Texila International Journal of Public Health
Special Issue 2023
DOI: 10.21522/TIJPH.2013.SE.23.01.Art003

Synthesis, Characterisation, and in Vitro Biocompatibility Studies of Selenium Nanoparticles Synthesized using *Hybanthus Enneaspermus* Plant Extract for Potential Biomedical Applications

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Abstract

Hybanthus enneaspermus (HE) is a traditional medicinal plant used for treating various disease conditions. Selenium nanoparticles (SeNPs) possess various properties such as anticancer, antioxidant, etc. The objective of the present study is to conduct green synthesis of selenium nanoparticles using Hybanthus enneaspermus(HE) and evaluate their biocompatibility. Leaves of HE are utilized for synthesizing SeNPs. Characterization studies of HE-SeNPs are carried out using UV spectrophotometry, FT-IR spectroscopy, and SEM. To check the biocompatibility, hemolytic assay, and Annexin V-PI assays are carried out. A change in color is observed after the addition of sodium selenite to the leaf extract. UV spectrophotometry gives a peak at 271 nm confirming the synthesis of SeNPs. FT-IR gives peaks at 3224, 1565, 1399, 1078, 784, and 717 cm^{-1} with a fingerprint of 3500 - 1000 cm^{-1}. SEM analysis shows the spherical morphology of the SeNPs. HE-SeNPs at lower concentrations cause less hemolysis. However, HE-SeNPs are found to be less biocompatible, so further studies are needed to confirm their biocompatible nature. SeNPs synthesized from HE can be ideal for biomedical applications but further studies are required to check its biocompatibility.

Keywords: *SeNPs, Hybanthus enneaspermus, green synthesis, biocompatibility.*

Introduction

In recent years nanotechnology has become a very prominent topic in the field of scientific research. Nanoparticles tend to be used in almost all fields including biomedical, biochemical, etc. Nowadays nanoparticles are even used as an alternative for drug delivery systems [1] Usually the conventional methods used for nanoparticle synthesis would be physical or chemical, but these methods have been shown to produce increased toxicity in the synthesized product. An alternative way to the physical and chemical methods for the fabrication of nanoparticles would be biosynthesis or green synthesis. Green synthesis involves the use of natural bio-reductants for the synthesis of eco-friendly nanoparticles. In green synthesis, the natural bioactive compounds found in plants and their derivatives act as excellent promotive agents in nanoparticle synthesis and this approach is considered an ecofriendly approach [2–5]. The phytochemical constituents are present in the plant’s aid and facilitate the nanoparticle synthesis. In the synthesis of nanoparticles, the bioactive compounds present in the plant not only act as a biocatalyst but also as a stabilizer [6–11]

Selenium is one of the essential trace elements required every day for the maintenance of proper body function. It is an organic compound that possesses the properties of both metals and nonmetals [12] Selenium

Received: 01.12.2023 ***Accepted: 07.12.2023*** ***Published on: 30.12.2023***
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nanoparticles (SeNPs) have gained huge importance due to their promising biological and physical attributes. The physicochemical properties vary greatly based on the methods utilized to synthesize the selenium [13–17] Both organic and inorganic selenium are biologically less active when compared to SeNPs. The extensive use of SeNPs in biological fields is mainly due to their potent properties such as antioxidant, anticancer, and antimicrobial, and also their very low toxicity [3] Selenium also plays an important role in immune system regulation, acts as a drug carrier in chemotherapy, and exhibits antioxidant activity in dietary allowance [18] [19–21]. Because of these excellent properties, selenium is used extensively in nanomedicine [22] Bioactive compounds from plants act as reducing and stabilizing agents in nanoparticles [23–25]

Hybanthus enneaspermus is one of the most important herbs in Indian medicine. It is commonly known as the 'spade flower' or 'pink ladies slipper' [26]. It grows mainly in the warmer parts of India [27] and is also found distributed in the subtropical and tropical regions of the world [28] Traditionally this herb is used to treat both simple and complicated diseases and disorders [29] such as urinary infections, diarrhea and leucorrhoea. The phytochemical screening of *Hybanthus enneaspermus* has interpreted the presence of different bioactive compounds, namely alkaloids, flavonoids, phenols, tannins, sterols, nitro compounds and amines [30, 31]. Similarly amino acids like leucine, valine and glutamic acid are found in higher proportions in the nectar of *Hybanthus enneaspermus* [32] The presence of all these bioactive compounds makes *Hybanthus enneaspermus* an excellent therapeutic in treating diseases. *Hybanthus enneaspermus* extract has been reported to possess excellent antioxidant, anti-arthritic, antispasmodic, anticonvulsant, aphrodisiac, free radical scavenging, and nephroprotective activity [33] [34].

The objective of this study is to synthesize SeNPS from *Hybanthus enneaspermus*, and to carry out characterization studies using UV spectrophotometry, FT-IR spectroscopy, and SEM. Synthesized SeNPs are also evaluted for their biocompatibility.

Materials & Methods

Collection of Hybanthus Enneaspermus Specimen

Fresh leaves of *Hybanthus enneaspermus* are collected from a herbal garden and identified by a botanist. The collected specimen is washed thoroughly with distilled water and air-dried in the shade to remove any moisture content. The dried leaf is ground into a coarse powder using a mortar and pestle and stored carefully away from moisture and direct sunlight.

Preparation of Hybanthus Enneaspermus Extract

1 g of the *Hybanthus enneaspermus* leaf powder is added to 25 mL of distilled water and boiled at 80°C for 20 mins. The mixture is then filtered using Whatmann's filter paper. The filtered leaf extract is used for the synthesis of selenium nanoparticles. To 10 ml of the filtrate, 20 ml of 50 mM sodium selenite is added and stirred continuously in a magnetic stirrer at 800 rpm for 24 hrs. The color change is observed with a gradual increase in time. After 24 hrs the solution is centrifuged at 8000 rpm for 10 mins. The centrifugation pellet obtained is lyophilized and used for further studies.

Characterization Studies

The synthesized SeNP powder is first dispersed in distilled water to carry out the characterization studies. Ultraviolet-visible (UV) spectrophotometry is carried out to confirm the presence of the synthesized HE-SeNPs and the absorbance was measured in the wavelength range of 200 - 700 nm.

To determine the presence of different functional groups in the green synthesized HE-SeNPs, Fourier Transform Infrared

Spectroscopy (FT-IR) is carried out in the wave number range of 3500 - 1000 cm^{-1}.

The size and structural morphology of the HE-SeNPs are analyzed using Scanning Electron Microscopy (SEM). The selenium nanoparticles are collected from the solution and then subjected to freeze drying. The sample is fixed in 4% paraformaldehyde solution overnight. Then the sample is dehydrated through successive graded ethanol baths (10% - 100%). The dehydrated sample is then fixed on an aluminum stub and coated with gold via a sputter coater at 37°C. Then the sample is examined under a scanning electron microscope *(JEOL JSM-IT800 FE-SEM, JEOL Ltd., Tokyo, Japan)* and the micrographs of the sample are taken.

Hemolytic Assay

To determine the amount of hemoglobin released from the blood post-treatment with green synthesized HE-SeNPs, hemolytic assay is performed based on the previously reported procedure by [35] Blood samples are collected from healthy volunteers after receiving approval from the Saveetha Dental College & Hospitals Institutional Review Board (SDCHIRB) (Ref. No.:- SRB/SDC/UG-2104/22/465). The collected blood is centrifuged at 1500xg for 5 mins to remove the plasma. The RBCs separated at the bottom are washed thrice with phosphate-buffered saline (PBS) with pH 7.4. The washed RBCs are diluted to 10% of their initial concentration using PBS. These diluted RBCs are used for hemolytic assays. The redispersed HE-SeNPs are taken in varying concentrations of 12.5, 25, 50, 100, and 200 µl/ml. 200 µl of the erythrocyte suspension is added to the samples of varying concentrations. The final volume is made up to 1 ml using PBS. An erythrocyte suspension treated solely with distilled water acts as the positive control, while an erythrocyte suspension treated solely with PBS acts as the negative control. All the contents are incubated at 37°C for 1 hr. After incubation, the contents in the tube are centrifuged at 1500xg for 5 mins. After centrifugation, the supernatant is loaded in a 96-well plate to read the absorbance at 540 nm using an ELISA plate reader.

$$Hemolysis\ (\%) = \frac{O.D\ at\ sample - O.D\ at\ Negative\ Control\ \times 100}{O.D\ at\ Postive\ Control}$$

Biocompatibility Assay

Annexin V-PI assay is utilized to determine the biocompatible nature of HE-SeNPs. Blood samples are collected from healthy volunteers after receiving approval from the Saveetha Dental College and Hospitals Institutional Review Board (SDCHIRB) (Ref. No.: - SRB/SDC/UG-2104/22/465). The blood sample is added to histopaque in equal volume and centrifuged using density gradient centrifugation at 2000 rpm for 40 mins to isolate the peripheral blood mononuclear cells (PBMCs). PBMCs are obtained as the white buffy coat above the histopaque layer.

The PBMCs are pipetted out and subjected to a biocompatibility assay. About 100 µl of green synthesized HE-SeNPs is added to the isolated PBMCs and cultured for 24 hrs in Roswell Park Memorial Institute (RPMI) media with 10% FBS (Fetal Bovine Serum) *(HiMedia RM9955 FBS, HiMedia Labs Ltd., Chennai, TN, India)*, 1% amino acid L-glutamine and 1% PenStrep. Cells without HE-SeNPs treatment are taken as control and the culturing is done in triplicate. 5µl of Annexin V fluorescein isothiocyanate (FITC) and 5µl of Propidium Iodide are added and incubated for 5 mins to strain the cells. 400 µl of 1X binding buffer is added and the cells are observed for apoptosis using a flow cytometer *(BD FASCLyric, BD Biosciences, United States)* Statistical analysis is done using BD FACSuite Software Ver. 4.1.

Results

Green Synthesis

Addition of sodium selenite to the HE leaf extracts changes the colour of the solution. The change in colour gradually increases with time indicating the synthesis of SeNPs.

UV Spectrophotometry

The UV spectrum of green synthesized SeNPs gives an intense absorption peak at 271 nm indicating the synthesis of SeNPs. Fig. 1 represents the UV spectrum of HE-SeNPs.

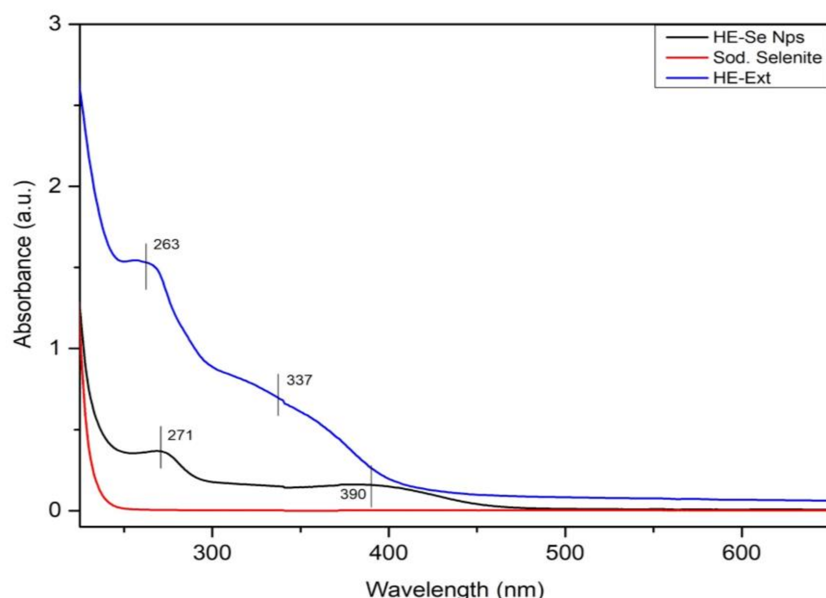

Figure 1. UV Spectrophotometry of HE-SeNPs

FT-IR Spectroscopy

FT-IR spectroscopy of HE-SeNPs gives strong peaks at 3224, 1565, 1399, 1078, 784 and 717 cm^{-1} with the fingerprint region ranging from 3500 - 1000 cm^{-1}. The peak at 3224 cm^{-1} corresponds to O-H stretching indicating the presence of alcohol or carboxylic acid group. The peak at 1565 cm^{-1} corresponds to C=C stretching indicating the presence of cyclic alkene. The peak at 1399 cm^{-1} corresponds to O-H bending indicating the presence of an alcohol group. The peak at 1078 cm^{-1} corresponds to C-N stretching indicating the presence of an amine group. The peaks at 784 and 717 cm^{-1} correspond to C-Cl stretching indicating the presence of halo compounds. Fig. 2 represents the FT-IR spectroscopy of HE-SeNPs.

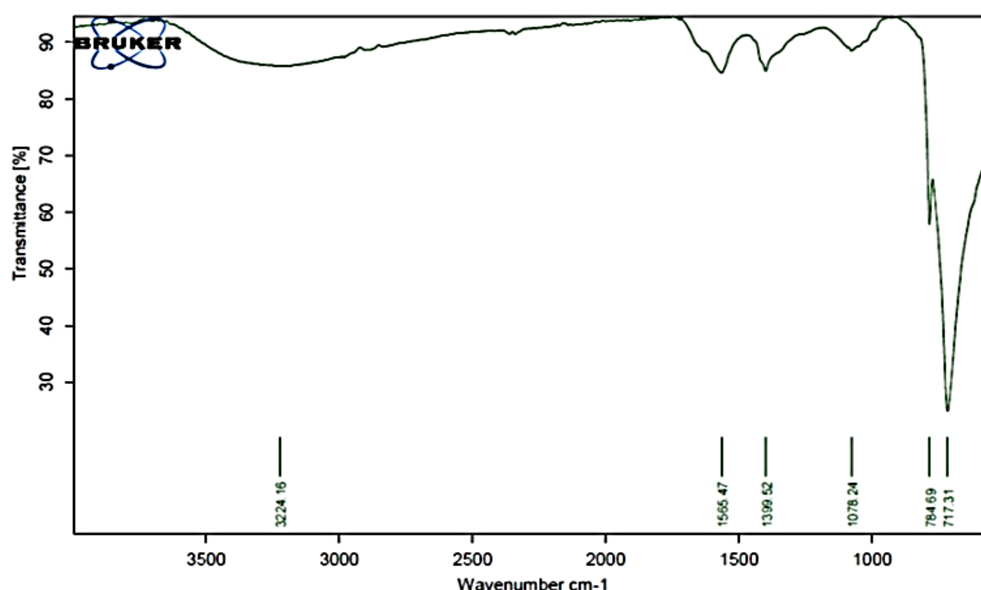

Figure 2. FTIR Spectroscopy of HE-SeNPs

SEM Imaging

The SEM analysis of HE-SeNPs shows spherical shaped Se NPs with a size range of 90 nm. Fig. 3 represents the SEM images of the HE-SeNPs. The large size spheres shown in the first image represent the capping of extract. Each sphere carries numerous SeNPs as shown in the second image.

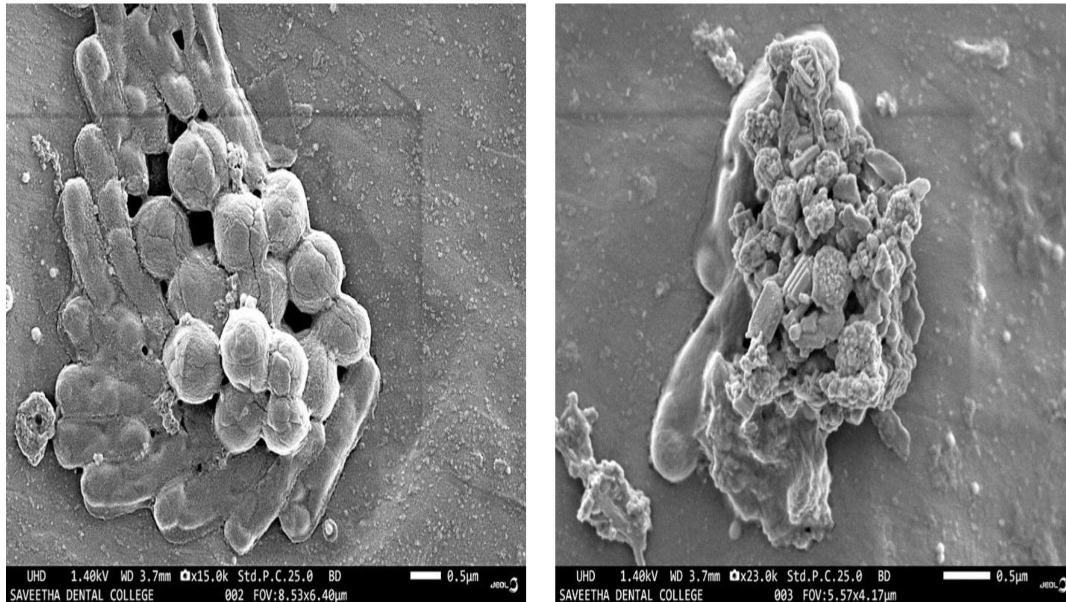

Figure 3. SEM Imaging of HE-SeNPs

Haemolytic Assay

The extend of hemolysis is directly influenced by the concentration of HE-SeNPs. The lower the concentration, the less the haemolysis. It is observed that greater amounts of haemolysis occurr in the 200 µl sample. This suggest that the concentration of HE-SeNPs increases the potential for hemolysis also increases. Fig. 4 represents the results of haemolytic assay of the HE-SeNPs.

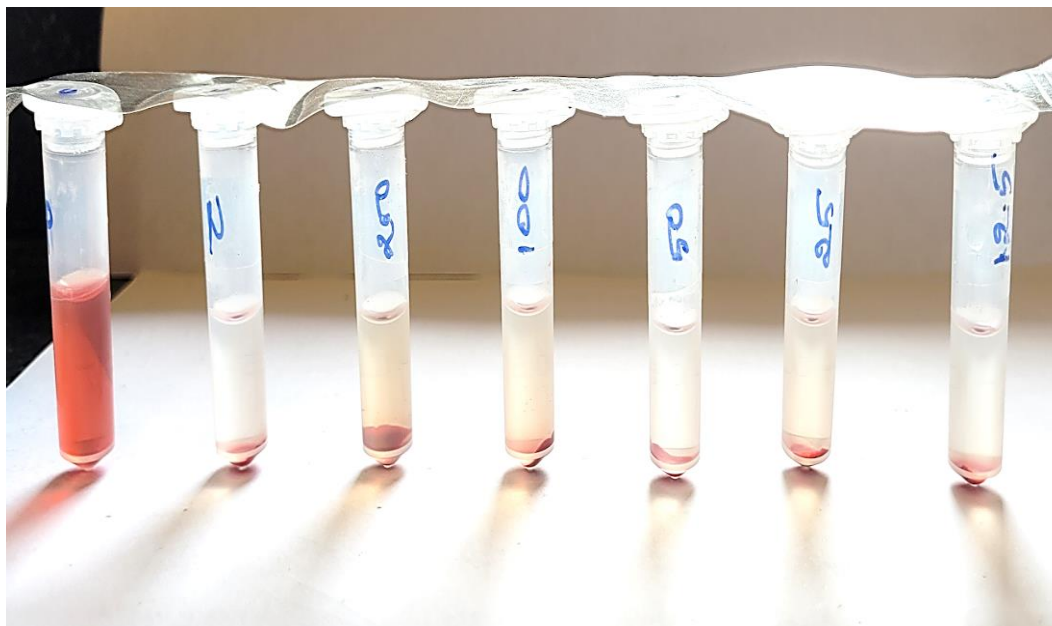

Figure 4. Haemolytic Assay of HE-SeNPs

Annexin V-PI Assay

The results of the biocompatibility assay are interpreted in four quadrants. The lower left (LL) quadrant represents the percentage of viable cells, while the lower right (LR) quadrant represents the percentage of cells in early apoptosis. The upper right (UR) quadrant represents the cells in late apoptosis, and the upper left (UL) quadrant represents the percentage of cells in necrosis. Post-treatment assessment of PBMCs with HE-SeNPs shows that 51.83% of cells are viable, 23.80% of cells are in early apoptosis, 16.77% of cells are in late apoptosis and 7.60% of cells are in the necrosis stage. Fig. 5 represents the results of the apoptosis assay of the HE-SeNPs.

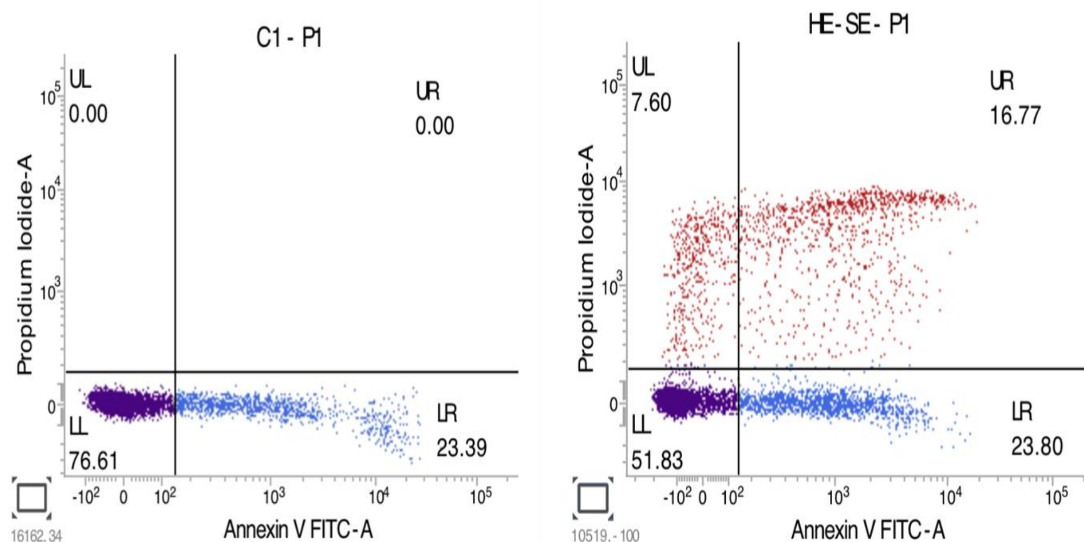

Figure 5. Annexin V-PI Assay of HE-SeNPs

Discussion

At present, nanotechnology is an emerging aspect of science that has its applications in almost all fields of life. The biocompatible nature of nanoparticles makes its applications extend to the medical field. Nanoparticles synthesized from plant extracts are ideal for biomedical uses. In this study selenium nanoparticles (SeNPs) are synthesized from *Hybanthus enneaspermus* leaf extract. Similar green synthesis of SeNPs has been previously reported in garlic [36].

In this study the UV spectrophotometry of HE-SeNPs shows an intense peak at 271 nm and this peak is concordant with the peaks obtained from the green synthesis of SeNPs from *Withania somnifera* at 320 nm [37] Similarly the FT-IR spectroscopy of HE-SeNPs gives a strong absorption peak at various regions indicating that different functional groups are present in the HE leaf extract. They act as capping and reducing agents in SeNPs synthesis. The SEM analysis of HE-SeNPs shows the spherical morphology of the nanoparticles with a size of around 90 nm.

The results of the hemolytic assay reveal that at low concentrations, minimal or no hemolysis occurs. So SeNPs at low concentrations are ideal for applications in the biological field. However, in the Annexin V-PI assay, HE-SeNPs show less biocompatibility as the percentage of viable cells is found to be only 51.83%. Hence further studies are needed to confirm the biocompatibility of the nanoparticles.

Conclusion

The synthesis of SeNPs from Hybanthus enneaspermus holds promise for potential biomedical applications, but it necessitates further experimental validation. Nonetheless, a comprehensive investigation is imperative to

precisely evaluate the full extent of its biocompatibility.

Limitations

The Annexin V-PI apoptosis assay shows that the selenium nanoparticles synthesized from *Hybanthus enneaspermus* show very little biocompatibility as the percentage of viable cells is found to be only 51.83%.

Scope of Future Research

From the results of the biocompatibility studies, we found that the percentage of viable cells is found to be only 51.83%. So further detailed studies were required to accurately assess the exact extent of the biocompatibility of the HE-SeNPs and the feasibility of using the nanoparticles in pharmaceuticals and therapeutics.

Author Acknowledgements

Aardra Binithadas Segin Chandran- Literature search, experimental data collection, analysis, manuscript writing; Hema Priya Manivannan- Study design, data verification, manuscript drafting; Dr. Vishnu Priya Veeraraghavan - Study design, data verification, manuscript drafting.

Conflict of Interest

The authors declare that there is no conflict of interest to report.

Funding

This research was funded by Kamala Dental Speciality Hospital, Thiruvananthapuram with a grant of ₹30,000/-.

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