Indian Journal of Biochemistry & Biophysics
Vol. 59, May 2022, pp. 558-564

Cytotoxic and genotoxic assessment of wastewater on HEK293 cell line

Akanksha Verma[1], Usha Singh Gaharwar[1,2], Anurag Maurya[1] & Paulraj Rajamani[1]*

[1]School of Environmental Sciences, Jawaharlal Nehru University, New Delhi- 110 067, Delhi, India

[2]Swami Shraddhanand College, University of Delhi (Alipur), New Delhi-110 036, Delhi, India

Received 09 March 2022; *revised 19 April 2022*

The increasing industrialisation and urbanisation have deteriorated the quality and quantity of water bodies, harming the surrounding flora and fauna. Therefore, in our studies, we have chosen the HEK293 cell line to examine further the level of wastewater toxicity to which living beings are exposed. The water samples were collected from various sites around the Agra Canal in the Faridabad region of Haryana. Furthermore, cytotoxicity and genotoxicity confirmation of wastewater samples were done by MTT and comet assay, respectively. The water quality of the Agra canal is heavily influenced by agricultural, domestic, and industrial waste, which may affect the genetic material of species exposed to contaminated water and the sustainability of the local environment. As a result, continuous environmental monitoring and proper policy formulation are required to minimise the adverse effects of pollutants in waste, which would further enrich India's preparation to take India a step ahead, and that could be the best possible way to commemorate India's 75th year of Independence with the Azadi Ka Amrit Mahotsav.

Keywords: Cell morphology, Comet assay, Health hazard, MTT assay, Water pollution

Pollution-causing activities have considerably altered aquatic habitats over India's 75-year development phase. Contamination of water reserves owing to anthropogenic wastes has become a severe concern in metropolitan cities, leading to changes in water composition that will undoubtedly have negative consequences for the species that dwell in these water bodies and health problems for humans[1]. The discharge of heavy metals in water bodies from agricultural land, domestic waste, municipal solid waste, and industrial waste get bioaccumulated in the marine organism has been reported in a previous study conducted on the southeast coast of India[2]. Urban garbage contributes the most to the cumulative genotoxic burden imposed on ecosystems. It includes pharmaceuticals, endocrine disruptors (EDCs), and other substances among the micropollutants found in wastewater that contribute to the most cumulative genotoxic burden imposed on ecosystems. As a result, there has been a surge in interest in learning more about the possible human health impacts of using reclaimed water containing known and undiscovered micropollutants directly or indirectly[3]. Significant health risks for humans include fertility issues and changes in the cellular, metabolic, and DNA level that has been documented among the lethal and non-lethal effects of this hazardous wastewater[4].

It is essential to assess the toxicity of reclaimed water in the natural and anthropogenic aquatic ecosystem, including wastewater-effluent-dominated streams. Bioassay using a cultured cell line is an effective method for determining the toxicity of hazardous substances. This approach is highlighted as a simple and less time-consuming toxicity monitoring technique for water body[5]. The impacts of wastewater and treated wastewater on different human cell lines have been reported in multiple studies. Furthermore, the cytotoxic and genotoxic nature of wastewater, drinking water, and surface water samples were examined on HepG2 cells[6-8]. Likewise, human Caco-2 cells were used to investigate the effects of wastewater generated from the textile industry and its cytotoxic and stress response disruption[9]. The impact of reclaimed wastewater on MCF-7 and Caco-2 cell lines in terms of cytotoxicity and estrogenicity was also studied[10,11]. This work evaluated wastewater samples' cytotoxic and genotoxic nature on the HEK293 cell line (Human embryonic kidney 293). Wastewater sampling was done from the Agra canal on the outstretch of the National Capital Region (NCR) of Delhi. NCR of Delhi is known for its high pollution level due to increased urbanisation and industrialisation, which motivates us to choose Agra

*Correspondence:
E-mail: paulrajr@yahoo.com

canal water for *in vitro* toxicity assessment. Our study aligns with the themes of Azadi Ka Amrit Mahotsav to commemorate the 75th anniversary of India's Independence, which includes identifying and resolving the issue by playing our part as individuals or as groups and taking appropriate action to overcome a problem. Thus, the focus of this study was to assess the toxicity of diluted wastewater samples of various concentrations (20%, 40%, 60%, and 80%) on the HEK293 cells and accordingly try to implement necessary measures to reduce water pollution.

Materials and Methods

Water Sampling

Water sampling was done as mentioned by Verma *et al*. (2022)[12]. In brief, water samples were collected from eight different locations of the Agra canal in pre-cleaned plastic reagent bottles, placed in an icebox, and brought to the laboratory for further analysis. Site 1 is the Yamuna river in the Okhla region from where the Agra Canal originates. Sites 2, 3, and 4 are located around the residential area where domestic waste releases significant contaminants. In contrast, sites 5 and 6 are located around the Industrial area, where nearby industries and garbage dumps are significant sources of pollutants. Sites 7 and 8 are located on the city's outskirts, mostly nearby farmland, containing organic waste and an important source of contaminants. The graphical representation of the sites has been mentioned in our previous work[12].

Cell Culture

National Centre for Cell Science, Pune, provided human embryonic kidney 293 (HEK293) cells. Adherent cells were cultured in sterile plastic flasks in Dulbecco's Modified Eagle's medium (DMEM).

MTT

The cytotoxicity of the wastewater was analysed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), where dehydrogenase enzymes convert MTT to insoluble formazan, which is measured in this test[6]. The HEK293 cells were grown in 96-well plates in three replicates in a DMEM medium in a 5% CO_2 incubator at 37°C. Wastewater samples were filtered with 0.2-micron cellulose membrane filter paper (Millipore), and the different dosages of wastewater samples (20%, 40%, 60%, and 80% by *v/v*%) were added to the wells. The plates were incubated for 24 h at 37°C. After 24 h, 90 µL freshly prepared medium and 10 µL MTT (5 mg/mL) were added and incubated for the next 4 h. Dissolution of formazan crystals was done by adding 100 µL of DMSO, and the 96-well plates were incubated at 37°C for 30 min. Absorbance was taken under an ELISA plate reader (Bio-Rad 840) at a wavelength of 570 nm[13].

Cell viability was calculated as per the given equation[13]:

$$\text{Cell viability} = \frac{\text{Test sample}}{\text{Control sample}} \times 100$$

Comet Assay

Comet assay was performed by standardised protocol[14,15]. After treatment of cells with 20%, 40%, 60%, 80%, and control samples for 24 h in 12 well plates. The cells were harvested and fixed in glacial acetic acid and methanol in a ratio of 1:3 and then washed in PBS. In short, slides were coated with agarose (0.5%), followed by the addition of 10 µL single-cell suspensions of treatment groups. The slides were dipped in a jar of cold lysis solution and left for 2 h. It was followed by electrophoresis at 25 V, 300 mA for 20 min. Cell's DNA was neutralised by placing the slides in tris buffer, it was followed by staining of the slides with ethidium bromide. DNA damage that occurs in cells was observed with the help of a fluorescence microscope (Carl Zeiss, Germany). Furthermore, cell scoring was carried out by Comet IV software (Perceptive Instruments Ltd, UK).

Statistical Analysis

Statistical data analysis was done using one-way ANOVA and Dunnett's test from Graph pad prism 5.03. All the values were expressed as Mean ± SD. A significant difference was observed concerning their control at $P< 0.05$.

Results and Discussion

Cell morphology

The HEK293 cell line is derived from epithelial cells and is an adherent. Embryonic kidneys primarily consist of endothelial, epithelial, and fibroblast cells, so HEK293 is likely to be one of these cell types[16]. In our findings, HEK293 cell culture showed a stellar shape growth pattern in the initial days of growth, followed by a spindle form of adherence, and finally, the confluence of cells on the culture plate (Fig. 1). However, it has been speculated that they were neuronal cells based on the presence of particular mRNA and gene products. In comparison cells were

spheroid in suspension culture, and cells grown in adherent monolayer as culture seem flattened and have a wider diameter[17].

Cell viability assay (MTT)

According to a prior study, toxicological studies using human cell lines could be a better alternative when sensitivity is crucial, like in recycled water and drinking water assessment[18]. To assess toxicity at the cellular level, cell line toxicity assay has been performed on multiple cell lines like SH-SY-5Y human neuroblastoma cell line, Vero monkey normal kidney epithelial cell line, and Chang liver cell line[19].

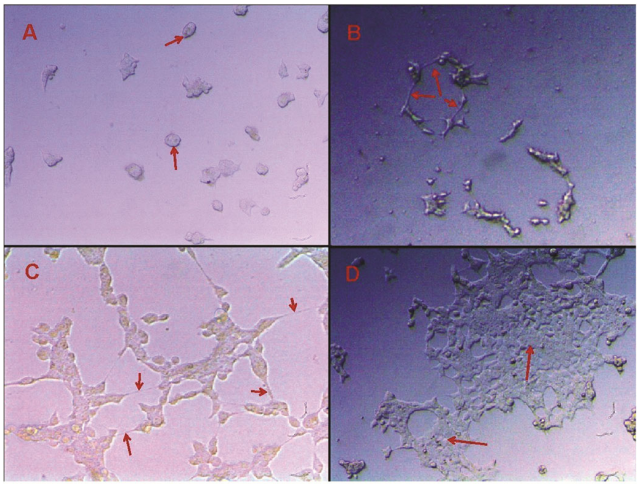

Fig. 1 — Different stages of HEK293 growth at 20x magnification. Where, (A) Arrow depicts spheroid shape; (B) Arrow represents stellar shape growth of cells; (C) Spindle shape adherence among cells; and (D) Adherent monolayer culture appear flattened cells

The result of cell viability of various treatment groups (20%, 40%, 60%, and 80%) is presented in (Fig. 2). It shows that at 20% by volume wastewater treatment on HEK293 cells, cell viability was in the range of 65-89%; at 40% doses, it was in the range of 63-87%; and at 80% doses, it was in the range of 55-74%. All sites show significantly lower cell viability than control (Milli Q) treated cells. Also, site 8 at 20% wastewater treatment shows a relatively less significant decrease in cell viability than control-treated cells. Our results align with the previous findings, which reported significantly lower cell viability at higher doses of wastewater treatment on HepG2 cells[7]. The same study reported that at 20-40 *v/v*% of industry effluent-treated cells show 50-80% cell viability. In wastewater treatment plant effluents, cell viability is 65-96%, which is in agreement with our findings.

Furthermore, cells treated with wastewater from each of the collection sites show significantly lower cell viability than control cells, which conforms with previous studies, which have reported reduced HepG2 cell viability in a maximum number of wastewater samples after 24 h exposure at 100% concentration[20].

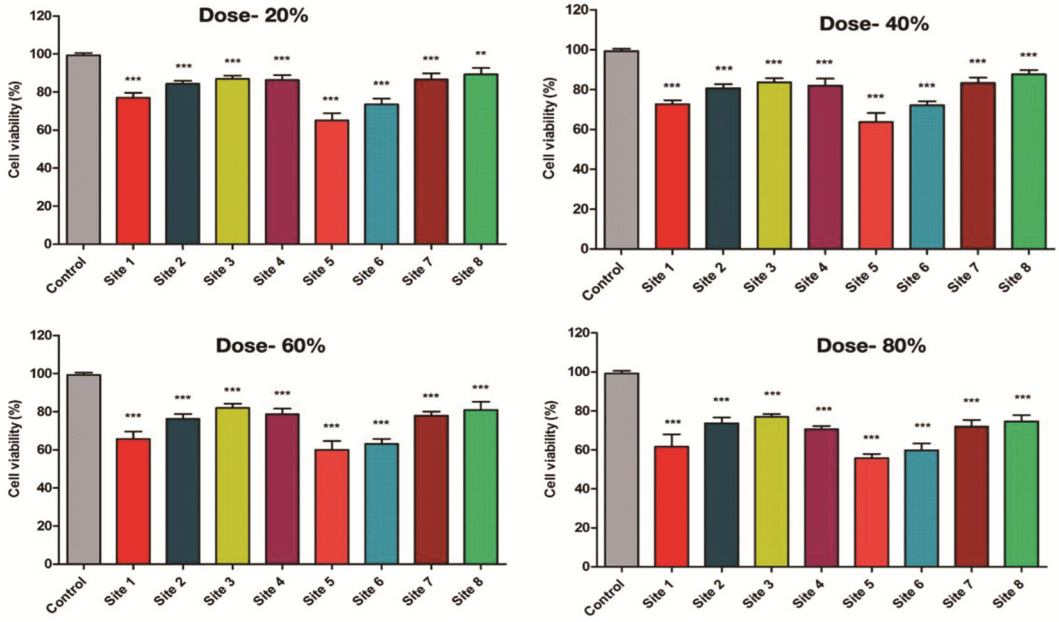

Fig. 2 — Cell viability (%) of HEK293 cells induced by the wastewater treatment of 20%, 40%, 60%, and 80% by *v/v*. Data represented as Mean ± SD. Where *** represents statistically significant ($P< 0.001$) and ** ($P< 0.01$) difference between canal water sites and control/Milli Q (one-way ANOVA, Dunnett's multiple comparison test)

Overall, results show a considerable reduction in cell viability with an increase in the concentration of wastewater, which is consistent with a previous study on Caco-2 cells. Values were statistically significant compared to control[11]. In our findings, sites 5 and 6 show very low cell viability due to their industrialised location. Similarly, site 1 shows lower cell viability, as this is the water collected from the Yamuna River, known for its high pollution level. Sites 2, 3, 4, 7, and 8 show comparatively higher cell viability due to the self-purification nature of the water body as it flows downstream. A previous study shows that wastewater effluents were genotoxic and industrial discharges were identified as the primary sources of genotoxic contaminants in wastewater, which concur with our findings[21]. Similarly, an *in vitro* study conducted on Ganga river water demonstrates the cytotoxic nature of water due to various toxic pollutants. The study illustrates the significance of using bioanalytical methods to monitor water quality and the necessity of regulating the contaminants discharged from Industrial and urban waste into the water body[22].

DNA Damage (Comet assay)

The cytotoxic results of our study revealed that site 5 is the most toxic site among all studied sites. So accordingly, site 5 is further chosen for examining the genotoxic effect by comet assay, also known as single-cell gel electrophoresis. Wastewater samples from site 5 in various dilutions (20%, 40%, 60%, and 80%) assessed for their genotoxicity. Wastewater treated and control HeK293 cell outcomes were represented by percent DNA tail and olive tail moment and shown in (Figs 3 & 4). The visual images of the comet assay have been presented in (Fig. 5). In our results, DNA damage increases in a dose-dependent basis except at 40% of wastewater, where a reduction in DNA damage is observed. According to our findings, the highest tested concentration (80%) caused a significant ($P< 0.05$) increase in DNA damage.

Similarly, a previous study revealed that at the maximum tested dose (30%), there was a significant increase in DNA damage[6]. The substantial DNA damage seen in the current research via comet assay was anticipated given the poor water quality of canal water contaminated with heavy metals due to industrial and municipal waste discharge, as evaluated in our previous research[12]. Metals such as iron, aluminium, chromium, and zinc have been shown to cause genotoxicity[23]. Likewise, the genotoxic nature of the effluents released from the paper mills causes DNA damage in human hepatocellular carcinoma (HepG2) cells mainly due to the presence of various endocrine-disrupting compounds (EDCs)[24].

As site 5 is near industrialised areas, certain mutagenic chemicals may be generated during the biological treatment of industrial wastewater, which is why the notable genotoxicity of wastewater effluents was observed[6,25]. The single-cell gel electrophoresis revealed that when cells were exposed to wastewater samples, DNA damage increased significantly more than when they were exposed to distilled water[26].

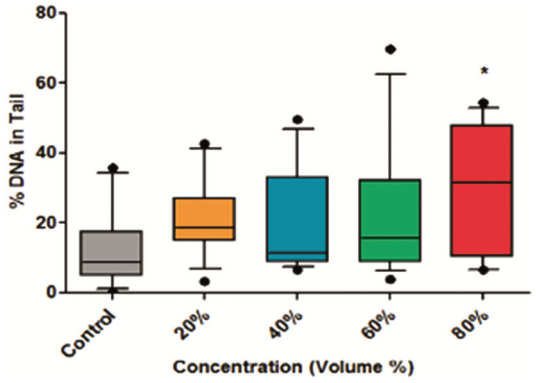

Fig. 3 — Quantile box plots show the DNA damage (% of DNA in the tail) in HEK 293 cells treated with wastewater samples at 20, 40, 60, and 80 (*v/v*%). The box's edges show the 25th and 75th percentiles, while the median value is represented by a black line inside the box. The error bars represent the 90th & 10th percentiles, respectively, while the circles denote outlying points outside the 10th and 90th percentiles, where * represents the statistically significant ($P< 0.05$) difference between doses of wastewater and control (one-way ANOVA, Dunnett's test)

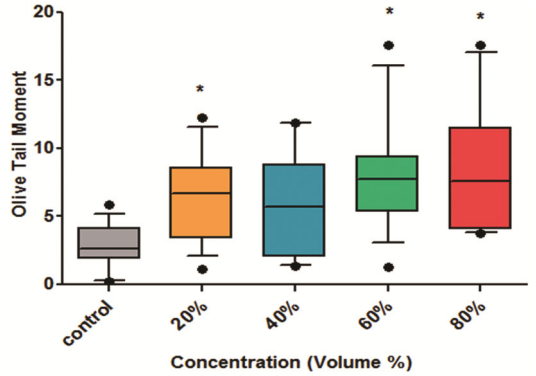

Fig. 4 — Quantile box plots show the Olive tail moment (OTM) of the wastewater-exposed HeK293 cells treated with 20, 40, 60, and 80 (*v/v*%). The 25th and 75th percentiles are shown by the box's edges, while the median value is represented by a black line inside the box. The error bars represent the 90th & 10th percentiles, respectively, while the circles denote outlying points outside the 10th & 90th percentiles, where * represents a statistically significant ($P< 0.05$) difference between doses of wastewater and control (one-way ANOVA, Dunnett's test)

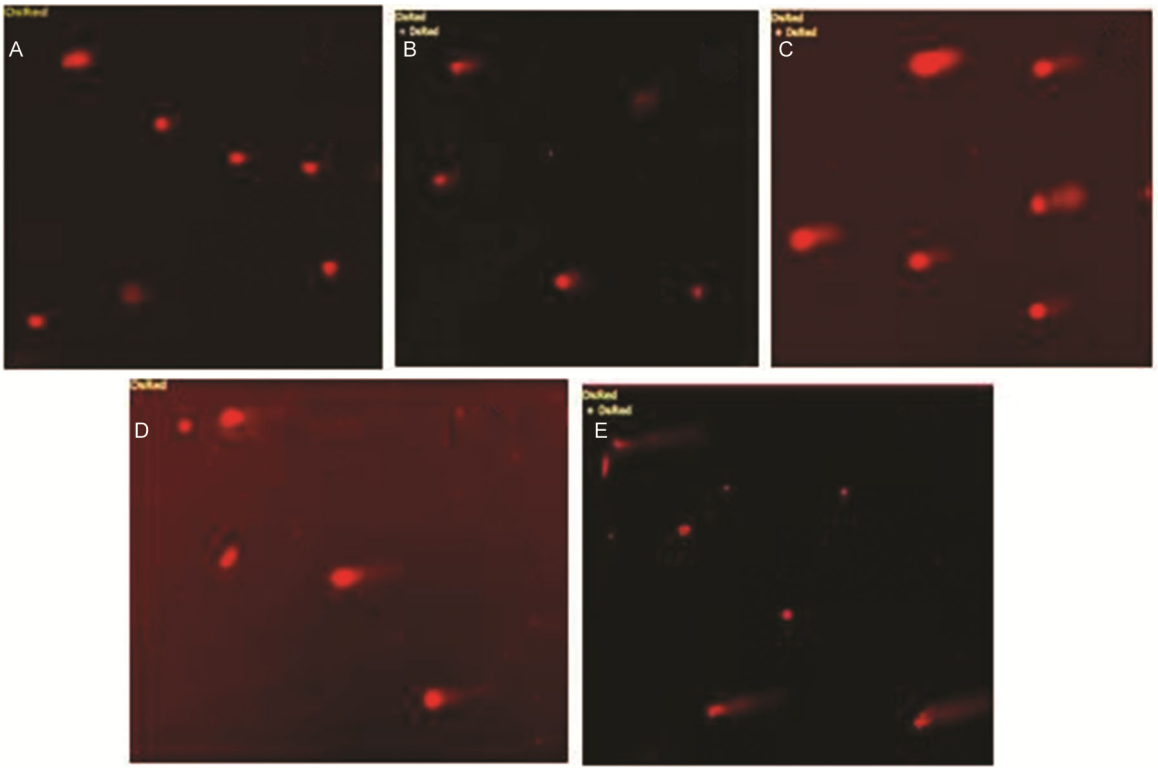

Fig. 5 — Comet images (A) control group (Milli Q water) and Wastewater treatment group by *v/v* (%) where, (B) 20%; (C) 40%; (D) 60%; and (E) 80%

Olive tail moment (OTM)

The application of the comet assay involves measuring the level of damage caused to DNA at contaminated sites in the environment compared to reference sites, and it has also been popularly utilised in the laboratory for assessing the impact of pollutant and processes involved in DNA damage[27]. To further evaluate the level of damage caused to DNA, another parameter, olive tail moment (OTM), has been examined in our study. The finding shows an increase in OTM with an increase in the dose of wastewater exposure except at 40% dose, which shows marginally lower OTM than 20% dose treatment, the possible rationale behind this is seen in a previous study, which states that DNA strand breaks detected by the Comet assay can sometimes be reversed when repaired by the cell's repair system[28]. Moreover, statistically significant ($P< 0.05$) OTM was seen at 20%, 60%, and 80% of wastewater treated cells than control. Similarly, a statistically significant increase in OTM with the rise in the toxicity of wastewater pollutants such as leachate[29]. In concurrence with our study, previous literature has shown that the Olive tail moment was statistically significant in wastewater treated effluents in blood cells than in control. Moreover, there is an increase in the Olive tail moment in a dose-dependent manner[33]. *E. gracili* cells treated with effluents and influences of wastewater treatment plants showed a significant increase in OTM compared to the control[20]. Our findings show DNA damage in the early stages of its occurrence, indicating the recent exposure to the pollutants and the possibility of repair[30]. In line with previous investigations, our result shows that comet assay was sensitive enough to identify genotoxicity in Agra canal wastewater which is caused due to the discharge of various pollutants in the waterbody. Likewise, wastewater samples collected from the sewage treatment plant (STP) located in the Vasant Kunj region in New Delhi, India, show the genotoxic nature of wastewater when exposed to HepG2 cell line, which may be caused due to the presence of various organic compounds like phenols, phthalates and polycyclic aromatic hydrocarbons (PAHs)[31]. Similarly, genotoxicity and sensitivity of comet assay in Ribeirao Tata river water samples have been reported[32]. In agreement with our findings, a study on water samples collected from Esteio and Sapucaia streams (Rio Grande do Sul; Brazil) induced DNA damage in a HepG2 cell line. These streams act as a

significant source of water supply for the surrounding population. Also, the *in vitro* study shows that urban and industrial pollutants had a similar impacts[34]. The toxicity of the pollutants released in the wastewater is not limited to human cell lines, a recent study conducted on hemocyte cells of mollusk *Biomphalaria glabrata* also showed genotoxic and cytotoxic damage when cells were exposed to domestic sewage sludge[35]. Industrial wastes, among other pollutants, are the most common contributors to water pollution and can cause biochemical and structural alterations in living species' tissues[36]. The uncontrolled discharge of contaminants poses a serious threat to humanity and the surrounding flora and fauna. To overcome this problem government of India has launched the ODF Plus program under Phase II of the Swachh Bharat Mission (Grameen), wherein ODF Plus activities will promote behavioural change among the masses along with focusing on initiatives for the safe disposal of solid and liquid waste management techniques which also forms the part of Azadi Ka Amrit Mahotsav[37].

Conclusion

The *in vitro* toxicological studies of canal water confirm that site 5 is one of the most polluted sites. Moreover, the cytotoxicity of wastewater was established by the MTT assay, which shows a dose-dependent decline in cell viability in HEK293 cells. The lowest cell viability was found at site 5, followed by sites 6 and 1. Similarly, the genotoxicity of wastewater was confirmed by comet assay, which shows an increase in DNA damage with an increase in wastewater concentration. The toxicity of wastewater collected from site 5 further confirms the presence of cytotoxic and genotoxic chemicals in canal water due to its core location amidst an industrialised town. As a result, it may be inferred that physical, chemical, and biological characterisation is essential for effective water quality regulation, and regular monitoring is required. Considering the toxicity and contamination of water, the government of India has provided a water testing facility at the village level and provided clean tap water connection to every household to commemorate 75 years of Independence in the form of Azadi Ka Amrit Mahotsav.

Acknowledgement

The author is thankful to the Council of Scientific & Industrial Research (09/263(1072)/2015-EMR-I), Government of India, for providing financial assistance in completing the work. Author (USG) thankfully Acknowledge ICMR (Indian council of medical research for providing Research Associate fellowship (Sanction number: 45/02/2018/-NAN/BMS).

Conflict of interest

All authors declare no conflict of interest.

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