

Original Article

Dynamized Preparations in Cell Culture

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Although reports on the efficacy of homeopathic medicines in animal models are limited, there are even fewer reports on the *in vitro* action of these dynamized preparations. We have evaluated the cytotoxic activity of 30C and 200C potencies of ten dynamized medicines against Dalton's Lymphoma Ascites, Ehrlich's Ascites Carcinoma, lung fibroblast (L929) and Chinese Hamster Ovary (CHO) cell lines and compared activity with their mother tinctures during short-term and long-term cell culture. The effect of dynamized medicines to induce apoptosis was also evaluated and we studied how dynamized medicines affected genes expressed during apoptosis. Mother tinctures as well as some dynamized medicines showed significant cytotoxicity to cells during short and long-term incubation. Potentiated alcohol control did not produce any cytotoxicity at concentrations studied. The dynamized medicines were found to inhibit CHO cell colony formation and thymidine uptake in L929 cells and those of *Thuja*, *Hydrastis* and *Carcinosinum* were found to induce apoptosis in DLA cells. Moreover, dynamized *Carcinosinum* was found to induce the expression of p53 while dynamized *Thuja* produced characteristic laddering pattern in agarose gel electrophoresis of DNA. These results indicate that dynamized medicines possess cytotoxic as well as apoptosis-inducing properties.

Keywords: apoptosis – cytotoxicity – p53 – thymidine uptake

Introduction

Homeopathy is a system of alternative medicine that has been practiced for more than 200 years. The homeopathic model of treatment is based on three pillars: 'principle of similitude', 'experimentation of substances in healthy individuals' and 'dynamized medicines'. For a drug to be considered 'homeopathic', it should be potentiated (dynamized), experienced in healthy individuals and applied according to the principle of the similarity of symptoms.

In order to escape from the aggravation and intoxication caused by substances used in ponderal doses, Hahnemann started to dilute and agitate them (method of dynamization), observing that through this process the substances produced the same symptomatic manifestations. In this

way, a dynamized preparation had the same effect as that of the mother tincture and produced similar reactions in the organisms. However, many drugs prepared in this manner are diluted to such an extent that they go beyond Avagadro's number (1,2). Hence the action of homeopathic drugs, even though clinically manifested, was always debated in scientific community.

Recently, limited investigations on the efficacy of dynamized medicines in animal models as well as clinical trials have reported that potentiated *Lycopodium clavatum* has protective action against CCl₄-induced liver damage in rats (3) and that chelidonium 30C could ameliorate both *p*-dimethylaminoazobenzene and azodye-induced hepatocarcinogenesis in mice (4,5). Antigenotoxic effect of different dynamized medicines has also been reported (6,7): *Arsenicum album* was found to ameliorate arsenic-induced toxicity in mice as well as in clinical studies and could reduce the elevated antinuclear antibody titer and hematological toxicities

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(8,9). Homeopathic therapy for asymptomatic HIV carriers has also proven beneficial (10) and recently Rajendran (11) reported homeopathy as a supportive therapy in cancer. Pathak *et al.* (12) investigated Ruta 6 on regression of human glioma brain cancer cell growth clinically and found that Ruta induces severe telomere erosion in MGRI brain cancer cells but has no effect on B-lymphoid cells and normal lymphocytes. Banerji and Banerji (13) reported that Ruta was effective for intracranial cysticercosis.

Very few investigations have explored the action of dynamized medicine in *in vitro* cell culture systems. Podophyllum has been shown to inhibit adhesion of neutrophils to serum-coated micro plates (14). Traumeen S, a homeopathic formulation used clinically to relieve trauma and inflammation has been shown to inhibit the production of Interleukin- β , TNF- α and Interleukin-8 by human T cells and monocytes in culture (15). Many homeopathic drugs at low potencies were found to potentiate oxidative metabolism in cultured cells (16).

We tried to evaluate the activity of selected homeopathic medicines *in vitro* for their cytotoxic and apoptosis-inducing activity by cell culture methods. Since the medications are not being used on animals we have used the term dynamized instead of homeopathic medicines (We are indebted to a referee for this suggestion). We have also compared the effect of dynamized medicines with that of mother tinctures.

Methods

Medicines

Our choice of medicines extensively used for cancer treatment was suggested by renowned homeopathic practitioner Dr Banerjee P, Kolkata, India. The dynamized medicines and their mother tinctures were procured from Willmar Schwabe, Germany.

	Mother tincture	30C	200C
1. <i>Thuja occidentalis</i>	*	*	*
2. <i>Hydrastis canadensis</i>	*	*	*
3. <i>Lycopodium clavatum</i>	*	*	NA
4. <i>Conium maculatum</i>	*	*	*
5. <i>Carcinosinum</i>	NA	*	*
6. <i>Ruta graveolens</i>	*	*	*
7. <i>Podophyllum peltatum</i>	*	*	*
8. <i>Phytolacca americana</i>	*	*	*
9. <i>Chelidonium majus</i>	*	*	*
10. <i>Marsdenia condurango</i>	*	*	*

NA—not available

Ethanol content in the preparations may vary and no attempt was made to determine the exact content. Maximum ethanol concentration used in the experiment was 2%, which will not produce any effect on cells.

Moreover results were compared with dynamized vehicle control or non-dynamized alcohol control.

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM) were purchased from Himedia laboratories, Mumbai, India. Fetal Calf Serum was purchased from Biological Industries, Israel. MTT and ethidium bromide were obtained from Sigma Aldrich, USA. Agarose was purchased from Sisco Research Laboratories, Mumbai. Primers were purchased from Maxim Biotech, Inc. USA. All other reagents used were of analytical grade.

Cells

Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells were originally obtained from Cancer Institute, Adayar, Chennai and are maintained in the peritoneal cavity of Swiss Albino mice.

L929 cells (mouse fibroblasts) and Chinese Hamster ovary (CHO) cells were obtained from National Centre for Cell Sciences, Pune. They were grown and maintained in Minimum Essential Medium containing 10% fetal calf serum.

Cytotoxicity of dynamized medicines in cells

Trypan blue exclusion method

Tumor cells were aspirated from peritoneal cavity and pelleted by centrifugation ($1000 \times g$, for 10 min). The cells were washed with sterile phosphate-buffered saline (PBS) and counted. They were made up at a concentration of 10 million cells per milliliter. DLA and EAC cells (1 million cells/0.1 ml) were incubated with dynamized medicines (20 μ l) in a total volume of 1 ml made up with PBS. Cells were incubated at 37°C for 3 h. After incubation, 0.1 ml of trypan blue (1%) was added and cytotoxicity was determined by counting live and dead cells using a haemocytometer. Untreated cells and cells treated with the same volume of potentiated diluent were used as controls.

MTT assay

L929 cells (5000 cells/well) were seeded in 96-well flat-bottom titer plates containing 0.2 ml of the medium and allowed to adhere for 24 h at 37°C in 5% CO₂ atmosphere. Dynamized medicines (20 μ l/ml) were added and incubated for 48 h. Twenty microliters of MTT (5 mg/ml) was added at 44th hour and the incubation was continued for another 4 h. After incubation plates were centrifuged and the pelleted cells were dissolved in 0.1 ml of DMSO and optical density was measured at 570 nm.

using a plate reader. Untreated cells and cells treated with same volume of diluent were used as control.

CHO-cell colony formation

CHO cells (500 cells/plate) were plated in 25 mm petridish and incubated with Dulbecco's Modified Eagles Medium containing 10% FCS (5 ml) in the presence and absence of various dynamized medicines (20 µl/plate) for 10 days at 37°C in atmosphere of 5% CO₂. After incubation, plates were washed and fixed with 10% formaldehyde for 10 min and stained with 1% crystal violet for 10 min. Plates were washed and the colonies were counted under microscope. Untreated cells and cells treated with same volume of diluent were used as control.

H³-thymidine uptake

L929 cells (5×10^6 cells/well) were plated in 96-well flat-bottom titer plates and incubated for 24 h at 37°C in CO₂ atmosphere in MEM with 10% FCS. Dynamized medicines (4 µl each/well) were added and further incubated for 24 h. After that (*H³) thymidine (1 µCi) was added and the incubation continued for another 16–18 h. Then the plates were centrifuged and the supernatant was removed. The wells were washed with cold PBS for 3 times and 200 µl of 6N NaOH was added and incubated at 37°C for 2 h. Contents were further added into 5 ml scintillation fluid and kept overnight in dark and c.p.m. was determined using a Rack β counter (LKB-Wallac). Untreated cells and cells treated with same volume of diluent were used as control.

Induction of apoptosis

Mother tincture and dynamized medicines (200C and 30C) 20 µl/ml were added to a MEM with 10% goat serum (5 ml) containing 1 million DLA cells/ml (suspension culture) and incubated for 48 h. Further, the cells were washed thrice with PBS, centrifuged and the pellet was separated. A small portion of the pellet was suspended in PBS and cell smear was prepared on a clean glass slide and stained with haematoxylin and eosin. Apoptosis was detected by the morphologic changes (chromatin condensation, nuclear condensation and formation of apoptotic bodies).

Cells were further treated with 1 ml of lysis buffer (10 mM Tris-HCl, pH 8, 10 mM EDTA, 0.9% NaCl, 0.2% Triton X-100) for 20 min on ice and centrifuged and at 10000 r.p.m. from 10 min at 4°C. Lysates were incubated sequentially with 20 µg/ml RNase at 37°C for 60 min and 100 µg/ml Proteinase K at 37°C for 3–5 h. DNA was extracted with phenol-CHCl₃-isoamyl alcohol (25:24:1) and precipitated by adding 1/10th volume of 3.5M sodium acetate pH 5.2 and equal volume of isoamyl alcohol. DNA was placed on 1.8% agarose gel in TBE buffer in a horizontal gel support apparatus and the

DNA ladder patterns were viewed under UV light followed by photography.

Apoptotic gene expression—p53, Bcl-2 and caspase 3

Gene expression analysis was carried out by RT-PCR method. Cell to cDNA TM11 kit, Ambion Inc, USA, were used for producing cDNA from DLA cells in culture without isolating mRNA. DLA cells (1×10^4 cells/well) were seeded in the 96 well 'U' bottom titer plate using MEM with dynamized medicines 20 µl/ml and incubated for 4 h at 37°C in CO₂ atmosphere. After incubation medium was removed and the cells were washed with ice-cold PBS. Ice-cold cell lysis buffer 100 µl were added to the cells and immediately transferred to a water bath, incubated for 15 min at 75°C and transferred to 200 µl holding nuclease-free micro centrifuge tubes. To this 2 µl DNase-1/100 µl cell lysis buffer were added and incubated for 15 min at 37°C. DNase was inactivated by treating at 75°C for 5 min.

PCR was performed with primers obtained from Maxim Biotech, Inc., USA. All reagents provided in the kit were assembled in nuclease-free micro centrifuge tube according to the protocol of primer kit. This master mixture (40 µl) was mixed with 0.2 µl *Taq* DNA polymerase and 10 µl cDNA sample. Reaction mixture was vortexed and centrifuged and PCR thermal cycling was performed according to the protocol of Maxim Biotech, Inc. The PCR products (8 µl) separated by submerged agarose gel electrophoresis (1.8%) was visualized in a UV chamber and documented with gel documentation system.

Statistical analysis

Data was expressed as mean ± SD. Significance levels for comparison of differences were determined using Student's *t*-test.

Results

Comparison of cytotoxic action of mother tincture with dynamized medicines

DLA and EAC cells

Cytotoxicity of dynamized medicines and their mother tinctures to DLA cells and Ehrlich cells are given in Figs 1 and 2. Results indicated that mother tincture of *Thuja* and *Lycopodium* was highly cytotoxic to DLA cells and Ehrlich cells. Mother tincture of *Condurango* and *Hydrastis* showed less effects.

It was also noted that 200C samples of *Thuja* and *Hydrastis* showed higher cytotoxicity compared to 30C. Samples of *Conium* and *Carcinosinum* showed

cytotoxicity only after potentiation indicating that these samples increased their cytotoxic potential after potentiation. None of the others including diluent control had any apparent cytotoxicity at short incubation. Results on DLA cells and EAC cells were almost similar.

L929 cells

Most of the mother tincture showed cytotoxicity when incubated at longer period (Fig. 3). Interestingly many of

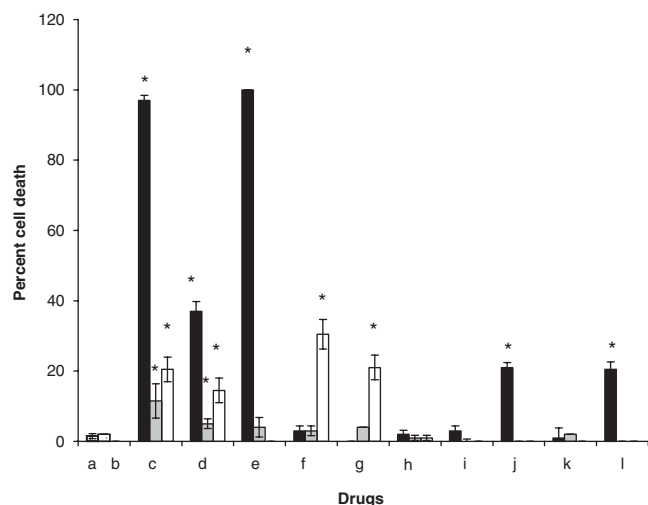


Figure 1. Effect of mother tincture and dynamized medicines on Dalton's lymphoma ascites cells. (a) Untreated Control, (b) Potentiated diluent, (c) Thuja, (d) Hydrastis, (e) Lycopodium, (f) Conium, (g) Carcinosinum, (h) Ruta, (i) Chelidonium, (j) Condurango, (k) Podophyllum, (l) Phytolacca. Filled square—Mother Tincture; Shaded square—30C; Open square—200C. * $P < 0.01$.

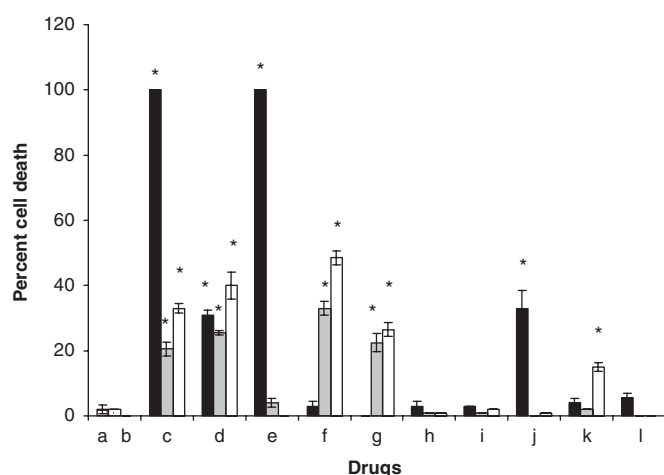


Figure 2. Effect of mother tincture and dynamized medicines on Ehrlich ascites carcinoma cells. (a) Untreated Control, (b) Potentiated diluent, (c) Thuja, (d) Hydrastis, (e) Lycopodium, (f) Conium, (g) Carcinosinum, (h) Ruta, (i) Chelidonium, (j) Condurango, (k) Podophyllum, (l) Phytolacca. Filled square—Mother Tincture; Shaded square—30C; Open square—200C. * $P < 0.01$.

the dynamized samples (30C and 200C) also showed varying degrees of cytotoxicity. *Conium* showed 31.2% cytotoxicity with 30C and 42.5% with 200C. Similarly *Carcinosinum* showed 40.5% cytotoxicity with 30C and 39.2% with 200C. In the case of *Thuja*, cytotoxicity produced at 200C was higher than 30C. Diluent control had a cytotoxicity of only 5.7%. These results point to the fact that many of the dynamized medicines induce cytotoxicity to tumor cells *in vitro*.

CHO-cell colony formation

The effect of dynamized medicines on Chinese Hamster cell colony formation is shown in Fig. 4. All the mother

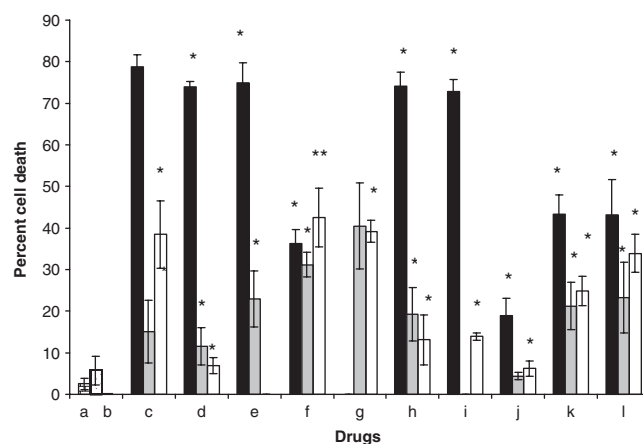


Figure 3. Effect of mother tincture and dynamized medicines on L929 cells. (a) Untreated Control, (b) Potentiated diluent, (c) Thuja, (d) Hydrastis, (e) Lycopodium, (f) Conium, (g) Carcinosinum, (h) Ruta, (i) Chelidonium, (j) Condurango, (k) Podophyllum, (l) Phytolacca. Filled square—Mother Tincture; Shaded square—30C; Open square—200C. * $P < 0.01$.

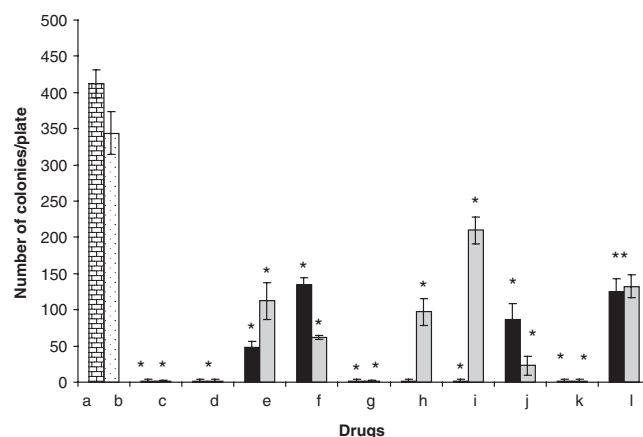


Figure 4. Effect of mother tincture and dynamized medicines on CHO cell colony formation. (a) Untreated Control, (b) Potentiated diluent, (c) Thuja, (d) Hydrastis, (e) Lycopodium, (f) Conium, (g) Carcinosinum, (h) Ruta, (i) Chelidonium, (j) Condurango, (k) Podophyllum, (l) Phytolacca. Filled square—Mother Tincture; Shaded square—30C; Open square—200C. * $P < 0.01$.

tinctures inhibited colony formation. Mother tinctures of *Thuja*, *Hydrastis*, *Ruta*, *Podophyllum* and *Chelidonium* showed 100% inhibition of colony formation. *Conium* (61%), *Lycopodium* (87%), *Condurango* (75%) and *Phytolacca* (64%) showed moderate activity. Interestingly, 200C preparation of many dynamized medicines also inhibited the colony formation. *Thuja*, *Hydrastis* and *Carcinosinum* inhibited the colony formation by 100%, while *Lycopodium* (68%), *Conium* (82%), *Ruta* (72%), *Condurango* (94%) and *Phytolacca* (62%) showed significant activity. As in the other studies 200C of *Conium* inhibited the colony formation more than mother tincture. Diluent control did not show any inhibition of colony formation.

Inhibition of thymidine uptake

The effect of dynamized medicines on the thymidine uptake during L929 cell proliferation is shown in Fig. 5. All the mother tinctures (except *Phytolacca*) inhibited the thymidine uptake significantly. Inhibition of thymidine uptake in many cases was more than 95%. Dynamized preparation of *Thuja* (30C and 200C) produced nearly 43%, *Hydrastis* 40%, *Conium* produced more inhibition at 200C. *Ruta* 30C produced inhibition of 50%. Other potentiated preparations did not produce any inhibition of thymidine uptake.

Dynamized medicines and the induction of apoptosis

Morphology and DNA laddering

The effect of dynamized medicines on induction of apoptosis is given in Table 1. Results indicate that most

of the mother tinctures as well as some potentiated preparations induced apoptosis as seen by their morphological features. DNA isolated from cells treated with potentiated preparations produced typical DNA laddering after electrophoresis indicating the presence of apoptotic cells (Fig. 6).

Expression of apoptotic genes

The expression of proapoptotic genes p53, Caspase 3 and antiapoptotic gene Bcl-2 was checked with *Ruta* 200C, *Thuja* 200C and *Carcinosinum* 200C. Although there was no expression of p53 and Caspase 3 when incubated with *Ruta* and *Thuja*, *Carcinosinum* induced significant

Table 1. Induction of apoptosis by mother tincture and dynamized medicines

Dynamized medicines (20 µl/ml)	Induction of apoptosis		
	MT	30C	200C
<i>Thuja</i>	+	+	+
<i>Hydrastis</i>	+	+	—
<i>Conium</i>	—	—	—
<i>Lycopodium</i>	+	+	ND
<i>Carcinosinum</i>	ND	+	+
<i>Ruta</i>	+	—	+
<i>Chelidonium</i>	+	—	—
<i>Condurango</i>	+	ND	—
<i>Podophyllum</i>	+	—	—
<i>Phytolacca</i>	+	—	—

Diluent control did not produced any apoptosis. MT, mother tincture; ND, not determined.

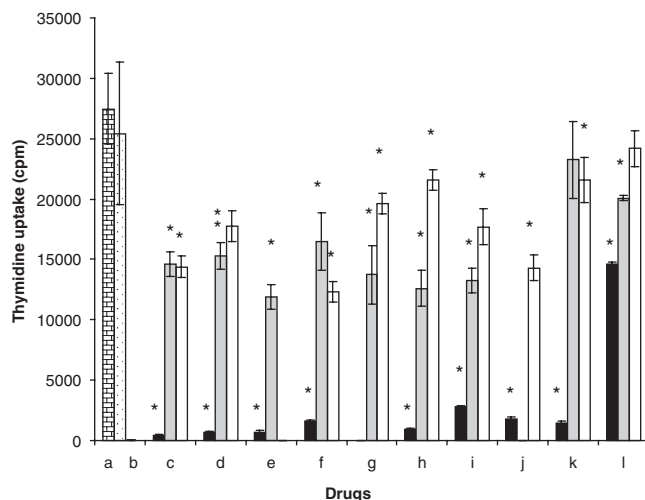


Figure 5. Effect of mother tincture and dynamized medicines on thymidine uptake. (a) Untreated Control, (b) Potentiated diluent, (c) *Thuja*, (d) *Hydrastis*, (e) *Lycopodium*, (f) *Conium*, (g) *Carcinosinum*, (h) *Ruta*, (i) *Chelidonium*, (j) *Condurango*, (k) *Podophyllum*, (l) *Phytolacca*. Filled square—Mother Tincture; Shaded square—30C; Open square—200C. * $P < 0.01$.

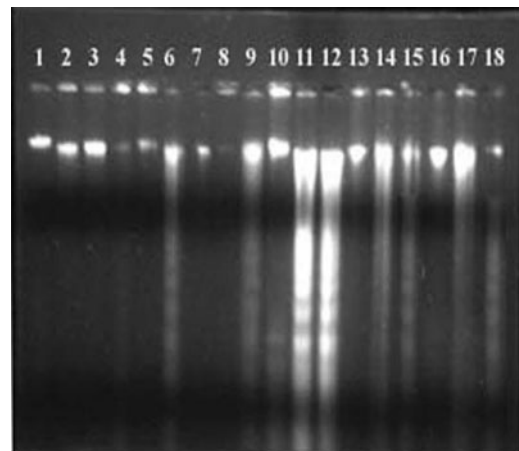


Figure 6. Effect of mother tincture and dynamized medicines on DNA laddering. Lane 1: Control. Lane 2: *Hydrastis* MT. Lane 3: *Hydrastis* 30C. Lane 4: *Hydrastis* 200C. Lane 5: *Lycopodium* MT. Lane 6: *Lycopodium* 30C. Lane 7: *Conium* MT. Lane 8: *Conium* 30C. Lane 9: *Conium* 200C. Lane 10: *Thuja* MT. Lane 11: *Thuja* 30C. Lane 12: *Thuja* 200C. Lane 13: *Chelidonium* MT. Lane 14: *Chelidonium* 30C. Lane 15: *Chelidonium* 200C. Lane 16: *Ruta* MT. Lane 17: *Ruta* 30C. Lane 18: *Ruta* 200C.

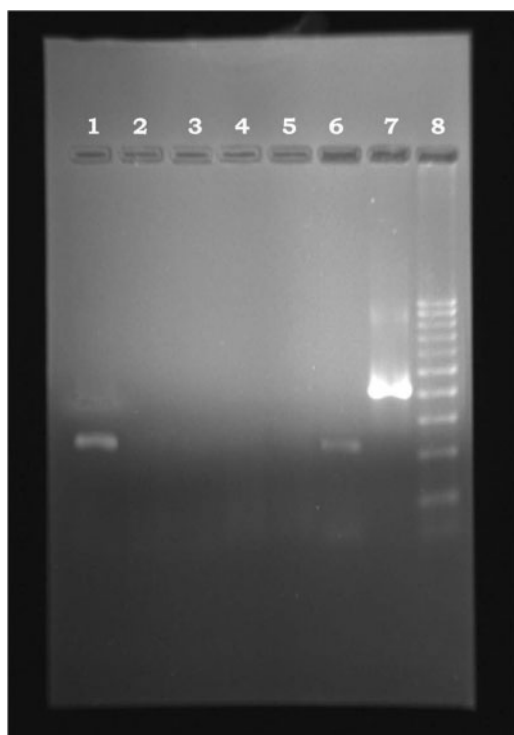


Figure 7. Effect of dynamized medicines on p53 expression. Lane 1: Positive control. Lane 2: Untreated control. Lane 3: Succussed alcohol control. Lane 4: Thuja 200C. Lane 5: Ruta 200C. Lane 6: Carcinosinum 200C. Lane 7: GAPDH. Lane 8: Mol. Wt markers.

expression of p53, which is a proapoptotic gene (Fig. 7). Bcl-2 gene (anti-apoptotic) was not expressed by any of the drug treatment while internal control GAPDH was expressed in all the samples.

Discussion

Although the healing potential of homeopathic drugs is widely accepted, the exact mechanism of action is still unclear. In paragraphs 63–69 of *Organon*, Hahneman describes the mechanism of action through the ‘primary action’ of the medicine (dynamized or not) and the ‘secondary and curative reaction’ of the organism: ‘Every agent that acts upon the vitality, every medicine, deranges more or less the vital force, and causes a certain alteration in the health of the individual for a longer or a shorter period. This is termed primary action. Although a product of the medicinal and vital powers conjointly, it is principally due to the former power. To its action our vital force endeavors to oppose its own energy. This resistant action is a property, is indeed an automatic action of our life-preserving power, which goes by the name of secondary action or counteraction’. We have tried to explain the mechanism of action of the dynamized preparations taking into consideration the original proposition by Samuel Hahnemann and have approached this problem by investigating the action of

dynamized drugs in various cultured cells in a systematic scientific manner.

Cytotoxic activity of a drug is often considered a first step towards elucidating its possible use against cancer and all of the drugs selected are being used by homeopathic practitioners against cancer. We found that in short-term cytotoxicity research, some of the dynamized preparations showed significant cytotoxic actions against cancer cell lines and at times the activity was higher than that of the mother tinctures. For example, *Conium* at 200C potency was more cytotoxic than its mother tincture and that the cytotoxicity induced by *Carcinosinum* was higher at 200C than at 30C potency indicating that dynamization induces the cytotoxic potential of these medications. Results were more pronounced during MTT assay in which a longer period of incubation was involved. Many dynamized preparations at potency of 200C inhibited the growth of L929 cells. Clonogenic assay using CHO cells is a standard method to determine growth inhibitory activity of the drugs and we found that dynamized preparations of *Thuja*, *Hydrastis*, *Carcinosinum* and *Podophyllum* at 200C potency almost completely inhibited the CHO colony formation. As in other cases, *Conium* 200C was more active than 30C. We have confirmed the cytotoxic potential of dynamized preparations by thymidine uptake, for the marker of the inhibition DNA synthesis. As in the case other experiments, DNA synthesis was significantly inhibited by several dynamized preparations.

Cytotoxicity could be produced in cells either by necrosis or by apoptotic induction. Apoptosis, which is known as programmed cell death is highly regulated by events taking place within the cell and is highly relevant with respect to the destruction and removal of transformed cells from the body. The induction of apoptosis could be an external agent and a cascade of reactions taking place within the cell produces an ultimate cell death. Some of the events occurring during apoptosis include morphological changes in the cell, production of apoptotic bodies, damage to genetic material and finally induction of proteolytic enzymes, which produces cellular destruction. Apoptosis could be visualized by morphology and DNA laddering. In the present study, dynamized preparations induced apoptosis as observed from their morphology and DNA laddering. Moreover, dynamized preparation of *Carcinosinum* could induce the p53, which is considered to be a proapoptotic protein and involved in signal transduction pathway.

The mechanism of action of some of the homeopathic drugs has been proposed. Potentiated preparation of *Ruta* possesses protective action on normal B-lymphoid cells against H_2O_2 -induced chromosomal damage (13). Moreover, the telomere erosion was enhanced in cancer cells by treatment with *Ruta* while normal cells showed no change. Thus, the telomeres that protect individual chromosomes of cancer cells are damaged by *Ruta*, which

may be the mechanism of its therapeutic action in brain cancer (13).

The protective effect of *Chelidonium* against p-DAB-induced hepatic cancer may occur by the modulating effect of the drug on restoration of damage caused to several gene-regulated phenomena like enzyme activities and chromosomal abnormalities. This gives insight into the mechanism of action, which may be by means of interfering with the process of carcinogenesis by actively modifying actions of oncogenes or by activating tumor suppressor genes (5). Another mechanism of actions of homeopathic drugs may occur through immune modification. Benveniste (17) has shown that human basophils undergo degranulation not only at usual anti-IgE antibody doses but also at extremely high dilutions. Bastide (18) has shown the therapeutic effect of high dilution of α - β interferon and thymic hormones on cellular immunity and had good therapeutic effect in immunodepressed patients. Similarly Bentwich *et al.* (19) revealed that small amounts of antigens are capable of specific antibody response. The role of immunity in the therapeutic efficacy of homeopathic medicines has also been reviewed (20).

Our results indicate that the dynamized preparation initially produces a secondary action on cells that is in line with the original proposition by Hahnemann on the mechanism of action of medicines dynamized or not. However, our limited knowledge in this area does not fully explain the mechanism of action of all drugs that we investigated. More scientific analyses are warranted to elucidate these interesting preparations of ultra dilutions.

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