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Research Article

The Effect of Scrophularia Striata on the Nitric Oxide (NO) Production in the Rat Hypocampal Cell: In Vitro Study Running Title: Scrophularia Striata and Nitric Oxide Production

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2. Keywords

Scrophularia striata; Sequential Extract; Nitric Oxide; Rat Hypocampal Cell

1. Abstract

Effect of sequential extract of aerial part of plant on the nitric oxide production in rat CA1 hippocampal cells was investigated.

1.1. Method: S. striata was collected from the eastern part of Iran. Sequential extracts of aerial parts of S. striata were prepared with increase in polarity of the solvent. The CA1 hippocampal cells were obtained from the hippocampus of one-day old pops, cultured and exposed to sequential fractions at the second day of culture. NO content was measured after 7 days.

1.2. Results: Chloroform fraction which contains the non-polar components flavonoids and phenyl propanoid compounds had the most effect on increasing the production of NO followed by ethyl acetate and methanol fractions. Petroleum ether extract slightly reduced the NO production which was not significant compared to control group.

1.3. Conclusion: According to our data, extract from aerial parts of Scrophularia striata increases the production of Nitric Oxide in Rat Hypocampal Cells.

3. Introduction

The genus Scrophularia (Scrophulariaceae), consist of about 300 species and Many of them have been used as folk remedies to cure some medical problems like scrophulas, scabies, tumors, eczema, psoriasis, inflammatory affections, etc [1]. Some species in this genus have shown antitumor activity [2]. Another species, S. frutescens showed a significant cytotoxic activity in Hep2 and McCoy cells [3]. S. striata and some other species have been reported for some centrally effect such as anti-fever and neurological disorders. Nitric Oxide (NO) is a diffusible intercellular messenger with multiple functions including CNS effects. CND development is one of the most important examples. Direct and indirect excitatory effects of NO in CNS have been reported repeatedly. It has also a vital role in early brain development and normal physiological function of the brain. Relationship between NO and synaptic plasticity which results in learning and memory has been established in different experiments. It has been demonstrated that NO has an important function as a retrograde messenger mediating Long Term Potentiation (LTP) in CA1 hippocampal cells which have been reported to show the most responsibility in synaptic plasticity [4,5]. Some experimental findings indicate that NO may be associated with the tolerance of pyramidal cells in the CA1 hippocampus to ischemia induced by Limb Ischemic Preconditioning (LIP) in rats [6]. In fact, physiological amounts of this gas are neuro protective, whereas higher concentrations are clearly neuro toxic [7]. Nitric Oxide Synthases (NOSs) are a family of enzymes that catalyze the production of Nitric Oxide (NO) from

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L-arginine. Inducible NOS (iNOS) is not found in any healthy cell but is instead, as the name implies, induced in various cell types, including macrophages and microglia, generally occurring in response to products of infection, such as bacterial endotoxin, or to inflammatory mediators, including cytokines, interleukin-1 and tumor necrosis factor, resulting in long-term NO production to cytotoxic levels [4]. In our previous study, neuro protective effects of methanolic extract prepared from the aerial parts of Scrophularia striata were observed [8]. Neuro protective effect has been also showed in primary cultures of rat cortical cells which were treated with methanolic extract of the roots of other member of this family, Scrophularia buergeriana. Reported anti-amnesic and cognitive-enhancing properties of this species [9,10] bring some idea in mind about any probable relationship between these effects and neuro protective effects of increased amount of NO in physiological range. Based on our knowledge about neuro protective role of NO and the mentioned properties of Scrophulariaceae family, we designed this study to investigate the hypothesis that the extract of Scrophularia striata may increase the amount of NO in CA1 hippocampal cells.

4. Materials and methods

4.1. Materials

Trypsin, Dulbecco Modified Eagle Medium (DMEM), Fetal Calf Serum (FCS) and horse serum were obtained from Gibco, UK. L- glutamine, Poly-D-Lysine, tetra hydro biopterin (BH4), FITC immuno flurescent anti mouse IgG, (3-[4,5-dimethyl thiazol-2yl]- 2,5 diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co., UK. 50 µm nylon filter was purchased from Portex, UK. ACBD were obtained from Tocris, UK. Cell culture plastic dishes purchased from Nunc, Denmark and Anti-MAP2 antibody obtained from Calbiochem, USA.

4.2. Plant collection

The aerial parts of S. striata Boiss were collected from the north-eastern part of Iran, Ruin region, and were air-dried at room temperature. A sample was authenticated by Dr F. Attar, and a voucher specimen was preserved in the Faculty of Sciences' Herbarium at Tehran University of Medical Sciences, Tehran, Iran (TUH No: 36501). Plant extracts and chemicals. Different extracts were used during this investigation. All experiments were performed based on the dry mass of the concentrated extract.

4.3. Sequential extracts

Different extracts were sequentially prepared using 500 g dried and powdered aerial parts of the plant with increase in polarity of the solvent: petroleum ether (A, 3.3 g dry weight corresponding to 0.6%) (bp. 70–100 ˚C), chloroform (B, 5.7 g dry weight corresponding to 1.1%), ethyl acetate (C, 6.4 g dry weight corresponding to 1.2%) and methanol 80% (D, 8.2 dry weight corresponding to 1.6%). For better extraction each step was performed three times and the final products of them were mixed together. Before change to the new, more polar solvent, the plant were being dried and weighted. Maceration period was 24 hours in all steps. The process was done at room temperature. **(Figure 1)** shows the sequence of extraction.

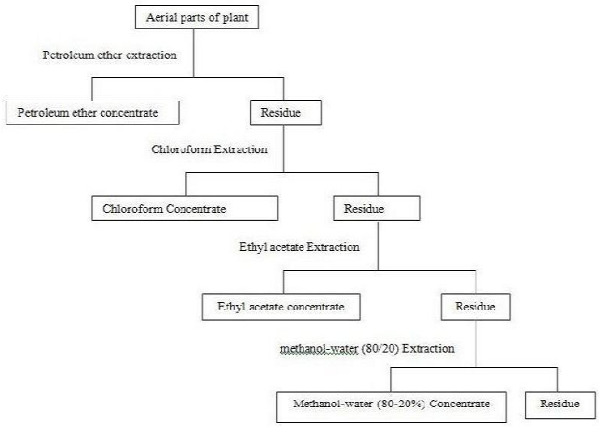

Figure 1: Sequential extraction of aerial parts of the plant Scrophularia striata

4.4. Preparation of CA1 hippocampal neural (CA1HP) cells

Pregnant Sprague-Dawley rats (300-400 g) were purchased from Iranian Pasteur Institute and housed in conditions of constant temperature (23 ± 2oC) with light-controlled conditions (12 hrs light and dark cycles). Food and water were provided ad libitum. The hippocampus of one-day-old pups were removed aseptically (10 pups in each experiment in three separate occasions). We chose one-old pops because based on previous studies, the cells of the brain of 1-old pops have the most recovery during the experiments. The tissue was then incubated in dissociation medium (90 mM Na2SO4, 30 mM K2SO4, 5.8 mM MgCl2, 0.25 mM CaCl2 and 10 mM HEPES with the pH adjusted to 7.4) containing 0.025% trypsin for 20 minutes. Cells were then filtered through 50 µm nylon filter followed by washing in Dulbecco Modified Eagle culture Medium (DMEM) containing 5% FBS, 5% HS, 400 µg L-glutamine and 17 mM D-glucose. The dissociated cells were plated at a density of approximately 5.6×105 cells/ml in 35 mm poly-D-Lysine coated plates. Non-neural cells were omitted by a 24-hr exposure to cytosine arabinoside (10µM solution). Culture medium was then replaced and cells were washed with pre-warmed PBS and were incubated for 7 days at 37oC with 5%CO2-95%O2 atmosphere. All the experiments were conducted in accordance with the accepted principles for laboratory animal use and care in TUMS Animal Research Ethics Committee.

4.5. Extract administration to the cultured cells

The CA1HP cells were purified as described above and were exposed to sequential fractions at the second day of culture for 7 days. The NO content was measured as explained before.

Cell viability tests

a) Cell viability was determined by use of Trypan blue dye exclusion method. The cells were trypsinized and 20µL of Trypan blue solution (0.4% v/v) was added to 20µL of cell suspension. Those cells which exclude the stain were considered as viable cell and counted by light microscopy. Five objects were counted. Number of cells per ml was estimated using this formula:

The number of counted cells/5×20000

The number 20000 has been measured based on dimensions of Neubauer lamel.

Cell viability was represented as the percentage of viable cells to total number of the counted cells.

b) Determination of Mitochondrial dehyrogenase activity (MTT)

100 µL of MTT (3-[4, 5-dimethyl thiazol- 2yl]-2, 5-diphenyl tetrazolium bromide) solution was added to each well. Mitochondrial dehyrogenases of viable cells cleave the tetrazolium ring of the yellow MTT to yield purple for mazan crystals which are insoluble in aqueous solutions. The crystals were dissolved in 300 µL of the acidified isopropanol and the absorbance of the resulting purple solution was measured at 570 nm against 690 nm for blank solution. The amount of produced formazan is proportional to the number of viable cells.

4.6. Immuno cyto chemistry

Cultured neurons were stained with monoclonal anti-MAP2 antibody that recognizes phosphate independent epitope of the 280 KD cytoskeletal MAP2 proteins. Briefly, cells were fixed in 4% paraformaldehyde at room temperature for 4 minutes followed by washing with PBS and incubation in blocking reagent for 30 min. Then, the cells were incubated with anti-MAP2 antibody (1:100) in blocking reagent for 3 hours at room temperature. Visualization was carried out using the FITC-immuno fluorescent anti-mouse IgG and the number of the immuno reactive neurons was determined under the Olympus B201 microscope **(Figure. 2)**. Five objects were selected under fluorescent microscope and at least 250 cells were counted at each one and the mean was calculated. Cell viability presents as percentage of viable cells to the mean of whole counted cells and the non-toxic doses of extracts were found out.

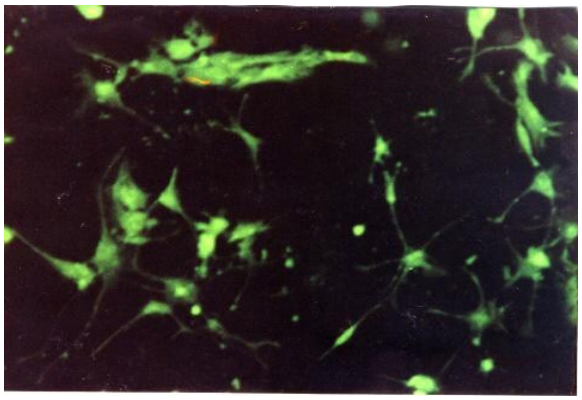

Figure 2: CA1 neuronal hipocampal cells stained by indirect FITC antibodies against MAP2 (X400)

4.7. Nitric oxide measurement

NO was measured according to the method of Richter et al [11]. Briefly, in a pre warmed (37°C) cuvette containing the buffer (0.1 M HEPES, pH 7.0), the NOS substrates and reagents were added as follows: 1mM L-Arginine, 1 mM CaCl2, 0.2 mM NADPH, 0.5 µM Flavine Mono Nucleotide (FMN) and 10 µM tetra hydrobiopterin (BH4). This mixture is highly unstable in a dilute solution due to auto-oxidation. To measure NO, 4 µM HbO2 (oxyhaemoglobin) - which was prepared according to Di Iorio method [12] was added to the cocktail, mixed gently and absorbance was recorded at 401 nm. This mixture was then added to the cells and incubated at 37°C for 20 minutes, and MetHb (methemoglobin) formation was measured by increasing in absorbance at 401 nm. NO content was estimated using this formula:

NO content (mM) = change in absorbance at 401 nm/49mM-1.

The number 49 is extinction coefficient of NO at 401 nm.

5. Statistical analysis

The data in each group were examined by one way ANOVA with Dunnett post test. Probability value less than 0.05 ($p<0.05$) was assumed significant.

6. Results

The present study was designed to show the effect of different non-toxic fractions of S. Striata extracts on NO production of CA1 neural cells in vitro. Pure neural hippocampal cells obtained from treatment of CA1 hippocampal cells with cytosine abrabinoside and were examined with antibody against MAP2 antigen by using fluorescent immuno cytochemical technique. Viability of cells remains unchanged at fractions range of administration. As previously reported, the optimum time for NO measurement is about 20 minutes after adding substrate for NOS. Results of this experiment showed that chloro formic fraction significantly increases NO production in the cells compared to control ($p<0.05$), while the other fractions did not show any noticeable effect. Indeed the petroleum ether fraction slightly decreased NO production in the cells **(Figure 3)**, although this change was not significant.

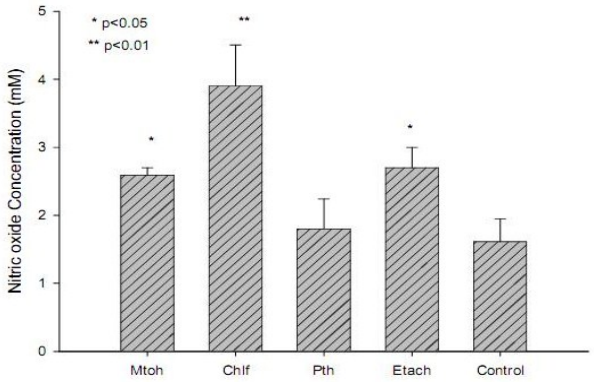

Figure 3: The effect of different extracts on Nitric Oxide production in CA1 hippocampus neural cells. (n = 3). Mtoh: methanolic extract, Chlf: chloroform extract, Pth: petroleum ether extract, Etach: ethyl acetate extract

*p<0.05

**p<0.01

F = 26.282

7. Discussion

The genus Scrophularia is represented by over 300 species. The present study was designed to investigate the effect of different non-toxic fractions of S. Striata extracts on the nitric oxide production of CA1 neural cells in vitro. Pure neural hippocampal cells obtained from treatment of brain extract cells with cytosine abrabinoside was examined by antibody against MAP2 antigen by fluorescent immuno cyto chemical technique. Viability of cells remains unchanged in the range of fractions administration. Different species of Scrophularia genus (Scrophulariaceae) have been traditionally used for several skin inflammatory ailments, e.g., scrofulas and different types of dermatosis including scabies, tumours and slough. Some of these species have shown a chemical profile characterized mainly by phenylpropanoid glycosides [12-15]. Neuro protective effects of this family has been also attracted much attention in folk medicine. The dried roots of Scrophularia spp. have been used in oriental medicine as a treatment for fever and neuritis [16,17]. Furthermore, several authors reported the neuro protective effect of Scrophularia species against neurotoxicity of glutamates [18]. CHCl3/ MeOH extract from the roots of Scrophularia buergeriana (Scrophulariaceae) exhibited significant neuro protective activity. It has been reported that many Scrophularia species have been investigated and found to contain many classes of secondary metabolites including irridoids, phenyl propanoides, phenolc acides, flavonoides and saponines [19,20]. In our previous study, one phenolic acid, two flavenoids and one phenyl propanoid glycoside were isolated and elucidated from S. striata. The phenolic acid, cinnmic acid, was purified from chloroform fraction of aerial parts of S. striata. Four compounds E -Harpago side, 8-O-(E-p-met h oxy cinnamoyl) harpagide (MCAHg), E-cinnamic acid (CA), and E-p – Methoxy Cinnamic Acid (MCA); extracted and purified from Scrophularia buergeriana root have shown neuro protective properties glutamate-induced neurotoxicity in primary culture o f rat cortical cells [9]. The results of our experiments showed that the chloro formic fraction increases the amount of NO and only the petroleum ether slightly reduced the total NO production which was not statistically significant. However, the percentage of component may change the total effect of extracts. In some model of brain ischemia, it has been shown that NO may play some kind of neuro protection. In this case increase of NO production in neuronal cell may in benefit of neuro protection. Increased level of NO after treatment with chloro formic fraction can explain the neuro protective application of this plant in folk medicine. Since increased level of NO more than physiological range can harm the nervous system because of exitotoxicity, it can be assumed that Scrophularia associated neuro protection is dose dependent, although this hypothesis needs more investigation in future. Furthermore, our future study will be focused on purification of active compounds of chloro formic fraction. Based on established data, iNOS has an important role in rapid increase in nitric oxide level in response to exogenous compounds. Therefore, the effect of chloro formic fraction on this enzyme is possible which should be investigated in future studies. In conclusion our results show that different fractions of S. striata may have different effects and these results may show some differences when other species of Scrophularia is investigated. In this study we showed that chloroform fraction had the most effect on production of NO and after that was Ethyl acetate and methanol fractions respectively. Petroleum ether fraction, which contains fatty acids and alkan compounds, didn't show significant different compared to control group ($P > 0.05$). The chloroform fraction contains flavonoid and phenyl propanoid compounds. In previous study, it was shown that phenyl propanoid compounds had similar effect on NO production which may related in some degree to influence on enzymes such as Nitric Oxide Synthases. However, more investigations are essential to understand perspective of this plant.

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