

EXTRA MURAL RESEARCH

Neuroprotective effect of *Bellis perennis* and *Hypericum perforatum* on PC12 cells

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The rat pheochromocytoma (PC12) cell line is widely applied as a model system to study a variety of neuronal functions. Upon addition of nerve growth factor (NGF), these cells undergo differentiation characterized by an increase in acetylcholinesterase (AChE) activity, and extension of neurite-like processes. Oxidative stress is implicated in the pathogenesis of neurodegenerative disorders and the purpose of this study was to evaluate the effect of *Bellis perennis* (Be) and *Hypericum perforatum* (Hy) (6C and 30C) on healthy neuronal cells. Both homeopathic medicines have been studied at three different concentrations of 2 µl/ml, 4 µl/ml and 8 µl/ml for 96 h in PC12 cells differentiated with NGF. The cell viability was tested by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and NRU (Neutral Red Uptake). To observe the oxidative damage and evaluate the antioxidative status after exposure to homeopathic medicines, the level of thiobarbituric acid reactive species (TBARS), glutathione (GSH) level, activities of glutathione peroxidase (GPx), glutathione reductase (GR), AcetylCholine Esterase (AChE), Na⁺K⁺ATPase and Monoamine Oxidase (MAO) were assayed. These results were compared with positive control (90% alcohol). The content of LPO was significantly decreased in drug treated groups as compared to positive control while the level of GSH was significantly increased. The activities of all other enzymes were significantly restored in drug treated groups as compared to positive control. In conclusion, these medicines have preventive role on differentiated PC 12 cells.

Keywords: homeopathic medicines; PC12 cell; NGF; oxidative stress

Introduction

Homeopathy is a form of alternative medicine that attempts to treat patients with heavily diluted preparations. Homeopathic remedies are prepared by serial dilution with shaking by forceful striking, which homeopaths term "succussion," after each dilution under the assumption that this increases the

effect of the treatment. Homeopaths call this process "potentization". Dilution often continues until none of the original substance remains.¹ It has been known for centuries that homeopathic remedy relieves the effects of stress, worry and nervous tension, plus supports nervous system health. There are hundreds of homeopathic medicines reported in homeopathic repertory for nervous system but there are very less preclinical or clinical data for these medicines.

Reactive oxygen species (ROS) have been implicated in more than one hundred diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer,

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and gastric ulcer.² In addition, excess production of ROS in the brain has been implicated as a common underlying factor for the etiology of a number of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and stroke. Therefore, the development and utilization of effective antioxidants are desired. The brain is very susceptible to the damage caused by oxidative stress, due to its rapid oxidative metabolic activity, high polyunsaturated fatty acid content, relatively low antioxidant capacity and inadequate neuronal cell repair activity.^{3,4}

For the experimental approach, rat pheochromocytoma PC12 cells were selected. PC12 line was established from rat adrenal pheochromocytoma cell⁵ and has been used as a model to study the cellular and molecular aspects of neuronal apoptosis. A notable characteristic of PC12 cells is that they can readily be induced to differentiate in culture with the neurotrophic factor, nerve growth factor (NGF) whereby cells cease to multiply, assume a neurite-bearing phenotype that resemble mature sympathetic neurons and exhibit firm attachment to the substratum.⁵

Biological effects of homeopathic potencies were thus studied in PC12 cell line. The aim of this study was to determine levels of lipid peroxidation measured as TBARS, glutathione (GSH), and the activities of key antioxidant enzymes including glutathione peroxidase (GPx), glutathione reductase (GR), Acetylcholine Esterase (AChE), Na⁺K⁺ATPase and Monoamine Oxidase (MAO) in PC12 cells supplied with different concentrations of homeopathic medicines. The positive control used in the study was 90% ethanol (vehicle of homeopathic medicines).

Materials and methods

Chemicals and reagents

Poly-L-lysine (PLL), [3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide tetrazolium] (MTT), Neutral red uptake (NRU), Oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB), bovine serum albumin (BSA), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sulfosalicylic acid (SSA), adenosine 5-triphosphate (ATP), ethylenediamine tetra acetic acid (EDTA), acetylthiocholine iodide, ouabain, benzylamine hydrochloride (BAHC), Dimethyl sulphoxide (DMSO), Sodium bicarbonate and protease inhibitor were purchased from Sigma-Aldrich chemicals Pvt. Ltd, India. Fetal Bovine Serum (FBS), Horse serum, Antibiotic-Antimycotic solution, Phosphate buffer saline (PBS), Nutrient F12 medium (NFM), Nerve growth factor (NGF) were purchased from Gibco, BRL, NY.

Cell Lines

PC12, rat pheochromocytoma cell line used in all experiments, was originally procured from Indian Institute for Toxicological Research (IITR), Lucknow and from 2008 it has been maintained in Neurotoxicology laboratory, Department of Medical Elementology and Toxicology, Jamia Hamdard. As per the standard protocols given by National Centre for Cell Sciences (NCCS), Pune, the PC12 cells were cultivated in F-12 Hams supplemented with 2.5% fetal bovine serum (FBS), 15% horse serum (HS), 0.2% sodium bicarbonate and 1.5% (100 x solution) of antibiotics and antimycotic. All treatments were performed when cells seeded at 80% confluence. Before experiments, the cells were seeded in 96 well micro plates (5000 cells/well) or 25 cm² flask (1 x 10⁵ cells) and precultured for 8 days with 50 ng/ml nerve growth factor (NGF 7.0S, Chemicon).

Cell Viability Assay

MTT reduction

After 96 h exposure to Be and Hy at various concentrations (2 µl/ml, 4 µl/ml, 8 µl/ml) and 90% alcohol, cell survival was determined by a standard colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]. Briefly, MTT was dissolved in phosphate-buffered saline (PBS, pH 7.4) at 5 mg/ml. From this stock, 10 µl per 100 µl of medium was added to each well and incubated for 4 h at 37°C. The medium was removed and 200 µl of dimethyl sulfoxide (DMSO) was added per well to dissolve the blue formazan product. Absorbance was measured at 570 nm in an automated plate reader (BioRad Model 680). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of control cells was 100%.

Neutral red uptake (NRU)

PC12 cells were seeded in 96-well culture plates and the cell viability was assessed by NRU assay as described by Borenfreund and Puerner [7]. The cells were exposed to Be and Hy at various concentrations (2 µl/ml, 4 µl/ml, 8 µl/ml) for same time period. Briefly 0.4% aqueous stock solution of the NRU dye was prepared and an aliquot was added to the complete medium to make the final concentration of 50 mg/ml. Addition of 200 µl of the neutral red medium to the wells and incubation for 3 h at 37°C, the viable cells stained with the dye. Thereafter the dye medium was taken and cells were washed rapidly with 40% formaldehyde. After the removal of the formaldehyde from the cells a mixture of

200 ml of 1% acetic acid and 50% ethanol was added, followed by an incubation of 20 min at 37°C. The plates were read at 540 nm using Multiplate Reader (BioRad Model 680). Results were expressed as percentage absorbance assuming that the absorbance of control cells was 100%.

Biochemical Studies

Preparation of cell lysate

For all enzymatic and non enzymatic assays 1×10^5 cells were taken and sonicated in SONICS vibra cells sonicator in 0.01 M Tris Buffer, pH 7.0 containing protease inhibitors (10 μ l/ml). The cell lysate was centrifuged at 800 x g for 5 min at 4°C to separate the nuclear debris and was used for estimation of lipid peroxidation (LPO). The supernatant (S1) obtained was again centrifuged at 10,500 x g for 15 min at 4°C to obtain post-mitochondrial supernatant (PMS) which was used for the estimation of GSH and antioxidant enzymes.

Estimation of thiobarbituric acid reactive substances

The assay of TBARS was done according to method of Islam et al.⁸ The cell lysate 0.25 ml was incubated at $37 \pm 1^\circ\text{C}$ in a metabolic shaker (120 strokes/min) for 1 h. Similarly, 0.25 ml of the same cell lysate was pipetted in a test tube and incubated at 0°C. After 1 h of incubation, 0.5 ml of 5% chilled TCA was added followed by 0.25 ml of 0.67% TBA in each test tube and proper mixing was done after each addition. The mixture was centrifuged at 3000 x g for 10 min. Thereafter, supernatant was transferred to another test tube and placed in boiling water bath for 10 min. The test tubes were cooled and the absorbance of the color was read at 535 nm. The rate was expressed as nmol TBARS formed/mg protein using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Reduced glutathione (GSH)

For estimating GSH content⁹, PMS was mixed with 4% sulfosalicylic acid in a 1:1 ratio (v/v). The samples were incubated at 4°C for 1 h and later centrifuged at $1200 \times \text{g}$ for 15 min at 4°C. The assay mixture contained 0.1 ml of supernatant, 1.0 mM DTNB and 0.1 M PB (pH 7.4) in a total volume of 1.0 ml. The yellow color developed was read immediately at 412 nm using spectrophotometer (UV-1601, Shimadzu, Japan). The GSH content was calculated as μmol of DTNB conjugate formed/mg protein using a molar extinction coefficient of $13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Glutathione peroxidase (GPx)

While estimating GPx activity¹⁰, the reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0), EDTA (1 mM), sodium azide (1 mM), glutathione reductase (1 EU/ml), glutathione (1 mM), NADPH (0.2 mM), hydrogen peroxide (0.25 mM) and 0.1 ml of PMS in the final volume of 1 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein by using molar extinction coefficient $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Glutathione reductase (GR)

While assaying Glutathione reductase¹⁰, the assay mixture consisted of phosphate buffer (0.1 M, pH 7.6), NADPH (0.1 mM), EDTA (0.5 mM) and oxidized glutathione (1 mM) and 0.05 ml of PMS in total volume of 1 ml. The enzyme activity was quantified at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Acetylcholinesterase (AChE) activity

In the method adopted for determining AChE activity¹¹, briefly 2.6 ml of Tris buffer (0.1 M, pH 8.0), 40 μ l of 0.075 M acetylthiocholine iodide and 0.1 ml of buffered Ellman's reagent (DTNB 10 mM, NaHCO_3 15 mM) were mixed and allowed to incubate for 5 min at room temperature. Enzyme sample (40 μ l) was added and optical density was measured at 412 nm within 5 min. AChE activity was expressed as nmol thiocholine formed/min/mg protein using a molar extinction coefficient of $1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Assay for Na⁺K⁺-ATPase

The Na⁺K⁺-ATPase activity was determined, with slight modification¹² in two reaction media, A and B. The reaction mixture A consisted of 0.2 M KCl, 1.0 M NaCl, 0.1 M MgCl₂, 0.2 M Tris-HCl buffer (pH 7.4), 0.1 ml of homogenate in a total volume of 2.0 ml. The reaction mixture B consisted of 0.1 M MgCl₂, 10 mM ouabain, 1.0 M NaCl, 0.2 M Tris-HCl buffer (pH 7.4), 0.1 ml of homogenate in a total volume of 2.0 ml. The enzyme reaction was started by adding 0.2 ml of 25.0 mM ATP at 37°C and terminated after 15 min by adding 1.0 ml chilled 10% TCA. The mixture was centrifuged and supernatant (0.5 ml) was used for the estimation of inorganic phosphorous.¹³

Mono amine oxidase activity

Monoamine oxidase activity was determined

spectrophotometrically.¹⁴ The assay mixture consisted of 0.2 ml cell lysate and 0.1 M BAHC (Benzylamine hydrochloride). The reaction was incubated for 30 min at 37 °C. Thereafter 10% perchloric acid was added to stop the reaction in a total volume of 3 ml. The mixture was then centrifuged at 1500 x g for 15 min to remove all the precipitated protein. The activity was recorded at 280 nm and expressed as nmol of benzaldehyde formed/min/mg protein with molar extinction coefficient of 7.6925 M⁻¹ cm⁻¹.

Determination of protein

Protein was determined.¹⁵ using bovine serum albumin (BSA) as a standard.

Statistical analysis

Results are expressed as mean±S.E.M. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's Kramer test for all parameters. The p-value <0.05 or less was considered statistically significant.

Results

Effect of *Bellis perennis* on the viability (MTT) of PC12 cells after 96h treatment

Cell viability was significantly decreased in the positive control (PC) group compared with the negative control (NC) group. *Bellis* (6C and 30C) treatment at concentration of 2 µl/ml, 4 µl/ml and 8 µl/ml significantly increased % cell viability (97.06%, 90.62%, 99.71% and 99.87%, 93.40%, 87.35% respectively). (Fig.1.)

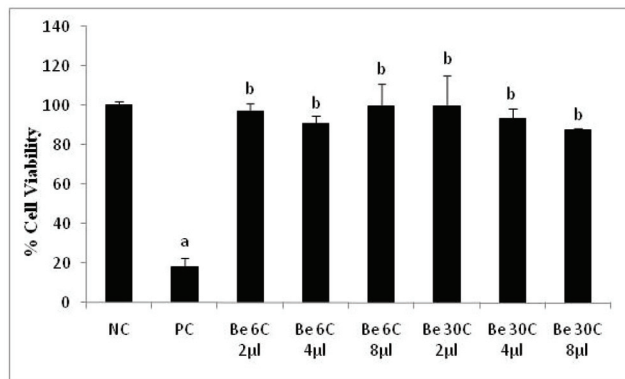


Figure1. Effect of *Bellis perennis* (6C and 30C) treatment on % viability (MTT) of PC 12 cells for 96 hr. Values are expressed as mean±S.E.M. (^ap<0.001 PC vs. NC; ^bp<0.001 Be vs. PC group).

Effect of *Hypericum perforatum* on the viability (MTT) of PC12 cells after 96 h treatment

Cell viability was significantly decreased in the

PC group compared with the NC group. *Hypericum* (6C and 30C) treatment at 2 µl/ml, 4 µl/ml and 8 µl/ml significantly increased the cell viability (94.01%, 92.1%, 91.53% and 94.82%, 91.76% , 83.18% respectively). (Fig. 2.)

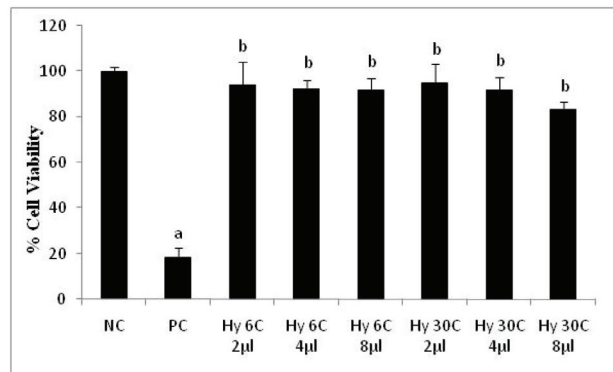


Figure 2. Effect of *Hypericum perforatum* (6C and 30C) treatment on PC 12 cells viability (MTT) for 96 h. Values are expressed as mean±S.E.M. (^ap<0.001 PC vs. NC; ^bp<0.001 Hy vs. PC group).

Effect of *Bellis perennis* on the viability (NRU) of PC12 cells after 96 h of treatment

Cell viability was significantly decreased in the positive control (PC) group compared with the negative control (NC) group. *Bellis* (6C and 30C) treatment at 2 µl/ml, 4 µl/ml and 8 µl/ml significantly increased the cell viability (92.16%, 78.14%, 79.38%, 89.53%, 83.20% and 81.50% respectively). (Fig. 3)

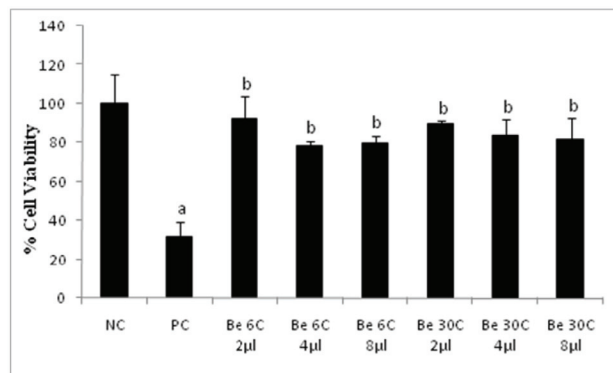


Figure 3. Effect of *Bellis perennis* (6C and 30C) treatment on the viability (NRU) of PC 12 cells for 96 hr. Values are expressed as mean±S.E.M. (^ap<0.001, Positive control, PC vs. negative control, NC; ^bp<0.001 Be vs. PC group).

Effect of *Hypericum perforatum* on the viability (NRU) of PC12 cells after 96 h of treatment

Cell viability was significantly decreased in the positive control (PC) group compared with the negative control (NC) group. *Hypericum* (6C and 30C) treatment at 2 µl/ml, 4 µl/ml and 8 µl/ml significantly increased the

cell viability (82.63%, 87.11%, 88.04%, 85.20%, 86.13% and 80.52% respectively). (Fig. 4)

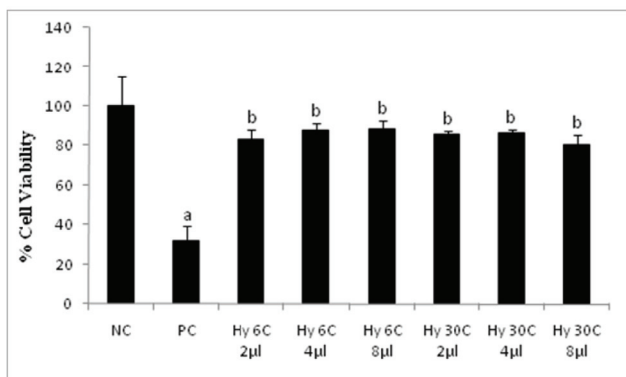


Figure 4. Effect of *Hypericum perforatum* (6C and 30C) treatment on the viability (NRU) of PC 12 cells for 96 hr. Values are expressed as mean±S.E.M. (^a $p < 0.001$ Positive control, PC vs. negative control, NC; ^b $p < 0.001$ Hy vs. PC group).

Effect of homeopathic medicines on TBARS content

The increase level of lipid peroxidation is toxic because it is formed with the generation of free radicals. It was observed that both the potencies of the Homeopathic medicines were quite effective in the depletion of the lipid peroxidation i.e. the reduction in the formation of free radicals. The level of lipid peroxidation was significantly increased in the positive control (PC) group compared with the negative control (NC) group. The treatment for 96 h with the Homeopathic medicines of 6C and 30C potencies has significantly decreased the level of lipid peroxidation at highest dose (8 µl/ml) for 96 h of treatment. (Fig. 5)

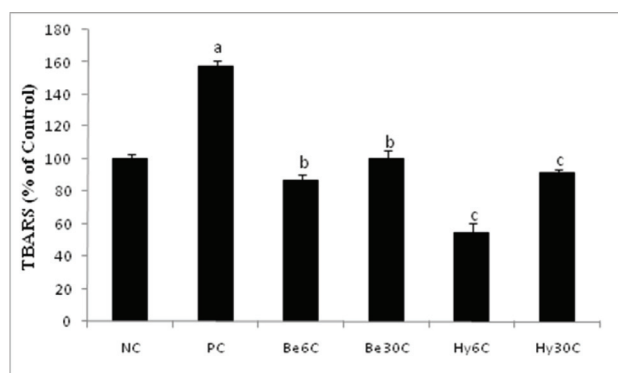


Figure 5. Effect of Homeopathic medicines (6C and 30C) treatment on TBARS (% of Control) in PC 12 cells for 96 hr. Values are expressed as mean±S.E.M. (^a $p < 0.001$, Positive control, PC vs. negative control, NC; ^b $p < 0.001$, Be vs. PC group, ^c $p < 0.001$, Hy vs. PC group).

Homeopathic Medicine restored GSH level

The increase level of glutathione (GSH), a –SH containing tripeptide play a protective role on the cells membrane. Its decreased level causes depletion of antioxidant enzymes in the cells. The GSH level was significantly decreased in the positive control (PC) group compared with the negative control (NC) group. Homeopathic medicines (6C and 30C) treatment significantly increased GSH level at highest dose (8 µl/ml) for 96 h. (Fig. 6)

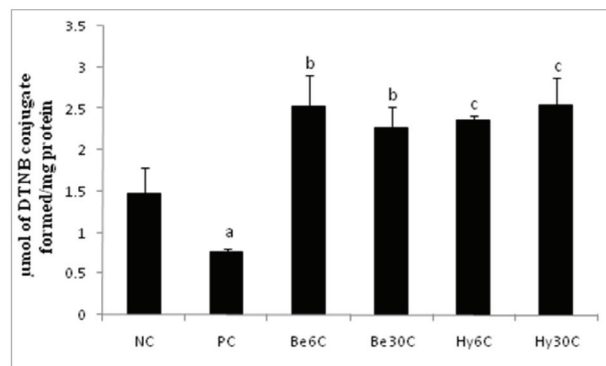


Figure 6. Effect of Homeopathic medicines (6C and 30C) treatment on GSH level in PC12 cells for 96 hr. Values are expressed as mean±S.E.M. (^a $p < 0.001$, Positive control, PC vs. negative control, NC; ^b $p < 0.001$, Be vs. PC group, ^c $p < 0.001$, Hy vs. PC group).

Effect of Be and Hy on antioxidant enzyme activities

Fig. 7 shows the effect of alcohol on the antioxidant enzymes (GPx and GR) activity. The alcohol has significantly decreased the activity of these enzymes compared with the negative control i.e. only culture medium. This is an indication of the toxicity of the alcohol when added in the incubation medium.

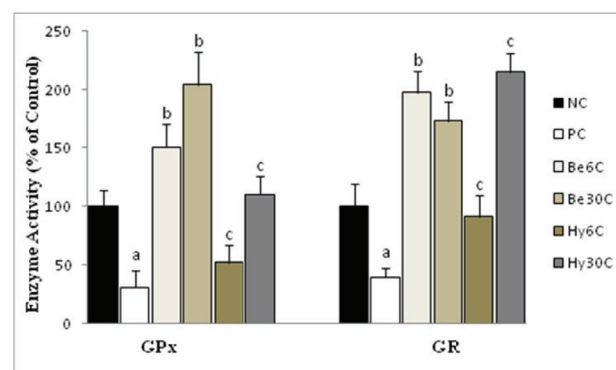


Figure 7. Effect of Homeopathic medicines (6C and 30C) treatment on GPx and GR level in PC12 cells for 96 hr. Values are expressed as mean±S.E.M. (^a $p < 0.001$, Positive control, PC vs. negative control, NC; ^b $p < 0.001$, Be vs. PC group, ^c $p < 0.001$, Hy vs. PC group).

The activity of these enzymes was increased significantly when treated with Be6C, Be30C, Hy6C and Hy30C. The GPx has shown highly increased activity of these enzymes with the dose of Be while GR has shown increased activity in both drugs. The graph shows that the activity of these enzymes was significantly decreased in the cells treated only with the alcohol (same conc and same amount) as compared with negative control. The Be and Hy having the same amount of the alcohol has protected the activity of these enzymes as compared to positive control.

Effect of homeopathic medicines on the activity of acetylcholine esterase (AChE)

The AChE activity was significantly increased in the positive control (PC) group compared with the negative control (NC) group. Homeopathic medicines (6C and 30C) treatment significantly decreased AChE activity at highest dose (8 µl/ml) for 96 h. (Fig. 8)

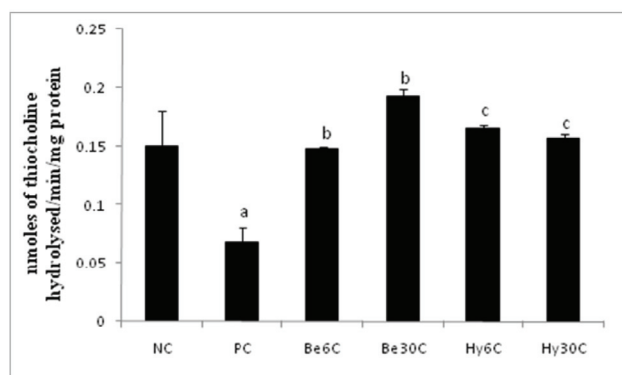


Figure 8. Effect of Homeopathic medicines (6C and 30C) treatment on the activity of AChE in PC12 cells for 96 hr. Values are expressed as mean±S.E.M. (^ap<0.001, Positive control, PC vs. negative control, NC; ^bp<0.001, Be vs. PC group, ^cp<0.001, Hy vs. PC group).

Effect of different homeopathic medicines on Na+K+ATPase levels after 96h treatment on PC 12 cells

The activity of Na+K+ATPase is responsible in the nerve transmission from one neuron to another. Its decreased activity may hamper the transmission of the impulse from one neuron to another neuron. Its activity was significantly decreased in the positive control (PC) group compared with the negative control (NC) group. Homeopathic medicines (6C and 30C) treatment significantly increased Na+K+ATPase activity at highest dose (8 µl/ml) for 96 h of treatment. (Fig. 9)

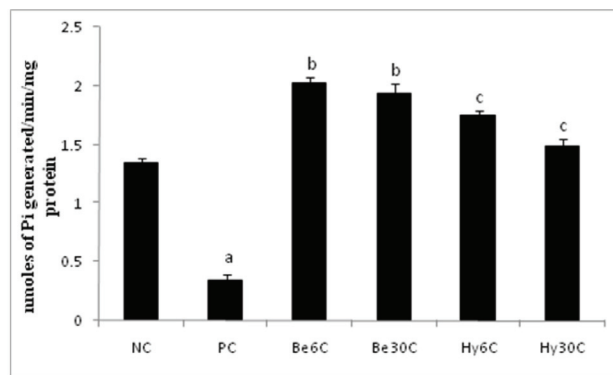


Figure 9. Effect of Homeopathic medicines (6C and 30C) treatment on the activity of Na⁺K⁺ATPase level in PC 12 cells for 96 hr. Values are expressed as mean±S.E.M. (^ap<0.001, Positive control, PC vs. negative control, NC; ^bp<0.001, Be vs. PC group, ^cp<0.001, Hy vs. PC group).

Effect of different homeopathic medicines on the activity of monoamine oxidase (MAO) after 96 h of treatment on PC 12 cells

The MAO plays a role in the synthesis and break down of the catecholamine. The activity of MAO was significantly decreased in the positive control (PC) group compared with the negative control (NC) group. Homeopathic medicines (6C and 30C) treatment significantly increased MAO level at highest dose (8 µl/ml) for 96 h. (Fig. 10)

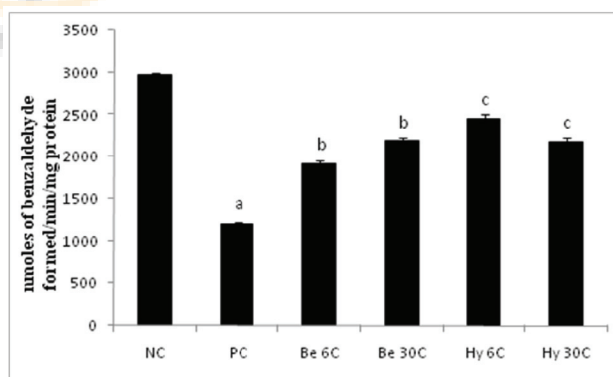


Figure 10. Effect of Homeopathic medicines (6C and 30C) treatment on the activity of MAO in PC 12 cells for 96 hr. Values are expressed as mean±S.E.M. (^ap<0.001, Positive control, PC vs. negative control, NC; ^bp<0.001, Be vs. PC group, ^cp<0.001, Hy vs. PC group).

Discussion

In this study, the effect of two homeopathic medicines *Bellis perennis* and *Hypericum perforatum* was investigated on healthy neuronal cell line. Alcohol (90%) was used as positive control as these medicines are formulated in the same vehicle. Two potencies (6C

and 30C) were used in the study. PC12 cell differentiated with NGF is used as neuronal model.³

The major findings of this study were that both these drugs had beneficial effect on cells and that the effect of alcohol present in the drug was somehow neutralized by these drugs. On the other hand only alcohol caused significant alterations in cell viability and oxidative stress parameters. The cell viability assay was done in order to assess any toxicity of medicines if observed. The MTT assay is based on formazon crystal formation by viable cells. Viable or live cells convert tetrazolium salt to formazon crystals (needle shaped and purple in colour) due to the activity of mitochondrial dehydrogenase. The purple colour is then read at 550 nm which indirectly gives the percentage of viable cells. Same principle is the basis of Neutral Red Uptake (NRU), another viability test where pink solution is obtained in case of viable cells. The live cells could retain the dye in lysosomes which is released on addition of lysosomes disruptors. The amount of dye retained is indirectly related to cell viability. The dead cells could not retain the dye and hence solution was clear. On treatment with 90% alcohol which serves as positive control, the cell viability was significantly decreased to 18% as compared to the negative control (only media) which was taken as 100%. *Bellis perennis* with 6C and 30C potencies at 2µl/ml, 4µl/ml and 8µl/ml significantly increased the cell viability as compared to the positive control. This result shows that the effect of alcohol present in *Bellis* as vehicle is neutralized by the medicine and the medicine shows same effect on cells as media only with no toxic effect. NRU also suggests the same results. On treatment with *Hypericum* 6C and 30C at different doses, same results were found as *Bellis* in MTT and NRU suggesting *Hypericum* also as a safe drug for neurodegenerative disorders.

Oxidative stress is defined as imbalance between the production of ROS and antioxidative defense mechanism. Mechanism is represented by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals. Lipid peroxidation is considered to be a marker of oxidative stress. If not terminated fast enough, there will be damage to the cell membrane, which consists mainly of lipids. The end products of lipid peroxidation may be mutagenic and carcinogenic.¹⁶ It has been found that 90% alcohol has significantly increased the lipid peroxidation suggesting oxidative stress as a mechanism in alcohol toxicity. Homeopathic medicines have shown results similar to negative control leading to a conclusion that these medicines are behaving as antioxidant. The enhancement of LPO may cause subsequent depletion in the content of GSH, which is often considered the first

line of defense against endogenous antioxidants. The loss of GSH in the brain results in various antioxidant dysfunctions including its dependent enzymes^{17,18} plasma membrane integrity, ATP levels^{19, 20} and Na⁺/K⁺-ATPase activity.²¹

GSH, a tripeptide binds with toxicants or exogenous substances in the presence of glutathione-S-transferase and facilitates its release from the body. GSH level was significantly depleted in positive control group suggesting toxic effect of alcohol on cells. Both homeopathic drugs have shown significant increase in GSH content than the negative control suggesting the beneficial effect of these medicines on the brain cells. GSH is converted to GSSG, which is reconverted to GSH by GR, thus maintaining the pool of GSH, which, in conjunction with the nicotinamide adenine dinucleotide phosphate (NADPH), can reduce lipid peroxidase, free radicals, and H₂O₂. The observed depletion of GSH, glutathione dependent enzymes (GPx and GR), suggest toxic effect of alcohol (90%). In contrast, all these enzymes were significantly exalted as compared to the positive control.

GPx, the major antioxidant enzyme, has the main function of reduction of lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water. GPx play an important role in the prevention of ROS-induced neuron damage.²² The decreased activity of GPx in case of positive control suggests induction of ROS and its overproduction in PC12 cells. *Be* (6C and 30C) showed significant elevation of GPx as compared to positive control. GPx levels were in fact better than the negative control. Same results were shown by *Hy* but 30C was better than 6C. Glutathione reductase is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant. In case of positive control its activity was depleted significantly and was restored by both the homeopathic drugs.

Cholinergic transmission is important for learning and memory processes, which require ACh for their proper functioning. Reduction of ACh below its normal level may lead to deficits in cholinergic neurotransmission, which is considered one of the main causes of cognitive deficits.²³ Acetylcholine (ACh) is necessary for memory formation and retrieval. Its synthesis depends on the availability of acetyl Co-A, provided by the breakdown of glucose and insulin, which controls the activity of AChE, a hydrolyzing enzyme for ACh. Traditionally AChE has been considered as best marker of neurotoxicity. This study shows significant increase of AChE activity in positive control. Different homeopathic medicines have restored the AChE activity in neurons.

It is obvious that Na^+/K^+ -ATPase activity is inhibited by free radicals during neurodegeneration as is also observed in our study.^{24,25} Thus, it can be speculated that alcohol treatment in cells causes an increased generation of free radicals, which may in turn lead to oxidative damage and ultimately to a decrease in Na^+/K^+ -ATPase activity. Supplementation by different homeopathic drugs has shown significant protection on cells as compared to positive control.

Monoamine oxidases (singular abbreviation MAO) are enzymes that catalyze the oxidation of monoamines. They are found bound to the outer membrane of mitochondria in most cell types in the body. The enzyme activity was markedly decreased by alcohol treatment in differentiated PC12 cells. It was suggested that alcohols can act as substrate analogues for MAO, and that the competitive nature of the inhibition of MAO activity by ethanol may be due to this capability. In contrast, homeopathic medicine treatment showed significant increase in MAO activity suggesting the protective role of these drugs.

Thus, these results suggest that alcohol treatment leads to oxidative stress while the homeopathic drugs have beneficial effects on cells as seen in negative control.

Conclusion

In the present study effect of Be and Hy was investigated, on healthy neuronal cell line. It was concluded that both these medicines were non toxic to cells even at higher concentration of 8 $\mu\text{l}/\text{ml}$. The results suggest that these medicines could be effectively used as preventive drugs for neurological disorders.

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Conflict of interest

The authors have no conflict of interest and certify hereby that this work has never been published. All the authors have fully consented to submit this manuscript in this journal.

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