and soxhlet Beetle extract at a concentration of 20 and 100 μg/mL, and cell death was measured by MTT assay. Beetle extract doesn't have the cytotoxicity profile of aqueous extracts and soxhlet extract test agents. Soxhlet extract shows more viability compare to control (Figure 2). Solvent extracts showed no toxicity at the indicated concentrations.

Screening of Beetle extract against to Erastin-induced ferroptosis:

A bioassay was conducted to evaluate the effectiveness of beetle soxlate extract as inhibitors of ferroptosis. The study aimed to assess the potential of the extract in preventing ferroptosis, a form of regulated cell death. When a low concentration (20μg/ml) of soxlate beetle extract is used, it demonstrates an inhibition of ferroptosis that exceeds 60%. On the other hand, when a high concentration (100μg/ml) of soxlate beetle extract is utilized, the inhibition of ferroptosis falls below 60% (Figure 3). The concentration of 20μg/ml of beetle soxlate extract shows promising results, indicating the need for further investigation to identify the bioactive compound of the medicinal plant piper beetle.

Assay of Optimum – dose of ferroptosis inhibition:

Pretreatment or co-treatment of BT^{SX} inhibited erastin-induced ferroptosis.

Efficacy of BT^{SX} was further characterized by dose-response effects (ranging from 1.56 to 50μg/ml) in pre-treatment and co-treatment protocol. In pre-treatment protocol, BT^{SX} effectively inhibited erastin-induced ferroptosis at doses beyond 12.5 μg/mL(Figure 4A). Interestingly in co-treatment protocol, BT^{SX} was more effective as it showed protection against erastin-induced ferroptosis at further lower doses of 3.12 ug/mL (Figure 4B).

BT^{SX} increase GSH levels: To understand the potential mechanism how BT^{SX} inhibit erastin induced ferroptosis, we measured total GSH levels in Beas2B cells 24h after BT^{SX} treatment. High GSH levels protect cells from erastin-induced ferroptosis. BT^{SX} treatment significantly increased GSH levels at concentrations 25 and 50μg/ml (Figure 5).

BT^{SX} activate Nrf2 pathway: Transcription factor Nrf2 regulates nearly all antioxidant defenses including GSH synthesis (20) (21). Activation of Nrf2 is shown to inhibit erastin-induced ferroptosis (22). We hypothesized BT^{SX} protects from ferroptosis through activation of Nrf2-dependent antioxidants. NQO1 is a transcriptional target of Nrf2 and increase in NQO1 mRNA levels and NQO1 enzyme activity is surrogate marker of Nrf2 activity. BT^{SX} treatment significantly increased expression of NQO1 mRNA (Figure 6A) levels as well as NQO1 enzyme activity (Figure 6B) suggesting that BT^{SX} activates Nrf2 pathway.

BT^{SX} increase GPX4 gene expression. GPX4 inhibits ferroptosis by detoxifying toxic lipid hydroperoxides. We tested whether BT^{SX} effects GPX4 expression. BTSX stimulated modest increase but not statistically significant in GPX4 mRNA expression **(Figure 7).**

Discussion

During the last few years, substantial progress has been made in the understanding of the molecular and metabolic underpinnings of ferroptotic cell death. Vitamin E is an natural antioxidant which can mitigate ferroptosis but was also shown to compensate for the conditional loss of GPX4 in endothelial cells and hepatocytes in vivo. [22] In pursuit of discovering novel herbal modulators to inhibit the ferroptosis we carried this study hence we used the Phytochemicals to upregulate Nrf2 pathway and protect from prooxidant induced cell death, inflammation and tissue injury by activating cytoprotective genes like GPx4,HO1&NQO1.

We prepared standardized extract of betel leaf for screening and identified betel to induced Nrf2 pathway. Although we noted the ability of selected plant extracts to significantly increase in Nqo1 activity, which was comparable to positive control sulforaphane, the Nqo1 mRNA expression was modestly induced as compared to sulforaphane . This discordance between Nqo1 mRNA expression and Nqo1 enzyme activity could be due to alteration in pathways affecting post-translational process including protein stability. Further, a time course analysis of NQO1 mRNA and protein expression post-treatment is warranted to explain this discordance.

Numerous studies have established that Piper betel contains a variety of bioactive compounds, including polyphenols, flavonoids, hydroxychavicol, and eugenol, all of which are known for their antioxidant properties. These compounds have been shown to activate the Nrf2 pathway, which enhances the expression of antioxidant enzymes and mitigates oxidative stress. For instance, research indicates that Piper betel leaves exhibit strong free radical scavenging activity, suggesting their capability to induce the Nrf2 pathway and provide protective effects against oxidative damage.[23,24]

The choice of solvent for extraction plays a significant role in determining the yield and antioxidant effectiveness of Piper betel extracts. In a recent study, it was found that the Soxhlet extract of betel leaf exhibits strong inhibition of ferroptosis. Related research by Jihye S et al in 2022 indicated that Soxhlet extracts produced the highest antioxidant activity, possibly attributed to the presence of bioactive compounds that activate the Nrf2 pathway. [25]These findings underscore the crucial role of extraction methods in maximizing the health-promoting properties of Piper betel.

Piper betel leaves contain a high amount of polyphenolic compounds, which have been found to possess significant antioxidant properties. These compounds are capable of influencing various signaling pathways associated with oxidative stress and inflammation. [26] A study on Beas2B cells revealed that the solvent extract of piper betel leaves has a notable impact on reducing ferroptosis, as indicated by the expression of the GPx4 gene. Similarly, a study by Young SC et al. in 2007 demonstrated that betel leaf extract can induce GPx4 gene expression in a rat model with carbon tetrachloride-induced liver injury. [27] The antioxidant activity of piper betel is believed to affect the GPx4 pathway, which plays a crucial role in shielding cells from oxidative damage. By scavenging reactive oxygen species (ROS) and bolstering antioxidant responses, piper betel may enhance the expression or activity of GPx4, providing a defense mechanism against diseases linked to oxidative stress, such as various forms of cancer. [28]

In our studies, we used erastin- induced ferroptosis model system for screening for ferroptosis inhibitors. Mechanistic studies have shown that erastin is chemical inhibitor glutamate-cystine antiporter system (X_{c-} system) which blocks import of cystine, depletes GSH synthesis and facilitate iron-induced lipid peroxidation.[29] We found that beetle leaf extracts significantly prevented erastin-induced ferroptosis in Beas2B cells (Figure 5). Interestingly, we also observed that co-treatment of beetle leaf extract was also effective in inhibiting erastin induced ferroptosis (Figure 6). GSH is a major cellular antioxidant that modulates ferroptosis [30]. We found that Beetle leaf extract significantly increased GSH levels in Beas2B cells. The enzymes, Glutamate-Cysteine Ligase Modifier Subunit (GCLM) and Glutamate-Cysteine Ligase catalytic Subunit (GCLC) synthesize GSH and transcription factor Nrf2 transcriptionally

regulates GCLM and GCLC expression [31]. Besides, GSH synthesis, Nrf2 regulates nearly all antioxidant proteins including glutathione peroxidase, hemeoxygenase1, and thioredox reductase [32]. Therefore, we tested if beetle leaf extract activates Nrf2 pathway. NQO1 gene is the key transcriptional target of Nrf2 and several studies have documented that upregulation of NQO1 has a surrogate marker of Nrf2 transcriptional activity. Hence, we measured NQO1 mRNA and enzyme activity as indicator of Nrf2 activation. Beetle leaf extract significantly increased NQO1 mRNA levels as well as NQO1 enzyme activity in Beas2B cells suggesting the potency of bioactive compound in beetle leave crude extract to activate Nrf2 pathway. Further studies are needed to characterize the bioactive compounds as well as Nrf2 activity.

Conclusion

To find out the herbal modulators of ferroptosis, we started with extraction of phytochemicals from piper betel. In this study, we used soxhlation extracts beetle medicinal plant, invitro screening was done to identify plant extracts to induce Nrf2 pathway using NQO1 enzyme and glutathione activity. First, Cytotoxicity of plant extracts were done using MTT assay in the concentration of 20ug/ml shown more cell viability compared to control. we considered below 50% cell viability compared to erastin is considered as good potent extract. We performed co & post treatment analysis of cytotoxicity of betel plant extract to determine the therapeutic window in the different concentration modest toxicity at the highest concentration 50ug/ml. NQO1 and GSH assay was done at different concentration and NQO1 and GSH induction was found maximum at the highest concentration of 12.5ug/ml compared to positive control. We identified betel plant have potential to activate Nrf2 pathway to confirm that thirdly, NQO1 and GPx4 mRNA expression was assessed with soxhlation extract of betel. We have identified potential betel extracts as Nrf2 inducers, further studies are required to test the specificity of the selected plant extract to upregulated Nrf2 pathway. Further research needed ,to formulating betel extract as neutraceuticals or dietary supplements to mitigate the diseases caused by oxidative stress.

Fig 1: Extraction Procedure.

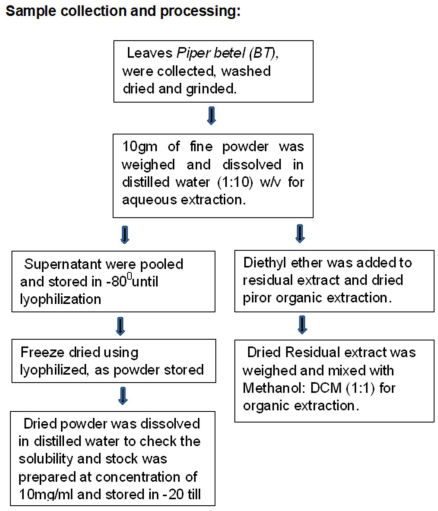

Figure 2: Cytotoxicity profile of Beetle extract.

Beas2B cell were treated with Beetle extract at indicated concentrations for 24h and cell death was assessed by MTT assay (superscript ^{AQ}, refers to aqueous extraction)

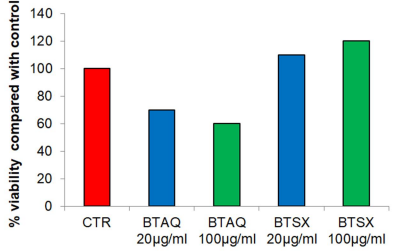

Figure- 3: Evaluation of beetle soxhlet extract against Erastin-induced ferroptosis. Beas2B cells were treated with beetle soxhlet extract for 24 h followed by erastin treatment for additional 24h. Cell death was assessed by MTT assay.

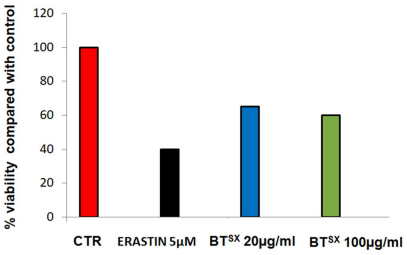

Figure 4: Pretreatment and co-treatment effects of BT^{SX} on erastin induced ferroptosis. Beas2B cells were pretreated with BT^{SX} for 24h (Figure 2A or 30 min (Figure 1B) prior to erastin and cell death was measured after 24 h by MTT assay.

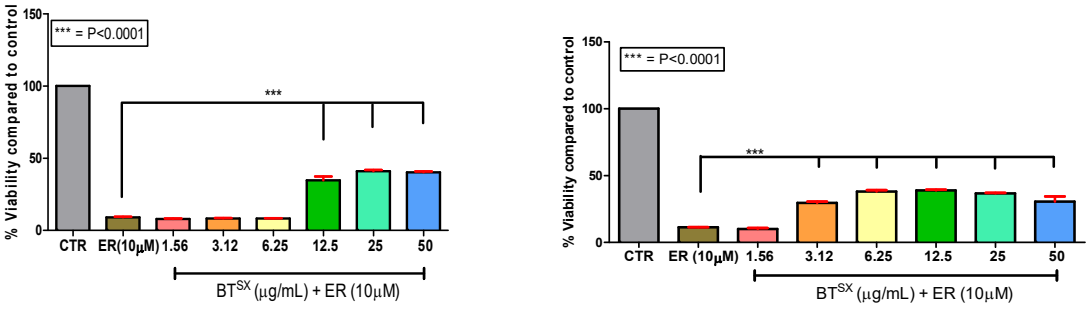

Figure 5: Effects of BT^{SX} on GSH levels in Beas2B cells. Cells were treated with BT^{SX} for 24h and GSH levels were measured in cell lysates.

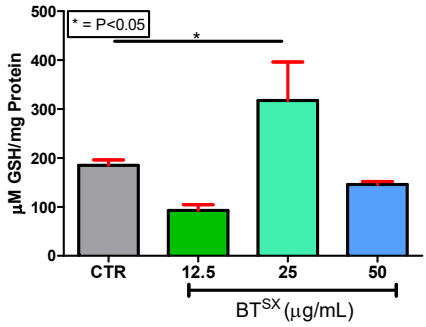

Figure 6 Effects of BT^{SX} on the expression of NQO1 mRNA levels and NQO1 enzyme activity in Beas2B cells. *Beas2B cells were treated with BT^{SX} for 24h and subsequently NQO1 mRNA (A) and enzyme activity (B) was measured (details presented in method sections).*

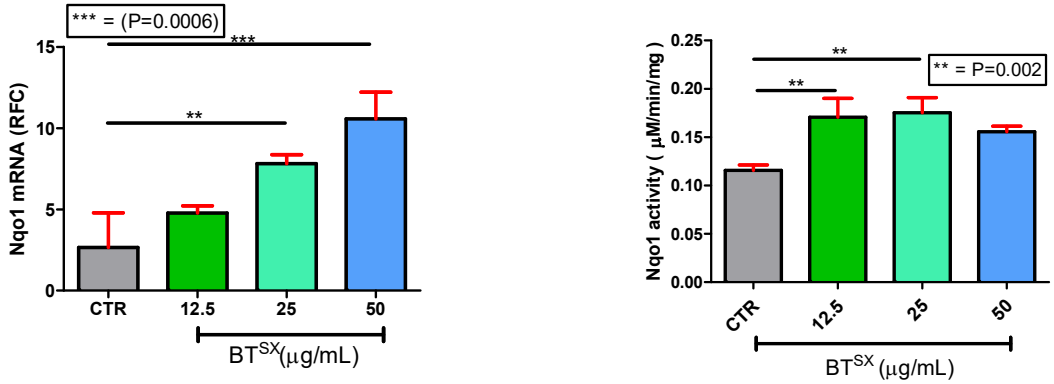

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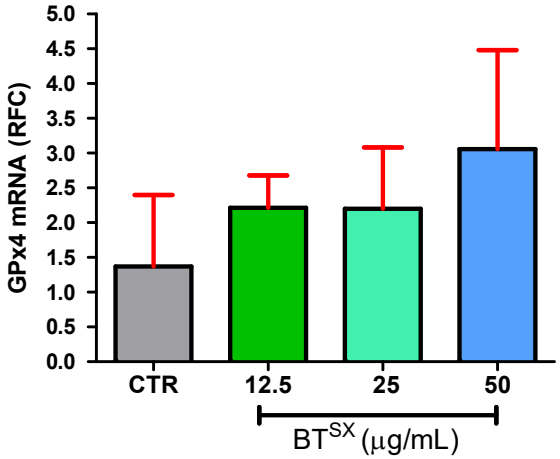
Figure7. Effect BT^{SX} on GPX4 expression in Beas2B cells. *Beas2B cells were treated with BT^{SX} for 24h and subsequently total RNA was extracted and GPX4 mRNA expression was measured by QPCR.*



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