**Antiproliferative effects of chlorogenic acid loaded chitosan nanoparticles:**

**An *in vitro* approach**

**ABSTRACT**

In recent years, chlorogenic acid has been reported to have multiple biochemical and pharmacological efficacy including anti-inflammatory and antioxidant properties. The poor solubility and low bioavailability are the major factors that reduce the therapeutic efficacy of the chlorogenic acid. The present study thus synthesized chlorogenic acid loaded chitosan nanoparticles (CACNP) using ion gelation procedure and evaluated its antiproliferative effect against Hep-2 cells under *in vitro* condition. MTT assay, ROS generation assay, MMP assay and Ao/EtBr assays are utilized to validate its antiproliferative potential in Hep-2 cells. CACNP significantly inhibited Hep-2 cell proliferation. The present study thus reveals the anticancer potential of CACNP, which open avenues for further research to assess its anticancer potential *in vivo* models.

**Key words:**  Cell proliferation, Chlorogenic acid, Chitosan, Hep-2 cell, Cell viability

**1.INTRODUCTION**

Carcinogenesis arises due to disturbances in the existing balance between the rate of cell proliferation and cell death. Cancer is a significant global public health issue and is among the leading causes of mortality. The number of deaths from cancer is projected to continue rising each year (Kashyap and Dubey, 2022). The effectiveness of conventional treatments, such as chemotherapy and radiotherapy, is limited due to their systemic toxicity (Liu et al., 2021). Consequently, there is a need for studies focusing on plant-derived compounds or their active components, which exhibit low toxicity and high selectivity in targeting cancer cells.

Hep-2 cell line, a widely recognized and utilized cell line in cancer research, originated from a laryngeal epidermoid cancer. Subsequent investigations, including isoenzyme analysis, HeLa marker chromosomes, and DNA fingerprinting revealed the presence of HeLa cell contamination and immunoperoxidase staining showed that the cells are keratin positive. American Type Culture Collection (ATCC) validated the presence of viral DNA sequences in Hep-2 cell line using PCR (Singh & Agarwal, 2006; Oğuz et al., 2023).

Reactive oxygen species play a dual role based on their concentration in the human body. While they play a vital role in apoptosis and other cell signalling pathways at physiological concentrations, over production of ROS could result in the genesis of various disorders including cancer (Huang et al., 2021). ROS mediated apoptotic induction in the cells has been widely utilized to test the antiproliferative efficacy of phytoconstituents. (NavaneethaKrishnan et al., 2019).

Chlorogenic acid, an important bioactive constituent of several medicinal plants, vegetables and fruits, possess multiple biochemical and pharmacological properties (Gupta et al., 2022). Research have explored its antioxidant, anti-inflammatory and anticancer effects in experimental animal models (Abd Elrazik et al., 2019; Li et al., 2024). However, chlorogenic acid has been reported to have low bioavailability which affects its therapeutic efficacy in experimental models. Recent studies have utilized chitosan polymer for the synthesis of nanoparticles using phytoconstituents to enhance bioavailability and ensure a sustainable release (Rosemol Jacob et al., 2024). The present study thus synthesized chlorogenic acid nanoparticles using chitosan polymer and assessed its antiproliferative efficacy against Hep-2 cells.

**2. MATERIALS AND METHODS**

**2.1 Chemicals**

For this study, Hi Media Laboratories, Sigma-Aldrich Chemicals Private Limited, and SD Fine Chemicals were the suppliers of the chemicals and biochemicals needed.

**2.2 Synthesis and characterization of chlorogenic acid loaded chitosan nanoparticles**

The synthesis of CACNP was carried out using the ionic gelation technique proposed by Calvo et al., (1997). Initially, the chitosan polymer (Mw: 1526.464 g/mol) was dissolved in acetic acid. Afterwards, chlorogenic acid was added into the chitosan solution at a 1:3 ratio. This blend was stirred non-stop for 60 min. In order to form nanoparticles, TPP was added to the chitosan-chlorogenic acid mixture in a 4:1 ratio with continuous stirring for 60 min, drop by drop. Ultracentrifugation at 15,000 rpm for 45 to 60 min was used to separate free chlorogenic acid from chlorogenic acid nanoparticles. The size of the synthesized nanoparticles was examined with a zeta sizer, while their morphology was studied using scanning electron microscopy (SEM). Following that, the lyophilized nanoparticles were utilized to assess their *in vitro* antiproliferative effects.

**2.3 Hep-2 Cells**

The Hep-2 cells was obtained from the National Centre for Cell Science, Pune, India, and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM). The cell line was maintained in the cell culture laboratory at Annamalai University.

10% Foetal bovine serum with 100 U/mL penicillin-G and 100 U/mL streptomycin was added to this medium as supplement. The studies were conducted after the confluence stage was reached, and the cells were kept in an incubator with 5% CO2 at 37ºC. Every two days, the medium was changed, and the regular procedures for maintenance were closely adhered to. Trypsin, phosphate, versene, and glucose in phosphate-buffered saline were used to dissociate the cells.

**2.4 Drug Exposure**

Hep-2 cells from log phase cultures were cultured for 24 hours at 37ºC in a CO2 incubator with different doses of CACNP (1.25, 2.5, 5, 10, 20, 30, 40, and 50 µg/mL). After dissolving CACNP in DMSO, the media's ultimate DMSO content was less than 0.1%.

**2.5 MTT assay**

The efficacy of CACNP on Hep-2 cell viability was assessed using the MTT assay by Mosmann, (1983) method. Hep-2 cells (5 × 10³ cells/100 µL) were cultured in 96-well plates and treated with varying CACNP concentrations (1.25, 2.5, 5, 10, 20, 30, 40, and 50 µg/mL) for 24 hours at 37ºC with 5% CO₂. After incubation, 10 µL of MTT (5 mg/mL) was added and incubated for 4 hours at 37ºC. Cell viability was determined by the color change from yellow to purple, indicating formazan formation by live cells. Absorbance was recorded at 570 nm using a multi-well plate reader. Cell viability (%) was calculated using the following formula:

% Cell viability = (OD value for test/OD value for control) × 100

**2.6 ROS generation**

The generation of intracellular ROS was examined using the fluorescent labeling of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Rastogi et al., 2010). In 6-well plates, the Hep-2 cells were cultured at 37ºC for 24 hours with 5% CO2. Each well contained 2×10 5 cells. After applying the IC50 value of CACNP, the cells were cultured for a whole day. Following a PBS wash, the cells were treated with 10µl of the fluorescent dye DCFH-DA and incubated at 37ºC for 10 min. Through the plasma membrane, the non-fluorescent dye DCFH-DA may readily enter a cell. After passing through the plasma membrane and being absorbed by the cells, DCFH (2',7'-dichlorofluorescin diacetate) is converted to DCF (2',7'-dichlorofluorescein) through intracellular oxidation. Stained cells were examined under a fluorescence microscope after two PBS washes. Fluorescence intensity was measured using a Shimadzu RF-5301 PC spectrofluorometer with excitation/emission filters at 485 ± 10 nm and 530 ± 12.5 nm.

**2.7 Determination of Mitochondrial membrane potential (MMP)**

The fluorescent dye rhodamine 123 is used to measure the potential of the mitochondrial membrane according to the procedure of Scaduto and Grotyohann's, (1999). Hep-2 cells (2×105 cells/well) were cultured for 24 hours at 37ºC with 5% CO2 to achieve the subconfluent stage. Hep-2 cells were treated with CACNP at an IC50 value and left to incubate for 24 hrs. The cells were then incubated with 5 mg/mL Rh-123 dye for 30 min. An aggregation of the dye-absorbed intact mitochondria produces a brilliant green and orange fluorescence. Conversely, disruption of MMP prevents accumulation in mitochondria; thus, it persists within the cytosol. The Hep-2 cells were then observed using a Floid cell image station Life technologies USA. The intensity of fluorescence was quantified at 485±10 and 530±12.5 nm using a Schimadzu spectrofluorometer.

**2.8 Acridine Orange/Ethidium Bromide (AO/EtBr) Dual Staining**

The morphological assessment of Hep-2 cell death was performed following the method of Baskic et al., (2006). Cells (2×10⁵/well) were seeded in a 6-well plate and incubated for 24 hours. Following incubation, the cells received a 24-hour treatment with CACNP. Following the treatment, the cells were removed and given three PBS washes. After staining the plate with acridine orange and ethidium bromide at a concentration of 1 mg/mL for five min, it was immediately viewed under a 200x fluorescence microscope. Acridine orange is absorbed by both living and non-living cells, while ethidium bromide is absorbed exclusively by non-living cells. When intercalated into DNA, ethidium bromide emits red fluorescence, whereas acridine orange emits green fluorescence. Cells were classified as live (green nuclei), early apoptotic (bright green, condensed/fragmented chromatin), late apoptotic (orange, condensed/fragmented chromatin), or necrotic (uniform orange nuclei).

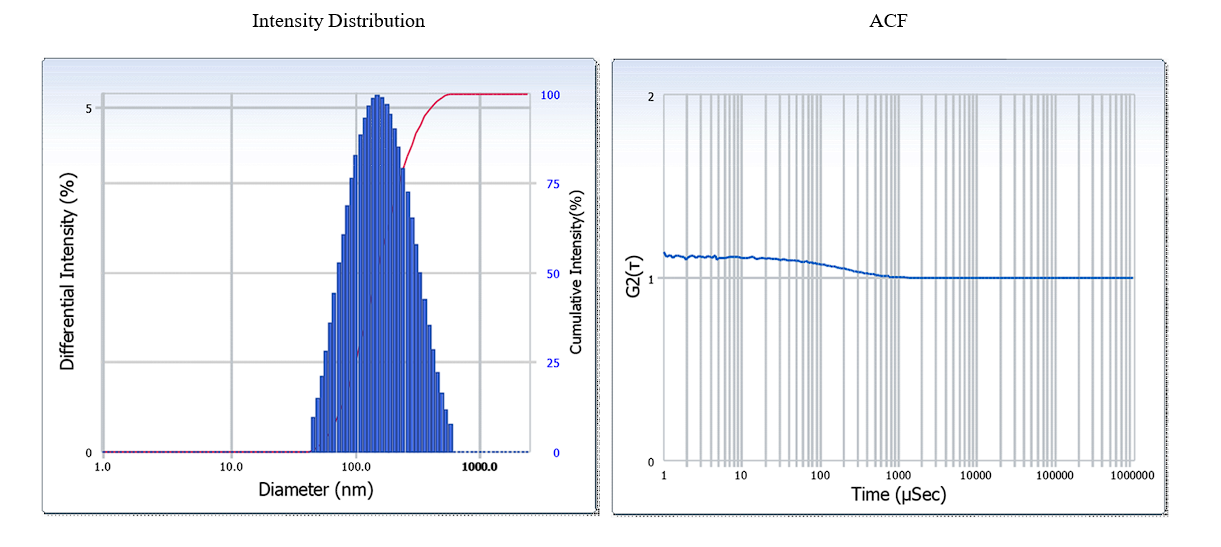
**2.9 Statistical analysis**

One-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to assess statistical significance, with *p* < 0.05 indicating significance between groups.

**3. RESULTS AND DISCUSSION**

**3.1 Analysis of Particle size**

The current study noted that the mean size of CACNP was 138.9 nm (Fig. 1.) with a polydispersity index (PDI) of 0.229. The Zetasizer measurements of nanoparticle size were in close coincidence with those obtained through SEM (137.14 nm; Fig. 2.).

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**Fig. 1.** Particle size of the CACNP.

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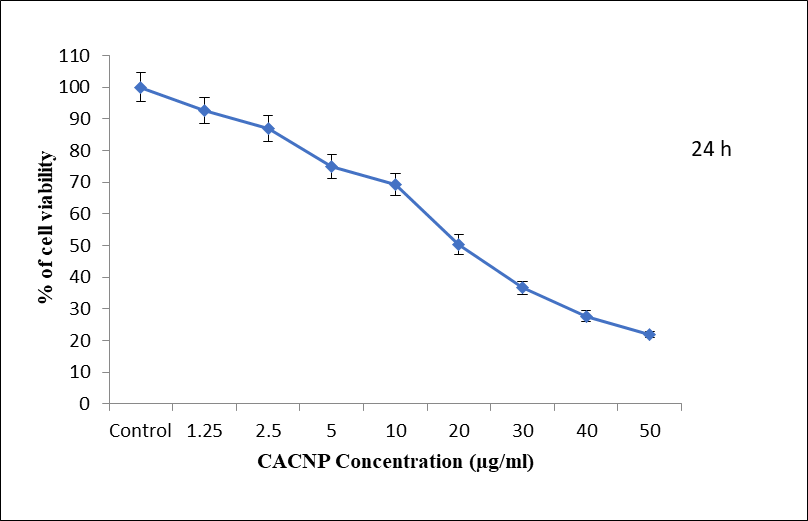
**Fig. 2.** SEM characterization of CACNP.

**3.2 Antiproliferative effects of CACNP**

Fig. 3. A and B illustrate the morphology of untreated and CACNP treated Hep-2 cells, after a 24-hour incubation. The current study demonstrated the *in vitro* anti-cell proliferative potential of CACNP against Hep-2 cells using the MTT assay. CACNP exhibited significant ability to reduce the cell viability of Hep-2 cells. The anti-cell proliferative efficacy of CACNP was found to be dose-dependent manner. CACNP reduced Hep-2 cell viability by 50% at 20 µg/mL (Fig. 4.).



**Fig. 3.** Morphology of untreated Hep-2 cells (A) and CACNP treated Hep-2 cells (B).

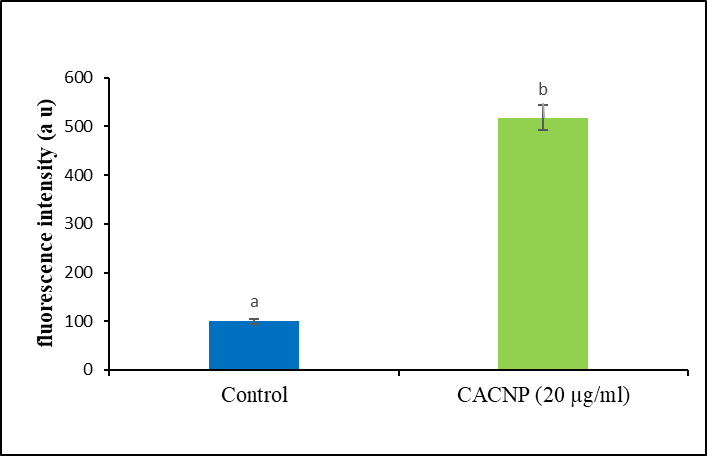
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**Fig. 4.** Measurement of antiproliferative effect of CACNP using MTT assay. The represented values (mean ± SD; n=3) are considered statistically significant at *p <* 0.05.

The effects of CACNP on ROS generation, MMP changes, and apoptosis in Hep-2 cells were studied using the IC50 concentration. Fig. 5. depicts the generation of ROS after a 24 hr incubation period in both untreated and CACNP treated Hep-2 cells and Fig. 6. shows the percentage of fluorescence intensity for ROS formation. Cytotoxic agents specifically kill cancer cells by inducing ROS-mediated DNA fragmentation and nuclear damage (Zhao et al., 2023). While malignant cells can tolerate high levels of ROS, there is a threshold beyond which ROS becomes toxic to them (Wang et al., 2017). Excessive ROS can cause mitochondrial damage and trigger cell death by acting as apoptotic signaling molecules (Brillo et al., 2021; Christidi & Brunham, 2021). Maximum ROS production and increased fluorescence intensity were observed in Hep-2 cells treated with CACNP (20 µg/mL). Treated cells showed excessive ROS production, indicated by intense green fluorescence from 2',7'-dichlorodihydrofluorescein) to DCF (2',7'-dichlorofluorescein). The present study thus suggests that excessive generation of ROS in CACNP treated Hep-2 cells which might have stimulated ROS-mediated apoptotic cell death.



**Fig. 5.** Measurement of ROS generation in untreated (A) and CACNP treated Hep-2 cells (B) using DCFH-DA staining.

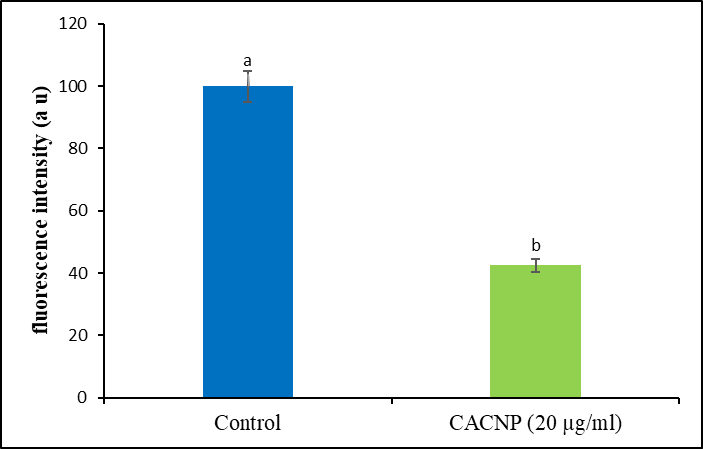
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**Fig. 6.** Measurement of fluorescence intensity for ROS generation in untreated and CACNP treated Hep-2 cells (mean ± SD n=3). Two different superscript letters (a and b) differ at p<0.5.

The MMP was examined in untreated and CACNP (20µg/mL) treated Hep-2 cells (Fig. 7.). The MMP was significantly altered in CACNP treated cells. This cells exhibited green fluorescence which indicated mitochondrial membrane polarization. Green fluorescence in Hep-2 cells treated with CACNP was reduced, indicating a decrease in mitochondrial membrane polarization. The percentage of fluorescence intensity was shown to be reduced at the IC50 dose (20µg/mL) of CACNP (Fig. 8.). Rhodamine accumulates in the membrane of living cells, causing Hep-2 cells to exhibit increased fluorescence and CACNP-treated Hep-2 cells to exhibit decreased fluorescence, which implies membrane polarization and depolarization, respectively. Depolarization of MMP triggers the release of pro-apoptotic proteins from the inner mitochondrial membranes, such as cytochrome c, which activates executioner caspase-3 to induce apoptosis (Urbani et al., 2021; Vassallo, 2025). The present investigation demonstrated that CACNP has the potential to enhance DΨm in Hep-2 cells.



**Fig. 7.** Measurement of MMP in untreated (A) and CACNP treated Hep-2 cells (B) Using Rhodamine-123 Staining.



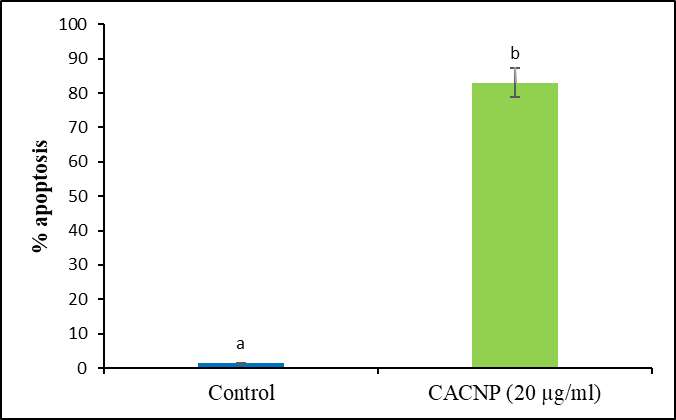
**Fig. 8.** Measurement of fluorescence intensity for MMP in untreated and CACNP treated Hep-2 cells. (mean ± SD n=3). Two different superscript letters (a and b) differ at p<0.5.

Fig. 9. illustrates the apoptosis-induced changes in untreated and CACNP treated Hep-2 cells. After treatment with CACNP for 24 hours at the IC50 concentration (20 µg/mL), Hep-2 cells exhibited increased apoptotic cell death index (Fig. 10.). Numerous techniques can be utilized to quantify apoptosis, focusing on the morphological, biochemical, and molecular changes that cells undergo during this process (Wang et al., 2015; Kari et al., 2022; Khodavirdipour et al., 2021). Fluorescent DNA-binding dyes like AO/EtBr can be used to evaluate cell membrane integrity and the apoptotic index (Sivasankaran et al., 2023). Acridine orange provides all cell types a green appearance by penetrating them (Manisekaran et al., 2024). Nevertheless, ethidium bromide (EtBr) can only cause the nucleus to become red in stained cells or ones where the membrane has become damaged (Karimian et al., 2022; Zheng et al., 2021). The current study assessed apoptotic inducing potential using AO/EtBr staining by which apoptotic, necrotic, and living cells may be identified. After staining, Necrotic cells showed uniformly orange nuclei without chromatin condensation, while viable cells had green nuclei. Apoptotic cells displayed orange nuclei with condensed or fragmented chromatin (Girija et al., 2024). In this study, untreated Hep-2 cells showed light green nuclei and CACNP treated Hep-2 cells exhibited orange chromatin and bright green nuclei. This study found that CACNP-treated Hep-2 cells exhibited chromatin condensation, nuclear fragmentation, and significantly reduced viability, confirming its apoptotic efficacy.



**Fig. 9.** Measurement of AO/EtBr on morphological changes in untreated (A) and CACNP

treated Hep-2 cells (B) using dual staining.



**Fig. 10.** Measurement of apoptotic index for AO/EtBr in untreated and CACNP treated Hep-2 cells. (mean ± SD n=3). Two different superscript letters (a and b) differ at p<0.5.

**4. CONCLUSION**

The current study thus explores the *in vitro* antiproliferative efficacy of CACNP on Hep- 2 cells. The antiproliferatiive efficacy of CACNP was validated by utilizing MTT assay, ROS generation (DCFH-DA staining), MMP assay and Dual staining (AO/EB). Based on our observations, the present study concludes that the cytotoxic potential of CACNP on Hep- 2 cells relies on its ROS-mediated apoptotic induction potential. Further studies will be conducted to explore the anticancer efficacy of CACNP using experimental animal model by analyzing spectrum of biochemical and molecular markers related to carcinogenesis.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

**COMPETING INTREST**

Authors have declared that no competing interests exist.

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