Homeopathy (2016) 105, 318–326

http://dx.doi.org/10.1016/j.homp.2016.02.004, available online at http://www.sciencedirect.com

ORIGINAL PAPER

Evaluating the anticancer activity and nanoparticulate nature of homeopathic preparations of *Terminalia chebula*

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Background: **Breast cancer is the most common cancer diagnosed among women and is the second leading cause of cancer death. Homeopathic medicines are part of the alternative medicines that are given as a supportive therapy in breast cancer. The objective of this study was to investigate the anticancer activity of commercially available homeopathic preparations of *Terminalia chebula* (*TC*) and evaluate their nanoparticulate nature.**

Methods: **Mother tincture (*MT*) and other homeopathic preparations (*3X*, *6C* and *30C*) of *TC* were tested for their effect on the viability of breast cancer (MDAMB231 and MCF7) and non-cancerous (HEK 293) cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell growth assay was performed to analyze the effect of the different potencies on the growth kinetics of breast cancer cells. *MT* and *6C* were evaluated for the presence of nanoparticles by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).**

Results: ***MT*** **decreased the viability of breast cancer (MDAMB231 and MCF7) and non-cancerous (HEK 293) cells. However, the other potencies (*3X*, *6C* and *30C*) decreased the viability of only breast cancer cells without affecting the viability of the non-cancerous cells. All the potencies, *MT*, *3X*, *6C* and *30C*, reduced growth kinetics of breast cancer cells, more specifically at 1:10 dilution at 24, 48 and 72 h. Under SEM, *MT* appeared as a mesh-like structure whereas under TEM, it showed presence of nanoclusters. On the other hand, *6C* potency contained 20 nm sized nanoparticles.**

Conclusion: **The current study reports the anticancer activity of homeopathic preparations of *TC* against breast cancer and reveals their nanoparticulate nature. These preliminary results warrant further mechanistic studies at both *in vitro* and *in vivo* levels to evaluate the potential of *TC* as nanomedicine in breast cancer.** *Homeopathy* (2016) **105**, 318–326.

Keywords: *Terminalia chebula*; Homeopathic preparations; Anticancer activity; Nanoparticles

Introduction

Breast cancer is the second leading cause of cancer death in women worldwide[1] and ranks as the first leading cause of death in India.[2] The current treatment methods for

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Received 29 June 2015; revised 8 February 2016; accepted 29 February 2016

breast cancer include chemotherapy, radiotherapy and hormone therapy. The serious side effects associated with conventional therapies have called attention towards natural products that are regarded safe and have been shown to exhibit excellent anticancer and immunomodulatory potential.[3]

Homeopathy is one of the areas of Complementary and Alternative (CAM) that has been used to treat various disease conditions including cancer. Homeopathic medicines are highly diluted preparations of a wide variety of natural products. Homeopathic drugs such as *Secale cornutum 30C*,[4] *Gelsemium sempervirens 2C*,[5] *sulfur*[6] and *Calcarea carbonica* (*1C*, *6C*, *12C*, *30C* and *200C*)[7] that were found to contain nanostructure, were reported to be effective against Papilloma in mice, cervical cancer cell line (HeLa), lung cancer cells as well as *in vitro* and *in vivo* model of breast cancer, respectively. Recently, the extremely diluted homeopathic medicines[8–13] including various plant[14–16] and mineral[17–19] based preparations were found to contain nanosized structures. Besides homeopathic remedies, bhasmas (very fine powders of metals and minerals) that form an important component of Ayurveda, have also been reported to exhibit nanoparticulate nature.[20] Few reports[8–13] have also shown the formation of nanosized superstructures of solvents prepared through ultramolecular dilutions according to homeopathic method of preparation suggesting that higher dilutions (potencies) of homeopathic medicines may exhibit higher activity.[21]

Terminalia chebula (*TC*) is a medium to large-sized tree distributed throughout Asia. It is an important medicinal plant that has found varied usage in Homeopathy and Ayurveda. Homeopathic preparations of *TC* have been prescribed by the clinicians for headache, gastrointestinal infections, indigestion, loss of appetite, dyspepsia and constipation.[22] It has been reported to exhibit variety of pharmacological[23] and biochemical properties[24] such as anti-inflammatory, anti-lipid peroxidative, antioxidant and membrane stabilizing activities.[25] It has also been reported to possess antibacterial,[26] antifungal,[27] antiviral,[28,29] anti-stress[30] and anti-diabetic[31] activities. Alcoholic extract of *TC* fruit has been reported to be effective in human (MCF7) and mouse (S115) breast cancer cell lines[32] as well as in Ehrlich Ascites Carcinoma induced breast cancer model in mice.[33]

In the present study, we have for the first time studied the effect of homeopathic preparations (*MT*, *3X*, *6C* and *30C*) of *TC* on the viability of breast cancer (MDAMB231 and MCF7) and non-cancerous (HEK 293) cell lines and have evaluated them for the presence of nanoparticles. Interestingly, *3X*, *6C* and *30C* of *TC* preparations significantly reduced the viability and growth rate of cancer cells at 1:10 dilution without affecting the viability of non-cancerous cell line (HEK 293). On the other hand, mother tincture (*MT*) reduced the viability of both cancerous and non-cancerous cells. Scanning electron microscopy (SEM) analysis of *MT* revealed the presence of a mesh-like structure whereas in transmission electron microscopy (TEM), it showed nanoclusters whereas *6C* potency showed presence of ~20 nm sized nanoparticles.

Materials and methods

Homeopathic samples

Homeopathic preparations of *Terminalia chebula* (*TC*) [*MT*, *3X*, *6C* and *30C*], were commercial preparations bought from Bakson's homeopathy, Pune, Maharashtra, India. For cell culture experiments, each potency (*MT*, *3X*, *6C* and *30C*) was diluted in Dulbecco's Modified Eagle's Medium (DMEM) as 1:10, 1:25, 1:50, 1:75 and 1:100.

Cell culture

Tissue culture plastic ware was purchased from BD Biosciences (CA, USA). The cell lines, MDAMB231, MCF7 and HEK 293, were obtained from National Centre for Cell Science (NCCS), Pune. The cells were grown in DMEM supplemented with 2 mM L-glutamine, 100 units/ml of penicillin/streptomycin, and 10% fetal bovine serum and were incubated in a humidified 5% CO_2 incubator at 37°C.

Cell viability assay

The effect of homeopathic preparations of *TC* (*MT*, *3X*, *6C* and *30C*) and ethanol on the viability of MDAMB231, MCF7 and HEK 293 was analyzed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye. To test the effect of solvent ethanol on the viability of breast cancer and non-cancerous cell lines, pure ethanol (100%) was diluted in DMEM as 1:2.5, 1:5, 1:10 and 1:20. The cells were seeded at 1×10^5 cells/ml density in 96-well plates. After 24 h, the cells were incubated with fresh medium containing either ethanol (1:2.5, 1:5, 1:10 and 1:20) or homeopathic preparations (1:10, 1:25, 1:50, 1:75 and 1:100 dilutions of potencies in DMEM). The plates were again incubated for 24 h. Next day, MTT solution (5 mg/ml) was added to each well, followed by 4 h incubation at 37°C in 5% CO_2 incubator. The intensity of colored formazan derivative was determined by measuring optical density (OD) with the ELISA microplate reader (Biorad, Hercules, CA) at 570 nm ($OD_{570-630\ nm}$). The cell viability was expressed in terms of percentage as

$$\%\ \text{Viability} = \frac{\text{Treated cells}}{\text{Untreated control cells}} \times 100$$

Cell growth analysis

Growth kinetics of breast cancer cells was monitored by cell growth assay. MDAMB231 and MCF7 cells were seeded at a density of 1×10^5 cells/ml in 24-well plates. Next day, the cells were treated with different dilutions (1:10−1:100) of the homeopathic preparations (*MT*, *3X*, *6C* and *30C*). The cells were harvested and counted for viability using trypan blue dye exclusion method for 24, 48 and 72 h.

SEM

SEM was performed with Zeiss EVO 50 equipment. To prepare specimen, one drop of *MT* was air-dried on a glass

cover slip and mounted on a holder. Since this is non-conductive solid specimen, this was coated with an ultra-thin layer of electrically conducting gold material laid on the sample by sputter coating (EMITECH K550X). Such coating prevented the accumulation of static electric charge on the specimen during electron irradiation and also increased the signal, thus improving contrast and resolution.

TEM

The size and morphology of *MT* and *6C* were evaluated using JEOL-1210 TEM (JEOL, Tokyo, Japan) operating at 60 kV. For these measurements, these preparations were carefully placed on 200 mesh formvar-coated copper TEM grid (grid size: 97 mm) (Ted Pella, Inc., Redding, CA, USA). The excess suspension on the grid was removed using a piece of fine filter paper and the samples were allowed to air dry for 10 h prior to imaging the particles under the microscope.

Results

Effect of homeopathic preparations of *TC* on the viability of breast cancer cells

Initially, we wanted to test whether the commercially available homeopathic preparations of *TC* (*MT*, *3X*, *6C* and *30C*) could reduce the viability of breast cancer cells (MCF7 and MDAMB231). Since *MT*, *3X*, *6C* and *30C* contained 59, 91.4, 91 and 91% v/v ethanol, respectively (as mentioned on the manufacturer's bottles), we initially tested the effect of the solvent ethanol on the viability of breast cancer and non-cancerous (HEK 293) cells. The cells were treated with different dilutions of pure ethanol (1:2.5, 1:5, 1:10 and 1:20 that contained 40, 20, 10 and 5% ethanol, respectively). Interestingly, ethanol was found to be safe to the cells at 1:10 and 1:20 dilutions while it reduced the cell viability at 1:5 and 1:2.5 dilutions (Figure 1).

To test the effect of homeopathic preparations of *TC* on the viability of breast cancer and non-cancerous cell lines, each potency (*MT*, *3X*, *6C* and *30C*) was diluted in DMEM as 1:10, 1:25, 1:50, 1:75 and 1:100 where dilutions of MT were calculated to have ≤6% ethanol and that of *3X*, *6C* and *30C* had ≤9.1% ethanol. *MT* decreased the viability of both the cancerous and non-cancerous cells significantly at 1:10 dilution compared to the untreated control cells or other dilutions (Figure 2A). It reduced the viability of the cells upto 1:75 dilution. Interestingly, other homeopathic potencies (*3X*, *6C* and *30C*) did not reduce the viability of HEK 293 cells at any of the dilutions indicating their safety for non-cancerous cells. However, at 1:10 dilution, *3X* (Figure 2B), *6C* (Figure 2C) and *30C* (Figure 2D) potencies of *TC* significantly reduced the viability of both MDAMB231 and MCF7 compared to other higher dilutions as well as untreated control cells. Thus homeopathic preparations, *3X*, *6C* and *30C*, exhibited anticancer activity against breast cancer cells.

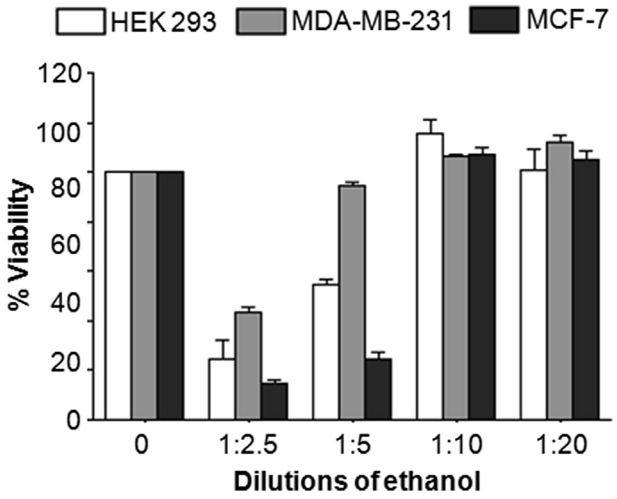

Figure 1 Effect of ethanol on the cell viability. The graph represents % viability of HEK 293, MDA-MB-231 and MCF-7 cell lines treated with different dilutions of ethanol (0, 1:2.5, 1:5, 1:10 and 1:20) for 24 h. All the data have been presented as mean ± SEM of three independent experiments. $p < 0.05$ indicates statistically significant differences compared to the control untreated group.

Homeopathic preparations of *TC* decreased the growth kinetics of breast cancer cells

Since *TC* potencies exhibited cytotoxicity against breast cancer cells, we wanted to observe their effect on the growth kinetics of the cells. MDAMB231 (Figure 3) and MCF7 (Figure 4) cells were treated with 1:10−1:100 dilutions of *MT*, *3X*, *6C* and *30C* potencies of *TC* for 24, 48 and 72 h. It was observed that at 24, 48 and 72 h, all the potencies significantly reduced the growth kinetics, more specifically at 1:10 dilution compared to either untreated control cells or with other dilutions (Tables 1 and 2). The growth reduction of breast cancer cells by *TC* potencies corroborated with the cell viability data.

Microscopic examination of homeopathic preparations of *T. chebula*

In cell viability and growth kinetics assay, *3X*, *6C* and *30C* potencies of *TC* reduced the viability and growth kinetics of breast cancer cells at 1:10 dilution. Therefore, we tested only *6C* for SEM and TEM studies. Since *MT* was the original source for other potencies and it reduced the viability of both the non-cancerous and cancerous cells upto 1:75 dilution, we also tested it for the presence of nanoparticles. *MT* showed a mesh-like structure under SEM on a scale bar of 20 μm (Figure 5A). However, under TEM, *MT* demonstrated the presence of nanoclusters (Figure 5B) that were not clear in SEM (Figure 5A) due to the latter's limited resolution. On the other hand, *6C* showed presence of distinctly separated nanoparticles having ~20 nm size (Figure 5C).

Discussion

In the recent times, various types of Complementary/Alternative Medicines are being used by cancer patient's

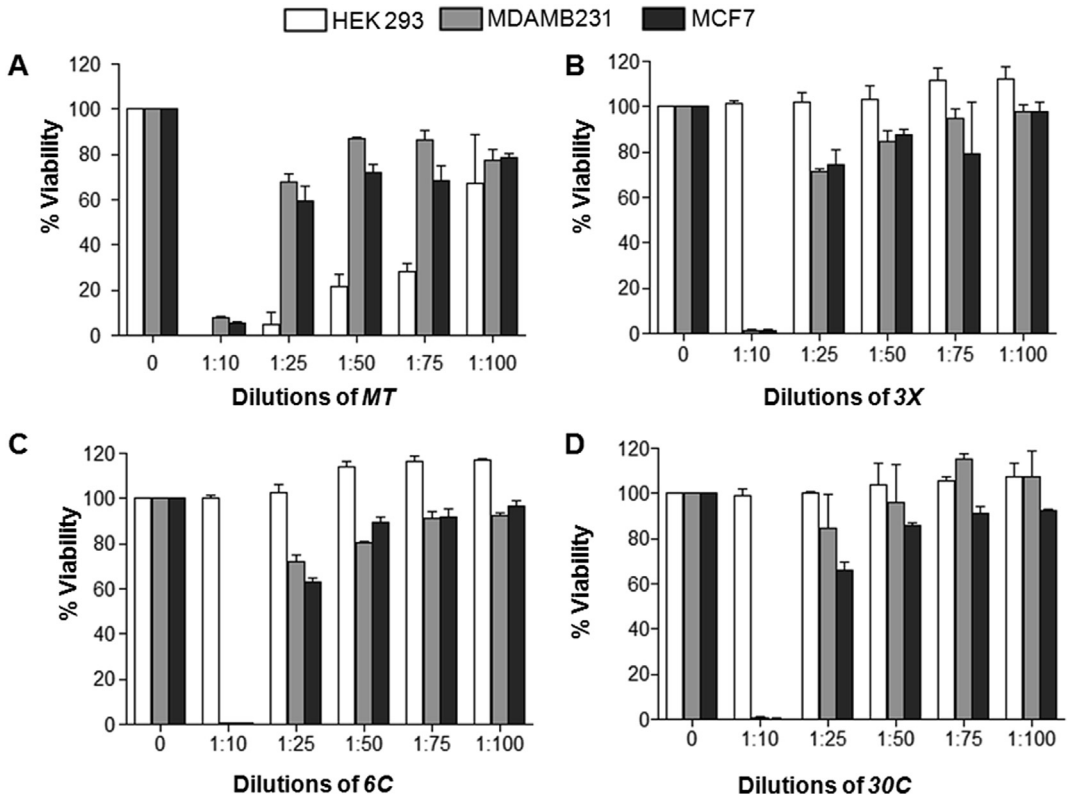

Figure 2 Effect of homeopathic preparations on cell viability. The graph represents % viability of HEK 293, MDAMB231 and MCF7 cell lines treated with different dilutions homeopathic preparations of *MT*, *3X*, *6C*, *30C* of *TC* in Figures A–D, respectively. All the data have been presented as mean ± SEM of three independent experiments, each performed in triplicates. $p < 0.05$ indicates statistically significant differences compared to the control untreated group.

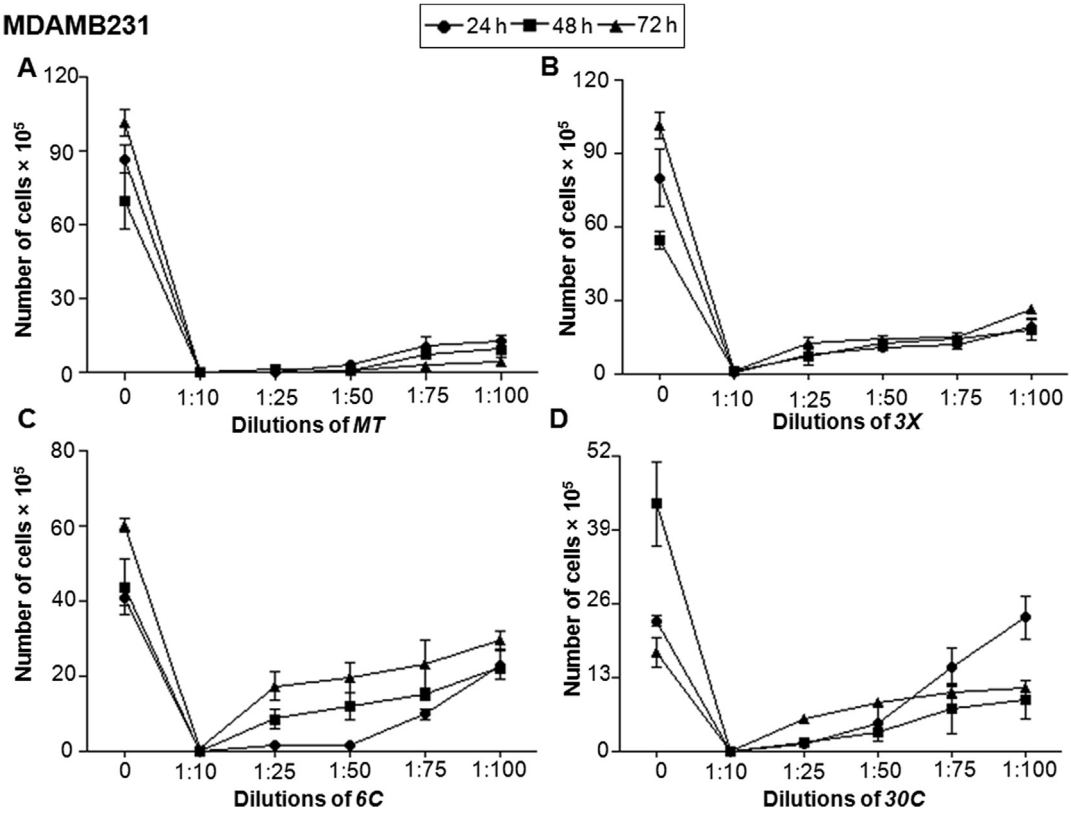

Figure 3 Effect of homeopathic preparations on growth kinetics of MDAMB231. Line graphs represent decrease in the number of MDAMB231 cells upon treatment with homeopathic preparations of *MT*, *3X*, *6C*, *30C* of *TC* in Figures A–D, respectively, for 24, 48 and 72 h by cell growth assay. The data represents mean ± SD of three independent experiments at $p < 0.0001$, indicating statistically significant differences compared to the control untreated group.

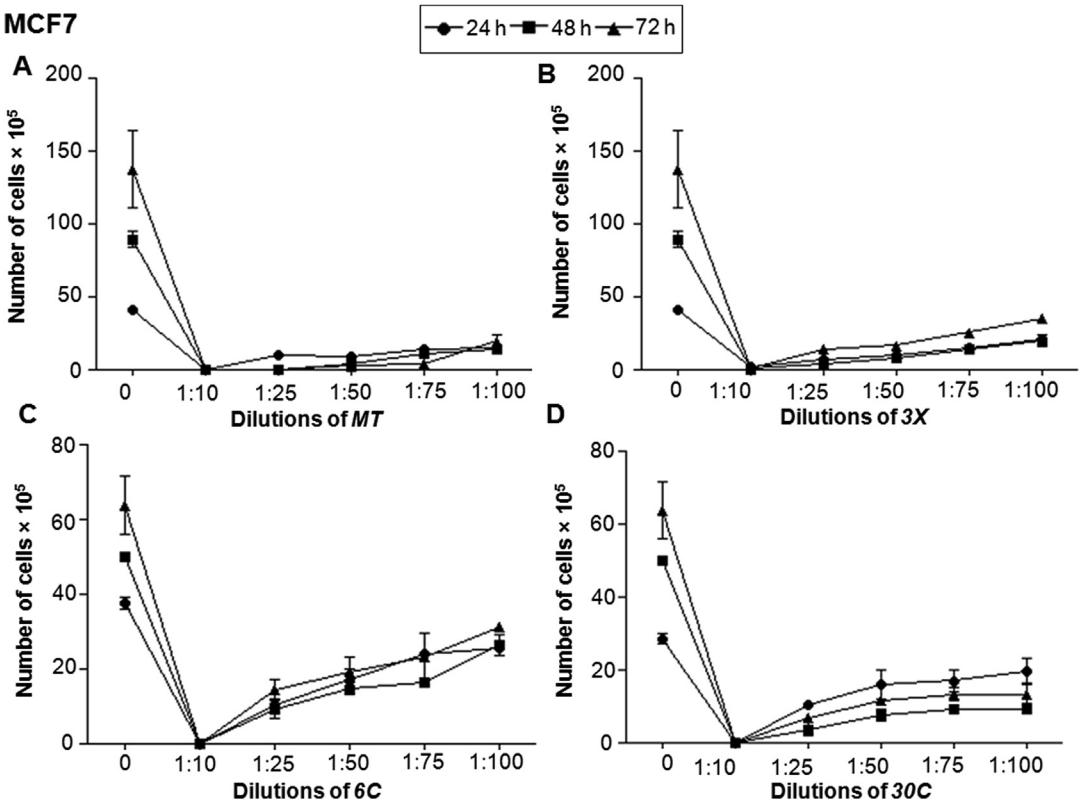

Figure 4 Effect of homeopathic preparations on growth kinetics of MCF7. Line graph represents decrease in the number of MCF7 cells upon treatment with homeopathic preparations of *MT*, *3X*, *6C*, *30C* of *TC* in Figures A–D, respectively, for 24, 48 and 72 h by cell growth assay. The data represents mean ± SD of three independent experiments at $p < 0.0001$, indicating statistically significant differences compared to the control untreated group.

along with the conventional treatments for improvement in overall Quality of Life (QOL). Homeopathic medicines are also being used as adjunct to conventional therapies by many cancer patients that have shown to improve their QOL.[34–36]

In the present study, the homeopathic preparations of *TC* (*MT*, *3X*, *6C* and *30C*), were tested for their effectiveness against breast cancer cells. Since *MT*, *3X*, *6C* and *30C* potencies contained 59, 91.4, 91 and 91% v/v ethanol, respectively, it was important first to evaluate the effect of various dilutions [1:2.5–1:20] of ethanol on the viability of breast cancer and non-cancerous cell lines. Interestingly, ethanol was found to be safe for the cells at 1:10 and 1:20 dilutions. For testing the effect of *TC* potencies (*MT*, *3X*, *6C* and *30C*) on cell viability and growth kinetics, each potency was diluted in cell culture medium [1:10, 1:25, 1:50, 1:75 and 1:100] which resulted into further dilutions of ethanol wherein dilutions of *MT* contained ≤6% ethanol and that of *3X*, *6C* and *30C* contained ≤9.1% ethanol. In spite of dilutions (in DMEM), the potencies *3X*, *6C* and *30C*, were significantly effective against breast cancer cells at 1:10 dilution and non-toxic to the non-cancerous cells, except for *MT*, which decreased the viability of the non-cancerous cells as well. Thus whatever effect of the potencies was observed on the breast cancer cells was purely due to the active principle present in them. Interestingly, *MT*, upon dilutions (in DMEM), contained less ethanol than other potencies and was toxic to all the cells thereby suggesting that its active principle was in higher amounts that could not be tolerated by the cells. These data reflected the effects of the homeopathic verum material and not the solvent ethanol in the remedy. The higher dilutions (1:25–1:100) of the potencies in DMEM, did not show appreciable anticancer effect since these were simple dilutions in culture medium and not potentized, resulting into loss of the active principle. Another important finding of the study was that *TC* could target both ER/PR positive (MCF7) and negative (MDAMB231) cancer cell phenotypes, the latter being insensitive to most of the chemotherapeutic drugs.[37]

Homeopaths usually suggest higher potencies of their formulations to their patients. In fact, many studies of nanoforms of herbs, nutraceuticals, vaccines and drugs have suggested that doses should be 10 to 1000 times lower than the typical bulk doses to produce direct biological effects.[38,39] In medium and high potencies of homeopathic formulation, which are commonly used in clinical practice,[40] the presence of starting material is usually negligible that could lead to the doubt of homeopathic preparations having placebo effect.[41] Owing to this fact, systematic studies on homeopathic medicines have suggested that extremely diluted homeopathic remedies contain nanosized structures.[8–13] A recent study on market samples of some metal-derived homeopathic medicines indicated that extreme homeopathic dilutions retained the starting materials in the form of

Table 1 Effect of homeopathic preparations of *T. chebula* on cell growth of MDAMB231. The table shows number of viable cells $\times 10^5$ represented as mean $\pm$ SD of three independent experiments

Dil^n	*Time*											
	24 h				*48 h*				*72 h*			
	MT	3X	6C	30C	MT	3X	6C	30C	MT	3X	6C	30C
Control	41 ± 0.25	41 ± 0.25	37.5 ± 1.5	28.5 ± 1.5	89 ± 5.5	89 ± 5.5	50.13 ± 0.63	50.13 ± 0.63	137.13 ± 26.63	137.13 ± 26.63	63.75 ± 7.75	63.75 ± 7.75
1:10	0 ± 0	1.75 ± 0.5	0 ± 0	0 ± 0	0 ± 0	0.63 ± 0.63	0 ± 0	0 ± 0	0 ± 0	0.75 ± 0	0 ± 0	0 ± 0
1:25	10.43 ± 0.08	6.75 ± 0.25	10.5 ± 1.5	10.5 ± 0.5	0 ± 0	3.50 ± 0.75	9.38 ± 2.63	3.75 ± 1.75	0 ± 0	14.25 ± 2.50	14.38 ± 2.63	6.88 ± 1.13
1:50	9.38 ± 1.63	9.75 ± 0.75	17 ± 3	16 ± 4	4 ± 0.25	7.88 ± 0.38	14.63 ± 0.88	7.63 ± 1.63	2.5 ± 2	16.63 ± 2.38	19.25 ± 3.75	11.75 ± 0.75
1:75	14.25 ± 1.5	15.25 ± 1	24 ± 1	17 ± 3	11 ± 1.75	13.88 ± 2.88	16.5 ± 0	9.25 ± 0.75	4 ± 1.25	25.63 ± 0.38	23.25 ± 6.5	13.38 ± 1.63
1:100	14.88 ± 0.38	20.75 ± 3.25	25.5 ± 0.5	19.63 ± 3.38	13.75 ± 1.25	19.63 ± 2.63	26.38 ± 2.63	9.25 ± 1.25	19.63 ± 4.38	35.38 ± 1.38	31.38 ± 0.63	13.38 ± 2.63

Table 2 Effect of homeopathic preparations of *T. chebula* on cell growth of MCF7. The table shows number of viable cells $\times 10^5$ represented as mean $\pm$ SD of three independent experiments

Dil^n	*Time*											
	24 h				*48 h*				*72 h*			
	MT	3X	6C	30C	MT	3X	6C	30C	MT	3X	6C	30C
Control	86.67 ± 5.97	80.00 ± 11.50	40.92 ± 2.16	23 ± 0.87	69.63 ± 11.38	54.63 ± 3.63	43.63 ± 7.38	43.63 ± 7.38	101.5 ± 5.50	101.50 ± 5.50	60.13 ± 1.88	17.38 ± 2.63
1:10	0 ± 0	0.5 ± 0.25	0 ± 0	0 ± 0	0 ± 0	1.375 ± 0.375	0 ± 0	0 ± 0	0 ± 0	1.125 ± 0.625	0.63 ± 0.13	0.13 ± 0.13
1:25	0.00 ± 0.00	7.75 ± 2.25	1.5 ± 0.25	1.42 ± 0.29	1.25 ± 0.00	7.38 ± 3.88	8.63 ± 2.63	1.5 ± 0.25	1.25 ± 0.00	12.88 ± 2.13	17.38 ± 3.88	5.75 ± 0.25
1:50	3.00 ± 1.00	10.63 ± 0.88	1.5 ± 0.25	4.92 ± 3.18	0.50 ± 0.25	12.63 ± 2.88	11.88 ± 3.63	3.38 ± 0.63	0.75 ± 0.25	14.13 ± 1.63	19.5 ± 4	8.5 ± 0
1:75	10.50 ± 4.00	12.00 ± 2.00	9.88 ± 1.38	14.92 ± 3.19	7.38 ± 0.88	14.13 ± 2.38	15 ± 1.5	7.5 ± 4.5	2.75 ± 0.25	15.25 ± 0.50	23.25 ± 6.5	10.38 ± 1.13
1:100	12.75 ± 2.50	19.38 ± 3.38	22.88 ± 3.88	23.58 ± 3.71	9.38 ± 3.13	17.88 ± 4.38	22.25 ± 0	9.13 ± 3.38	4.00 ± 1.00	26.63 ± 0.38	29.5 ± 2.5	11.25 ± 0.5

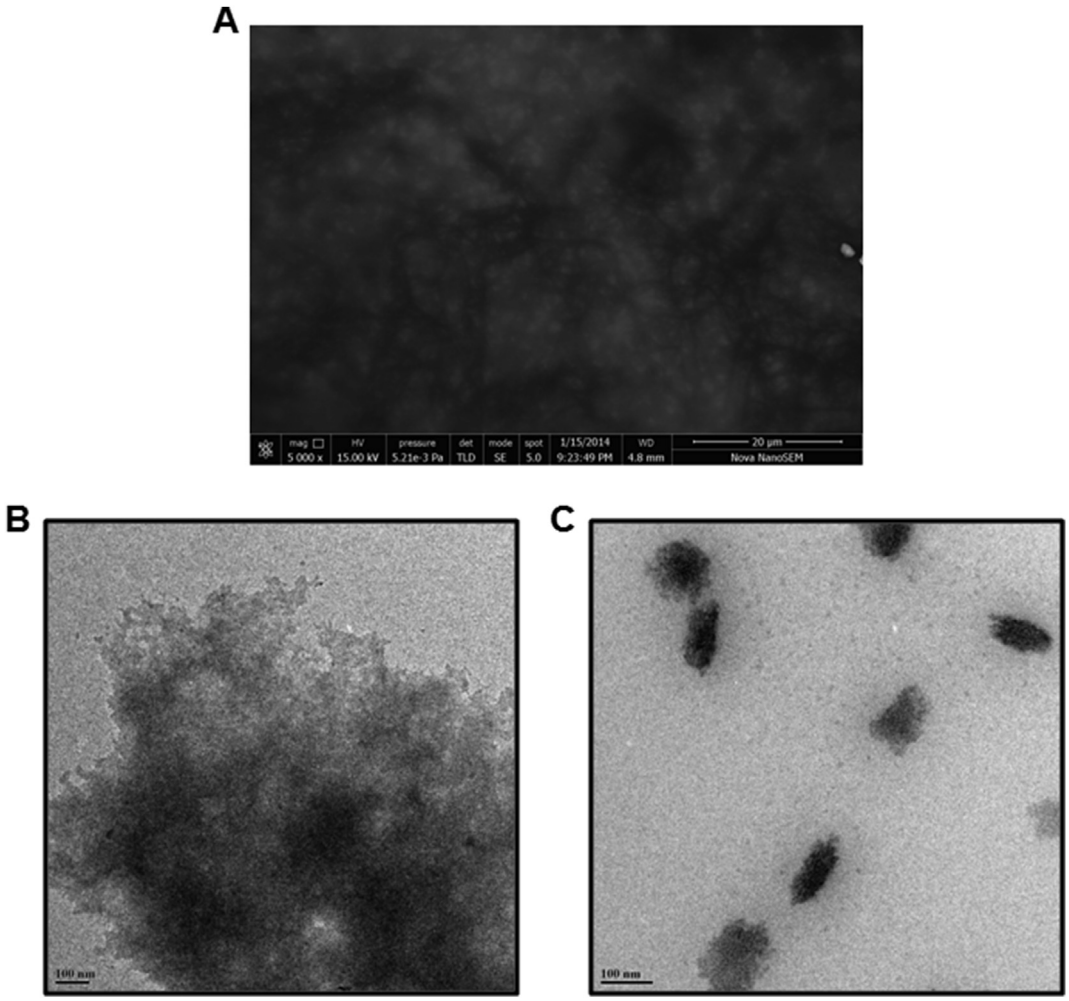

Figure 5 Microscopic analysis of homeopathic preparations of *T. chebula*. SEM image of *MT* (A); TEM images of *MT* (B) and 6C (C) potencies have been shown.

nanoparticles.[11] Chikramane et al. have demonstrated that six different commercial homeopathic metal remedies from two different manufacturers, at liquid potencies of *6C*, *30C* and *200C* existed as nanoparticles.[11] The same research group had conducted physicochemical studies of ultra-diluted homeopathic solutions and demonstrated the presence of nanoparticles in the starting raw materials.[12] We also found that *MT* and *6C* existed as nanoparticles. In SEM, *MT* showed a mesh-like structure. Since *MT* is a highly concentrated homeopathic preparation, it contains all the plant components (polyphenols, terpenes, anthocyanins, flavonoids, alkaloids, glycosides, etc.) in highly concentrated form that may have contributed towards its mesh-like appearance in SEM image. However, under TEM, *MT* showed the presence of nanoclusters that were not observed under SEM that could be due to latter's low resolution. Further succussions of *MT* to *6C* may have dispersed the mesh like structure (in *MT*) to distinctly separated nanoparticles (in *6C*). The formation of nanoparticles from larger particles highlights the importance of the trituration process in the generation of nanoparticles that could be attributed to the preparation method of the potentized drug.[7,42,43]

These preliminary results suggested that homeopathic potencies of *T. chebula* hold potential as nanomedicine and their anticancer activity could be further explored against breast cancer *in vivo*.

Conclusions

The current exploratory study evaluated the anticancer activity and nanoparticulate nature of different homeopathic potencies (*MT*, *3X*, *6C* and *30C*) of *T. chebula* in breast cancer cell lines. All the potencies exhibited anticancer activity; however, *MT* was toxic to the non cancerous cells as well. Interestingly, the tested potencies (*MT* and *6C*) exhibited nanoparticulate form. This preliminary data warrants further in depth *in vivo* studies for establishing the effective potencies that could hold promise for future clinical applications.

Conflicts of interest statement

Authors declare no conflict of interest.

Acknowledgments

The authors are thankful to Bharati Vidyapeeth Deemed University (BVDU), Pune, India for providing financial assistance.

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