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Original Article

Mode of action of *Chiang Tang Tong Fu*, a Chinese herbal supplement, against glutamate-induced toxicity

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ABSTRACT

Chiang Tang Tong Fu (CTTF), a Chinese herbal supplement, has been therapeutically employed as a laxative for constipation and for lowering the blood glucose level in diabetes. Recently, it has become widely used as a supplement for weight control. Despite its wide applications, its mode of action remains elusive. Here we report that CTTF contains Emodin and Physcion, which could account for many of its therapeutic effects including laxative effect and anti-diabetic function. Interestingly, in addition to the known effects, we have found that CTTF has an additional effect, namely, protection against glutamate-induced cell injury as demonstrated in PC 12 cells, suggesting that CTTF could also have a neuroprotective function.

KEYWORDS: *Chiang Tang Tong Fu* (CTTF), Physcion, Emodin, glutamate toxicity, PC 12 cells, cytoprotection.

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INTRODUCTION

Chiang Tang Tong Fu (CTTF), a Chinese herbal supplement, has been therapeutically employed as a laxative for constipation and for lowering the blood glucose level in diabetes. Recently, it has become widely used as a supplement for weight control. Despite its varied applications, its mode of action remains elusive. Among the natural products derived from plants, Emodin has been shown to attenuate lipid accumulation, and increase weight loss, while both Emodin and Physcion have been shown to regulate glucose utilization [1-3]. In addition, Emodin and Physcion have been shown to have a variety of pharmacological properties including anti-inflammatory, anti-bacterial, anti-cancer and hepatoprotective activities [4-7]. We have positively identified Emodin and Physcion as the active ingredients in CTTF and hence conclude that many of the pharmacological properties of CTTF could be attributable to Emodin and Physcion. Interestingly, in addition to the known effects, we have found that CTTF has an additional effect, namely, a protective function against glutamate-induced cell injury as demonstrated in PC 12 cells, suggesting that CTTF could also have a neuroprotective function. Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. Activation of glutamate receptors causes extracellular

calcium influx and the release of additional calcium from intracellular stores [8]. Excessive glutamate results in an overload of intracellular calcium *via* hyper-activated glutamate receptors, such as *N*-methyl-D-aspartate (NMDA) receptor. Excessive cytosolic calcium initiates cellular events, by activating catabolic enzymes such as proteases [9], phospholipases and endonucleases [10], which initiate the development of cellular injury and cell death. For example increases in intracellular calcium [[Ca^{2+}]i] activate phospholipase A2 (PLA2) which acts on membrane phospholipids, altering membrane structure and rendering it more permeable [11]. Excessive intracellular calcium also results in dysfunction of cellular metabolism and ultimately cell death by apoptosis and/or necrosis. Glutamate toxicity is a prevalent pathophysiological mechanism underlying several neurological disorders and diseases, such as stroke [12], Alzheimer's disease [13], Parkinson's disease [14] and Huntington's disease [15]. Therefore any compound that targets glutamate toxicity is a potential therapeutic agent for these diseases.

MATERIALS AND METHODS

Cell culture

Rat pheochromocytoma cell (PC 12 cells) [16] stock was purchased from American Type Culture Collection (ATCC, catalog no. CRL-1721). Cells were prepared and treated as previously described [17] with slight modifications. They were grown suspended in growth medium (RPMI 1640: Invitrogen; Carlsbad, CA, USA), supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum and penicillin (50 U/ml)/streptomycin (50 ug/ml) (all from Invitrogen; Carlsbad, CA, USA) in 25 cm^2 tissue culture flasks. Growth maintenance occurred in a humidified incubator at 37 °C, containing 95% air, 5% CO_2 and cells were fed three times per week. The cells were passed every 7 days or when they were at 80%-90% confluence, after which they were plated on poly-D-lysine-coated (10 ug/ml) (Sigma Aldrich; St. Louis, MO, USA) plates for experimental purposes. Cells between passages 2-7 were used in experimental protocols.

Glutamate cytotoxicity

Undifferentiated PC 12 cells were seeded on 96-well plates coated with poly-D-lysine at a density of 1.5 x 10^4. Twenty-four hours after initial seeding the medium was renewed with fresh culture medium containing various final concentrations (5, 10 and 20 mM) of glutamate (Sigma Aldrich; St. Louis, MO, USA), as described previously [18] with some slight modifications. Cell viability analysis was done after 12 or 24 hours of glutamate incubation.

Drug preparation and treatment groups

A stock solution (300 mg/ml) of CTTF was made and used in the experimental procedures. Undifferentiated PC-12 cells were divided into 4 groups. Group 1: control group. Group 2: glutamate group; cells were treated with 10 mM glutamate. Group 3: CTTF group; cells were treated with final concentrations of 0.640 µg/ml, 30 µg/ml, 500 µg/ml and 4000 µg/ml CTTF. Group 4: glutamate and CTTF group; cells were treated with final concentrations of 0.640 µg/ml, 30 µg/ml, 500 µg/ml and 4000 µg/ml CTTF, simultaneously with 10 mM glutamate. Stock solutions of 100 µg/ml Emodin and 100 µg/ml Physcion (Sigma Aldrich; St. Louis, MO, USA) were made. Experiments with Emodin and Physcion were done on undifferentiated PC-12 cells with a similar grouping as described for the CTTF experiments except that Group 3 received final concentrations of 50 ng/ml, 100 ng/ml, 500 ng/ml and 1000 ng/ml of either Emodin or Physcion and Group 4 were cells treated with Emodin or Physcion (50 ng/ml, 100 ng/ml, 500 ng/ml and 1000 ng/ml) simultaneously with 10 mM glutamate. Dilutions of all agents were made by using growth medium. All treatments were administered to PC12 cells after cells were seeded for 24 hours at 37 °C and 5% CO_2 incubation in 96-well plates. Twenty-four hours following treatment regimes, analyses of cell viability were performed.

Adenosine 5'-triphosphate (ATP) viability assay

The assessment of the number of cells that survived after each treatment was done using a Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega Corporation; Madison, WI, USA) which quantified the amount of ATP generated from cells that are metabolic active. The procedure was performed according to the manufacturer's protocol. Briefly, for analyzing the effect on

glutamate cytotoxicity, experiments were carried out in 96-well plates with undifferentiated PC12 cells incubated with various glutamate concentrations (5, 10 and 20 mM) for either 12 or 24 hours. After each respective time point, the 96-well plates were removed from the incubator and allowed to equilibrate at room temperature for 30 mins. Cells were then incubated in the ATP Kit's lysis buffer for 10-20 mins which released any ATP synthesized from viable cells. The ATP was then quantified by a luciferase reaction which produced a luminescence signal. The luminescence signal was detected at an absorbance of 550 nm by a microplate reader (Spectra Max, Molecular Devices) after transferring 100 ul of the lysate to a standard opaque-walled 96-well plate. The background luminescence of the culture medium was subtracted. Cell viability tests for other experiments were performed similarly to the glutamate cytotoxicity experiment, except that treatment conditions involving glutamate used only 10 mM glutamate and 24 hours was the selected time point for all experiments preceding the glutamate cytotoxicity experiment.

Identification of Emodin and Physcion by high-performance liquid chromatography (HPLC)

CTTF was first dissolved in acetonitrile and water-0.5% acetic acid solution with a ratio of 1:9. CTTF sample solution (20 μl) was loaded onto a Syncronis^{TM} C18 column (250 × 4.6 mm i.d., 5 μm) using Waters 717 auto-sampler. The column was eluted first with acetonitrile and water-0.5% acetic acid solution at a ratio of 1:9 (v/v) for 45 min, followed by elution with a mixture of acetonitrile and water-0.5% acetic acid solution (3.5:6.5, v/v) for 20 min. The column was further eluted with 100% acetonitrile for 15 min, followed by acetonitrile and water-0.5% acetic acid solution at a ratio of 1:9 (v/v) for 1 min. The eluate was monitored at 280 nm using Waters 2996 photodiode array detector. He Shu Wu, a well-characterized Chinese herbal medicine was used as a standard for confirming the presence of Emodin and Physcion, two well-characterized bioactive molecules in this well-known Chinese herbal medicine [18, 19].

Statistical analysis

Data were analyzed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). Statistical significance was determined by one-way analysis of variance (ANOVA) or two-way ANOVA, followed by Tukey post hoc test. Differences in P-values were considered significant if $P < 0.05$. Data are represented as the mean ± S.E.M.

RESULTS AND DISCUSSION

The clonal cell line of the PC12 cells, has long proven to be a well-established cell line for the investigation of neuronal injury and has been shown to be susceptible to glutamate toxicity [16]. Our first aim was to establish the concentration at which glutamate exhibited toxicity.

Glutamate cytotoxicity in undifferentiated PC12 cell

Glutamate toxicity was evaluated by the quantification of ATP in cells after incubating PC-12 cells for 12 or 24 hours with glutamate (5, 10 and 20 mM). Cell viability was then expressed as a percentage (%) of control. The decrease in cell viability was was both concentration- and time-dependent (Figure 1). There was a significant reduction in cell viability ($P < 0.05$) when the cells were exposed to 5, 10 and 20 mM glutamate (76 ± 5%, 69 ± 2%, and 67 ± 3% respectively) for 12 hrs. The decrease in cell viability observed at 12 hours glutamate incubation (5, 10 and 20 mM) was greatly potentiated at 24 hours of glutamate exposure (5, 10 and 20 mM: 72 ± 5%, 52 ± 6%, and 31 ± 2% respectively) when compared with control value. Although a significant decrease cell viability was observed at both 12 and 24 hours for the three glutamate concentrations (5, 10 and 20 mM), it was only at the 10 mM glutamate exposure for 24 hours that approximately 50% of the cells were viable. This concentration (10 mM) was representative of the half maximal effective concentration (the concentration where the response is reduced by a half) for glutamate in our toxicity experiment with undifferentiated PC12 cells. Based on this observation we decided to use 10 mM glutamate for 24 hours in subsequent experiments.

Effect of CTTF against glutamate-induced toxicity

The next step was to determine if CTTF had a potential protective effect against glutamate toxicity. First we exposed undifferentiated PC-12 cells to

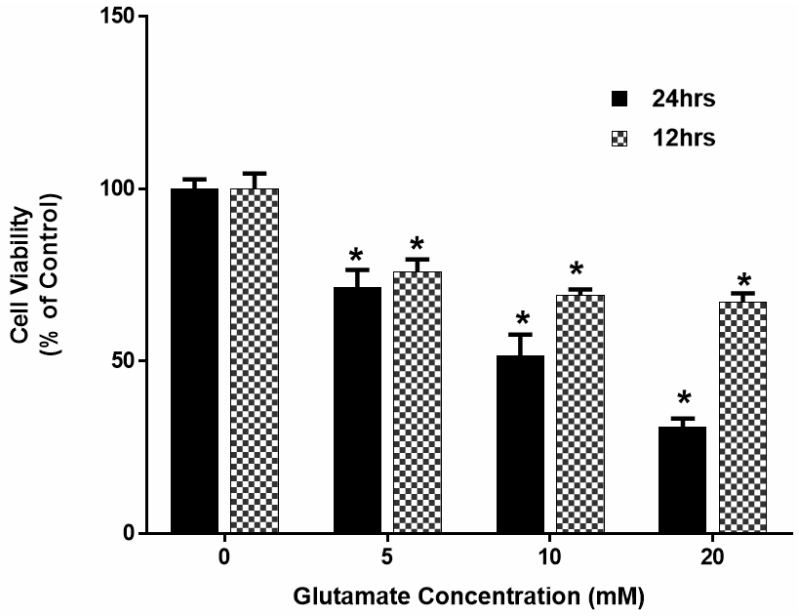

Figure 1. Glutamate-induced cytotoxicity in PC-12 cells.

Undifferentiated PC 12 cells were incubated with different concentrations of glutamate, 5, 10 and 20 mM, for 12 and 24 hours at 37 °C. Cell viability was measured using the ATP assay and expressed as a percentage (%) of control. Data expressed as the percentage (%) of control values are the mean ± SEM for triplicate determinations (n = 3). *P < 0.05; significantly different compared with control conditions. Statistical analysis was done with two-way ANOVA and Tukey as post hoc test.

various final CTTF concentrations (0.160 μg/ml, 0.640 μg/ml, 4 μg/ml, 30 μg/ml, 240 μg/ml, 500 μg/ml, 1000 μg/ml and 4000 μg/ml), and observed that concentrations below 500 μg/ml had no significant effect on undifferentiated PC-12 cells. The 1000 μg/ml and 4000 μg/ml significantly decreased cell viability $P < 0.05$ compared to control (Figure 2A). This suggested that CTTF concentrations at and above 1000 ug/ml would be toxic. Secondly, we investigated the potential for CTTF (concentrations lower than 1000 ug/ml) to rescue undifferentiated PC-12 cell from glutamate-induced toxicity. Our data indicated that CTTF rescued PC-12 in a dose-dependent manner from glutamate-induced toxicity, as observed in Figure 2B. We showed that there was a significant increase in cell viability ($P < 0.05$) from 0.64 μg/ml (66% ± 2.98%) to 30 μg/ml (73% ± 3.55%) by CTTF.

Identification of Emodin and Physcion in CTTF by HPLC

Our proceeding step was to identify the active agent(s) in CTTF. Both Emodin and Physcion are both well characterized Chinese herbal medicines [19, 20]. He Shu Wu, is well known to contain both Emodin and Physcion, and was therefore used as a standard to identify the possibility that both these compounds would be present in CTTF. Using HPLC, Emodin was detected with a retention time (RT) of 67.2 and Physcion at a retention time of 69.9 min in He Shu Wu (Figure 3A). When CTTF was analyzed under the same condition as He Shu Wu, two peaks with the RT of 67.2 and 69.9 min were identified, corresponding to Emodin and Physcion, respectively (Figure 3B).

The identification of Emodin and Physcion in CTTF is a critical finding since some of the therapeutic benefits of CTTF, such as its weight-control abilities, anti-diabetic and anti-constipation properties, could be attributed to the presence of Emodin and Physcion as active agents.

Emodin and Physcion potentiate cell survival against glutamate-induced toxicity

Since CTTF protected undifferentiated PC-12 cells against glutamate toxicity, we investigated

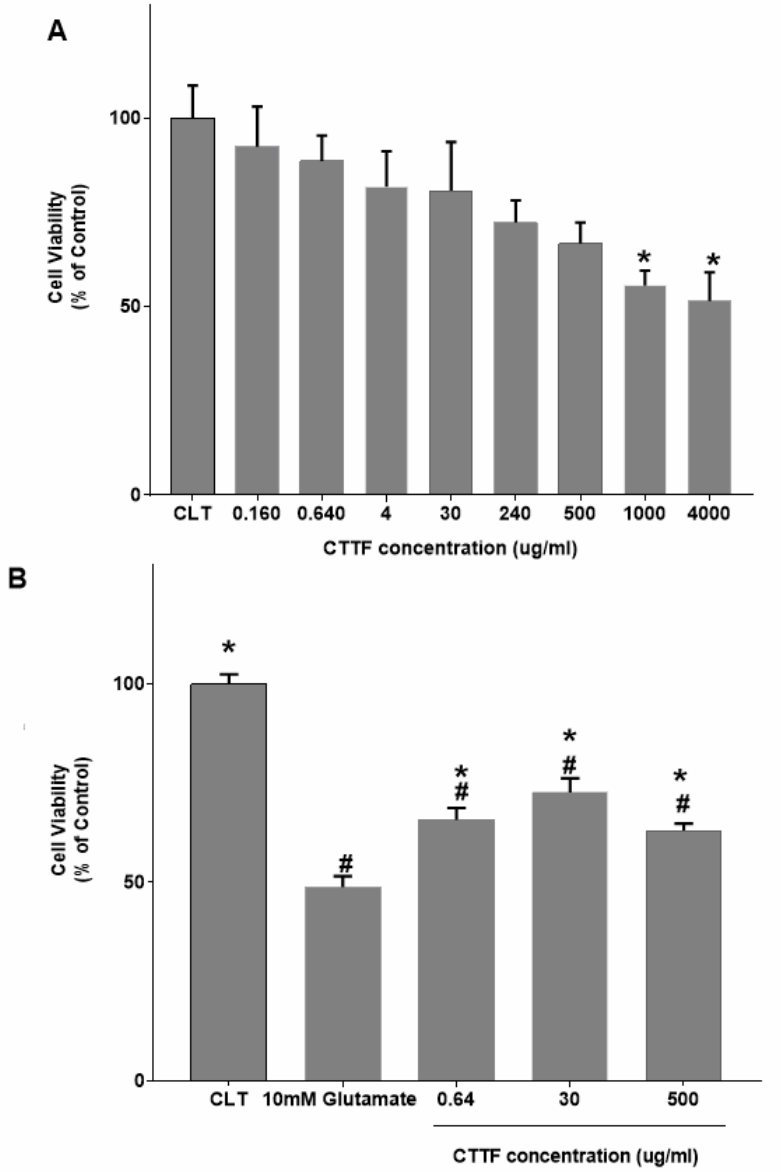

Figure 2. CTTF effect on cell viability against glutamate-induced toxicity.

(A) Various final concentrations of CTTF were administered to undifferentiated PC-12 cells. **(B)** Selected concentrations (0.64, 30 and 500 µg/ml) of CTTF against 10 mM glutamate were given to cells for 24 hrs. Cell viability was measured using the ATP assay, as a percentage (%) of control. Data expressed as the percentage (%) of control values are the mean ± SEM for triplicate determinations (n = 3). $^{\#}P < 0.05$; significantly different compared with control conditions, $*P < 0.05$; significantly different compared with cells treated with only glutamate. Statistical analysis was done with one-way ANOVA and Tukey as post hoc test.

the prospect of Emodin and Physcion (two compounds identified in CTTF) having a similar protective effect. We observed that the optimal concentration of unpurified CTTF was 30 µg/ml (Figure 3B); therefore using pure Emodin and Physcion (Sigma Aldrich), we administered various final concentrations (50 ng/ml, 100 ng/ml, 500 ng/ml and 1000 ng/ml) of Emodin or Physcion, against 10 mM glutamate. Our data indicated that Emodin at low concentrations (range of 50-1000 ng/ml) was non-toxic since cell viability was not significantly different from the

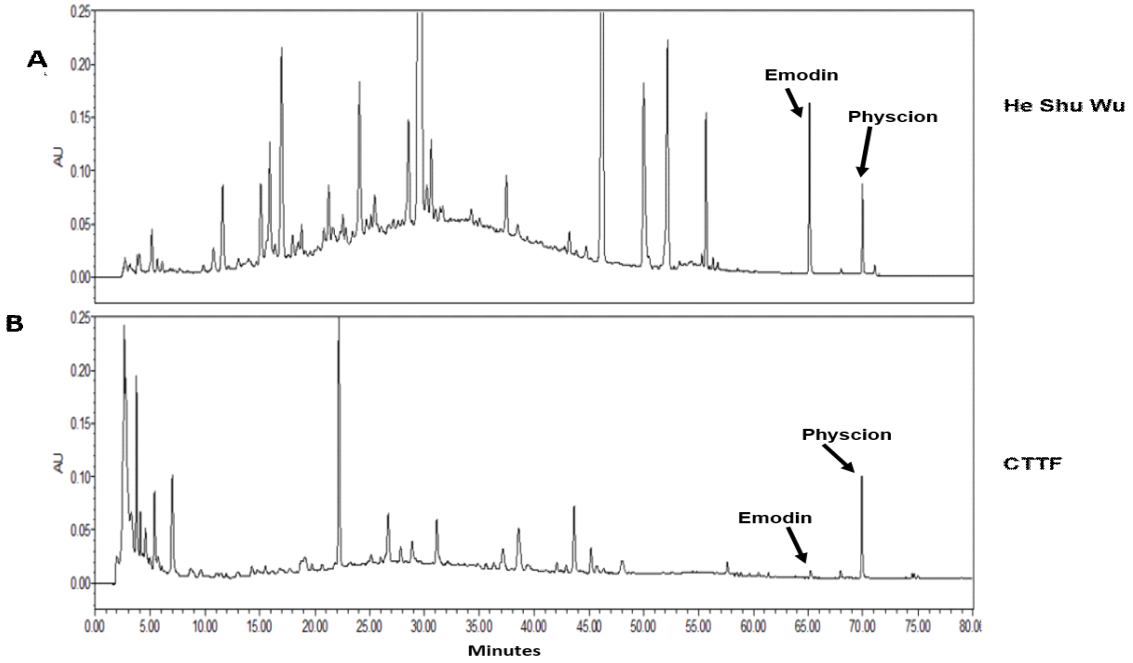

Figure 3. Chromatogram of Emodin and Physcion in CTTF.

(A) HPLC identification of Emodin and Physcion (indicated by arrows in upper panel) in He Shu Wu. **(B)** There was a similar detection of Emodin and Physcion (indicated by arrows in lower panel) in CTTF. The elutions were monitored at 280 nm using Waters 2996 photodiode array detector and expressed in absorption units (AU) on the X-axis. The elution time is expressed in mins. on the Y-axis.

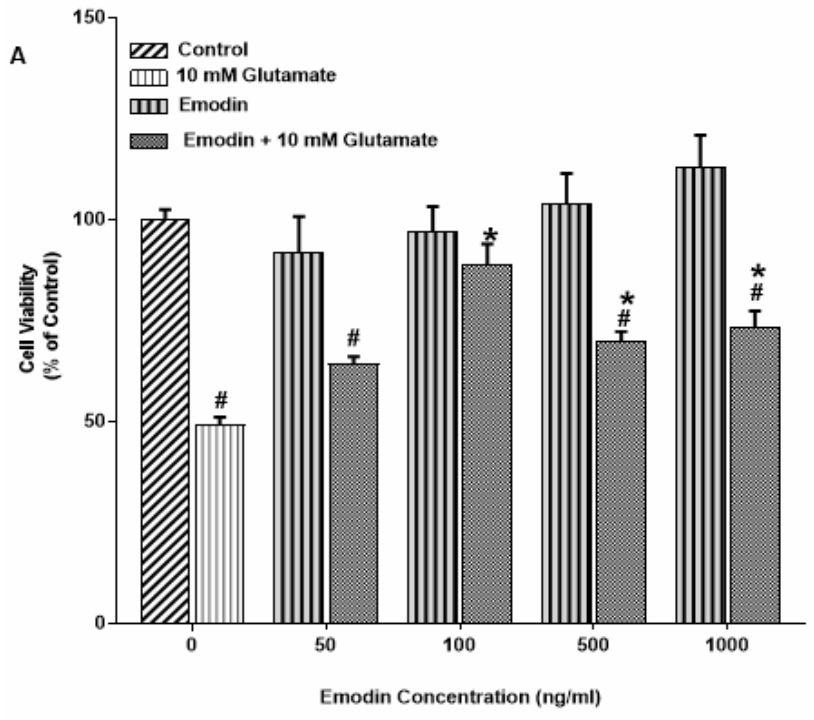

Figure 4

Figure 4 continued..

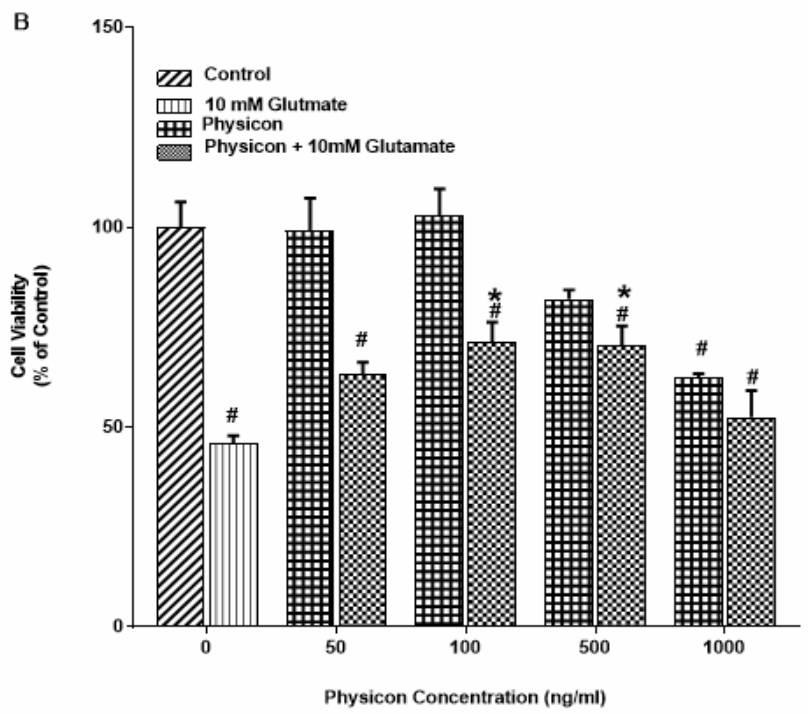

Figure 4. Emodin and Physcion effect on glutamate-induced toxicity.

Undifferentiated PC 12 cells were incubated with various final concentrations, 50, 100, 500 and 1000 ng/ml, of **(A)** Emodin and **(B)** Physcion against 10 mM glutamate for 24 hrs. Cell viability was measured using the ATP assay. Data expressed as the percentage (%) of control values are the mean ± SEM for triplicate determinations (n = 3). $^{\#}P < 0.05$; significantly different compared with control conditions, $^{*}P < 0.05$; significantly different compared with cells treated with only glutamate. Statistical analysis was done with one-way ANOVA and Tukey as post hoc test.

control group (Figure 4A). Emodin significantly ($P < 0.05$) rescued cells in a concentration-dependent manner with an optimal concentration of 100 ng/ml (88.79% ± 5.16%). Physcion was significantly non-toxic at concentrations below 1000 ng/ml (Figure 4B) and was able to rescue the cells, in a concentration range of 100-500 ng/ml ($P < 0.05$; 71.31% ± 4.93%-70.55% ± 4.81%). While both compounds were able to protect undifferentiated PC-12 cells from glutamate-induced toxicity, Emodin had a more potent effect, in which it rescued cells over a wider concentration range of 100 ng/ml-1000 ng/ml and showed no toxicity even at 1000 ng/ml. Our data indicated that Emodin would be the more active agent in CTTF.

CONCLUSION

While CTTF and its active ingredients, Emodin and Physcion, have several pharmacological properties [1-7], to our knowledge our findings are the first to demonstrate the ability of CTTF and its active agents to protect cells from glutamate-induced toxicity. CTTF could be a potentially therapeutic agent for neurological and neurodegenerative diseases, such as stroke, Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD), since one of the underlying pathologies of these diseases/ disorders is glutamate toxicity [12-15]. It is prudent that future research investigates the effect of CTTF in animal models of either stroke, AD, PD or HD.

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CONFLICT OF INTEREST STATEMENT

None.

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