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In Vitro Cytotoxic Potential of Different Extracts of *Cyclea peltata* in HepG2 Cell Lines

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ABSTRACT

This experiment was conducted during March-April, 2022 in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India to find out the cytotoxic potential of the different extracts and fractions of *Cyclea peltata* using MTT assay and AO/EB & JC-1 vital dye staining in HepG2 cell lines. *Cyclea peltata* also known as Padal belonging to the family Menispermaceae, was a slender twining climbing shrub, used largely for its antipyretic, diuretic, antioxidant, anti-inflammatory, anti-cancer and snake venom neutralising properties. The whole plant of *C. peltata* was collected from Thrissur, Kerala, was identified, dried and extracted using water and methanol and the methanolic extract was fractionated using n-hexane, dichloromethane, n-butanol and water. The cytotoxic potential was analysed using 3-(4, 5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay in HepG2 cell lines and its apoptotic nature was evaluated using acridine orange/ethidium bromide (AO/EB) and 5, 5', 6, 6' -tetrachloro-1, 1', 3, 3'-tetra-ethyl benzimidazol-carbocyanine iodide (JC-1) staining. The extracts and fractions showed a dose dependent decrease in the viability of the cells exposed to it. The plant extracts of *C. peltata* especially n-hexane fraction was indicating a vital cytotoxicity in HepG2 cell lines through intrinsic pathway of apoptotic cell death and may be further analysed for the presence of detailed bioactive anti-cancer compounds and its potency.

KEYWORDS: *Cyclea peltata*, neoplasia, HepG2 cell lines, MTT, AO/EB, JC-1

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

Neoplasia is one of the most destructive and fatal diseases creating a severe warning to the health of millions of individuals with rising incidence and fatality rates globally (DeSantis et al., 2014). Cancer arises when healthy cells lose their ability to regulate their growth, allowing aberrant cells to multiply uncontrollably and eventual spread to organs or other regions of the body via the circulatory and lymphatic systems (Huang et al., 2019). In the five years following a cancer diagnosis, there are approximately 18.1 million new instances of cancer cases, 9.6 million cancer deaths, and 43.8 million individuals living with cancer, according to the World Health Organization (WHO) and International Agency for Research on Cancer (IARC) (Ferlay et al., 2019). According to WHO statistics, 17.1 million people will die from cancer and 27 million new cases will be diagnosed by the year 2050, making cancer the second biggest cause of death behind cardiovascular illnesses (Saraswathy et al., 2013). The goal of current therapy is to eradicate or irreparably harm the malignant cells by administering strong chemotherapeutic agents often and in combination (Une and Bhagure, 2022).

Over the past 30 years, natural products have drawn more attention due to their potential as cancer preventative and therapeutic agents (Ali et al., 2023). The chemical compounds derived from plants targeting the signalling pathways of cell invasion and inhibit different cancer stages and associated inflammatory processes (Imtiaz et al., 2024 ; Cevatemre, 2018). As of now, more than 3000 plants have been found to have anticancer properties. Among FDA approved anticancer and anti-infectious drugs, drugs from natural origin have a share of 60% and 75% respectively (Lu et al., 2020). The presence of rich bioactive phytochemicals plays an important role as an alternative in cancer treatment (Asiimve et al., 2021) by inhibiting cell migration, invasion, apoptosis, proliferation, preventing angiogenesis, controlling immunity, reversing medication resistance, and having other impacts (Li et al., 2022). The rapid development of new technologies and analysis methods, such as combinatorial syntheses, nanoparticle drug delivery systems and high-throughput screenings, has brought a fresh perspective to the discovery and the design of new chemical constituents from natural sources (Lee et al., 2017; Khan et al., 2019).

The phytochemicals present in *C. peltata* were alkaloids, flavonoids, tannins, terpenes and saponins with diuretic activity similar to furosemide (Rajkumar et al., 2021). Alkaloid fraction of *C. peltata* exhibited its antioxidant and anti-inflammatory activity by scavenging free radicals generated *in vitro* (Shine et al., 2020) *C. peltata* plant extract revealed the presence of antivenom compounds such as tetradecanoic and octadecadienoic acid which have neutralizing properties on *N. naja* venom (Sivaraman et al., 2017). Due to its significant medicinal value, it was designated as "therapeutic plant species in high trade sourced from tropical woods"(Ved and Goraya, 2007). The HepG2 cell line (Human hepatocellular carcinoma cell line) was obtained from liver biopsy tissues of primary hepatoblastoma used as an experimental model for various *in vitro* studies and cellular reference models with the goal of developing new drugs and gaining insights into drug metabolism, including knowledge of involved enzymes and the drug's inhibition or induction potential. (Qiu et al., 2015). The study mainly aims at the development of plant based novel drug molecules which could serve as a reliable alternative source for cancer chemotherapeutic agents. Based upon the traditional knowledge, this study was focussed on the *in vitro* cytotoxic potential of aqueous, methanolic extracts and different fractions of methanolic extracts of whole plant of *C. peltata*.

2. MATERIALS AND METHODS

2.1. Collection and authentication of plant material

The whole plant of *C. peltata* was collected during March, 2021 from local areas of Mannuthy, Thrissur, Kerala, India. The plant material was authenticated by the herbarium department, St. Thomas College, Thrissur, Kerala and the voucher specimen of the plant was deposited in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy with accession no. HERB/ VPT/CVASMTY/3/2022.

2.2. Cell culture conditions

Human hepatocellular carcinoma cell lines (HepG2) was procured from National Centre for Cell Science, Pune, Maharashtra, India, was utilized for *in vitro* studies. Cells were adapted to grow in Rosewell Park Memorial Institute 1640 1X (RPMI-1640) media supplemented with heat inactivated foetal bovine serum with 1% Ceftriaxone (50 mg mL^{-1}) which were maintained in a humidified incubator at 37°C with 5% carbon dioxide (CO_2). Cell lines were sub cultured by enzymatic digestion with 0.25% trypsin/1 mM EDTA solution and with 70 to 80% confluency these trypsinized cells were used for various *in vitro* studies.

2.3. Plant extraction

2.3.1. Aqueous (AE) and methanolic (ME) extraction of C. peltata

The plant material was shade dried, pulverised and tightly packed in a muslin cloth and kept in beaker filled with distilled water using hot aqueous extraction process to derive aqueous extract. The plant material was packed in filter paper and kept in Soxhlet apparatus for methanolic extraction. Both the solvents were dried using rotary vacuum

evaporator under controlled pressure and temperature at 40°C. The extract was finally collected and the yield was calculated and kept under refrigeration in an airtight container until further use (Poonghuzhali et al., 2022)

2.3.2. Fractionation of methanolic extract of C. peltata

Methanolic extract (25 g) was mixed with 250 ml of hexane in a separating funnel and soluble fraction collected. The insoluble fraction was serially extracted with dichloromethane (100 ml), n-butanol and water (50 ml each) respectively, in the order of ascending polarity. The solvents were evaporated using rotary vacuum evaporator to obtain hexane fraction (HF), chloroform fraction (CF), n-butanol fraction (BF) and water soluble fraction (WF). The yield of the fractions was calculated and kept in an airtight container under refrigeration till further use (Nair et al., 2017).

2.4. Sample preparation

The aqueous and methanolic extracts of *C. peltata* were dissolved in distilled water at a concentration of 5 mg mL^{-1} and diluted with RPMI medium to the appropriate concentrations. The stock solution of fractions of *C. peltata* was prepared in 10% DMSO as described earlier and then were diluted with RPMI medium to the appropriate concentrations with DMSO concentration restricted to less than 0.1%.

2.4.1. Cytotoxicity studies of different extracts and fractions of Cyclea peltata in vitro

The cytotoxic potential of aqueous, methanolic extract and fractions of *C. peltata* in HepG2 cells was examined in vitro using 3-(4, 5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) reduction assay (Plumb et al., 2004) The cells from T25 flask were collected and concentration adjusted to concentration was increased to 1×10^4 cells ml^{-1} and 200 µl of cell suspension in RPMI-1640 media was added to all wells of a 96 well sterile tissue culture plate for 24 hours at 37°C in CO_2 incubator. The stock solution was diluted to 160, 80, 40, 20, 10, and 5 µg mL^{-1} solution using RPMI media and 200 µL of various dilutions of aqueous, methanolic extracts and fractions of *C. peltata* were added to the wells in triplicates and incubated for 24 hours at 37°C in CO_2 incubator. The plates were removed from the incubator, media removed and received ten microliters of MTT (5 mg ml^{-1} produced in DPBS) and 100 µl serum free media. The plates were covered with aluminium foil for 4 hours at 37°C in CO_2 incubator and MTT containing medium was removed after 4 hours. 200 µL DMSO was added to all of the wells to dissolve the formazan crystals that had developed and for 10 minutes, the plates were gently stirred on an orbital shaker. At a wavelength of 570 nm, the absorbance was measured using a microplate reader (Varioskan Flash, Thermofischer Scientific, Finland).

The per cent cell viability and per cent cell inhibition were calculated using the following formulae:

Per cent cell viability=(Average absorbance of treated cells /Average absorbance of untreated cells)×100

Per cent cell inhibition=100% cell viability.

The net absorbance from the control wells was taken as 100% viable. The IC50 (half maximal inhibitory concentration) values of extracts were calculated by plotting the concentration against per cent cell inhibition using Graphpad Prism V 5.0.

2.4.2. Acridine orange/ethidium bromide (AO/EB) staining

The acridine orange ethidium bromide (AO/EB) staining procedure was followed to differentiate the live, apoptotic and necrotic cells (Kumar et al., 2016). Trypsinized cells were seeded in 6 well culture plates at a concentration of 3×10^5 HepG2 cells for 24 hours. Cells were treated with IC50 of aqueous and methanolic extracts and fractions of *C. peltata*, with vehicle control and positive control for 24 hour at 37°C in CO_2 incubator. The cells were centrifuged at 3000 rpm for 3 min and pellet was re-suspended in 100 µL of fresh media. 25 µl of cell suspension was taken and stained to 5 µl of AO/EB solution (10 µg ml^{-1} of Ethidium bromide and 10 µg ml^{-1} of Acridine orange). The solution was analysed under Trinocular Research fluorescence microscope, DM 2000 LED, Leica with blue excitation (488 nm) and emission (550 nm) filters at 20X magnification.

2.4.3. JC-1 staining

A concentration of $3x10^5$ HepG2 cells were seeded into a six well cell culture plate in RPMI media for 24 hours, then treated with IC50 of test compounds as mentioned above and incubated for 24 hour at 37°C in CO_2 incubator. After, cells were incubated with five millimolar JC-1 for 30 min at room temperature in the dark. The cells were washed and analysed directly in the culture medium with filters having, emission spectra at 405 and 488 nm and excitation spectra at 530 and 595 nm filters using fluorescent microscope (DM 2000 LED, Leica) (Ovadje et al., 2007).

3. RESULTS AND DISCUSSION

3.1. Cytotoxicity studies of different extracts and fractions of Cyclea peltata in vitro

The per cent cell viability as studied by MTT assay 24 hours after treatment with aqueous, methanolic and different fractions of methanolic extract of *C. peltata* in HepG2 cell line is presented in table 1.

The results indicated that all the extracts and fractions produced a concentration-dependent reduction in cell

Table 1: Per cent cell viability of HepG2 cells after treatment with all the extracts and fractions of *C. peltata*

Concentration (μg ml^{-1})	AE	ME	HF	DF	BF	WF
5	87.84±1.81	75.40±2.92	94.12±0.53	95.45±5.42	102.83±1.01	98.04±0.95
10	68.99±1.40	74.74±2.16	55.40±1.53	85.95±4.09	98.62±2.17	92.52±1.23
20	62.23±1.50	58.08±2.68	51.28±1.89	83.47±5.76	88.44±5.30	86.67±0.38
40	53.06±0.56	45.00±2.73	48.58±2.19	81.31±5.48	88.27±5.72	75.17±0.96
80	48.60±0.73	38.16±2.52	42.07±0.96	66.81±7.57	85.77±6.04	68.50±4.76
160	39.91±2.07	23.29±1.84	34.30±0.85	66.41±5.29	74.06±4.94	66.19±1.51

AE: Alcoholic Extract; ME: Methanolic extract; HF: Hexane fraction; DF: Dichloromethane fraction; BF: Butanol fraction; WF: Water fraction

viability of HepG2 cells. Of these, methanolic extract, hexane fraction and aqueous extract showed maximum reduction in cell viability with 23.29%, 34.30% and 39.91% respectively, when compared with butanol, dichloromethane and water fractions of *C. peltata*.

The per cent cell inhibition as studied by MTT assay 24 hours after treatment with aqueous, methanolic and different fractions of methanolic extract of *C. peltata* in HepG2 cell line is presented in table 2.

The results indicated that all the extracts and fractions produced a concentration-dependent increase in cell inhibition of HepG2 cells. Of these, methanolic extract, hexane fraction and aqueous extract showed maximum cell inhibition with 76.71%, 65.70% and 60.08% respectively, when compared with butanol, dichloromethane and water fractions of *C. peltata*.

In MTT assay, nicotinamide adenine dinucleotide phosphate (NADPH)-dependant cellular oxidoreductase enzyme found in viable cells with active metabolism is effective in converting the yellow tetrazolium dye MTT to insoluble purple formazan crystals that are solubilized by DMSO and detected at 570 nm using an ELISA plate reader (Riss et al., 2004). The extracts and fractions of *C. peltata* showed a concentration-dependent reduction in cell viability and there was a maximum inhibition of 65.69% at 160 μg ml^{-1}. In comparison to other extracts and fractions, the n-hexane fraction of *C. peltata* displayed an IC50 value of 37.59 g ml^{-1}, showing that it possesses strong cytotoxic activity. Earlier findings confirmed that *C. peltata* roots showed dose-dependent anti-proliferative effect on HepG2 cell lines with a lower IC50 and higher Selectivity index value than cisplatin (Ng et al., 2006). Also, cytotoxic potential of *C. peltata* was evaluated in human colon carcinoma cell line HCT-116 in which inhibition of cell proliferation was seen with increasing concentration and time at 75% concentration (Jayaraman and Variyar, 2019). Another study on *in vitro* cytotoxicity of *Cyclea peltata* in L 929 cells lines showed 62.18% cell viability and 37.82% cell inhibition at initial concentration of 50 μg mL^{-1} (Anuja et al., 2021). The MTT assay is unable to distinguish between necrosis and apoptosis as the cause of the cell growth inhibition. Drugs cause destruction of proliferative cells mostly by inducing apoptosis whose sensitivity is directly proportional to the apoptotic levels. To examine the reason behind the cytotoxic potential of *C. peltata* plant extracts and fractions, we evaluated the apoptosis related morphological changes in HepG2 cells.

3.2. IC50 values of different extracts and fractions of C. peltata

The IC50 of *C. peltata* was calculated by using the per cent

Table 2: Per cent cell inhibition of HepG2 cells after treatment with all the extracts and fractions of *C. peltata*

Concentration (μg ml^{-1})	AE	ME	HF	DF	BF	WF
5	12.16 ±1.81	24.60±2.92	5.88±0.53	4.55±5.42	2.83±1.01	1.96±0.95
10	31.01 ±1.40	25.26±2.16	44.60±1.53	14.04±4.09	1.38±2.17	7.48±1.23
20	37.77 ±1.50	41.92±2.68	48.72±1.89	16.52±5.76	11.56±5.30	13.33±0.38
40	46.94 ±0.56	54.99±2.73	51.42±2.19	18.68±5.48	11.73±5.72	24.83±0.96
80	51.40 ±0.73	61.84±2.52	57.93±0.96	33.19±7.57	14.22±6.04	31.50±4.76
160	60.08 ±2.07	76.71±1.84	65.70±0.85	33.58±5.29	25.94±4.94	33.81±1.51

AE: Alcoholic Extract; ME: Methanolic extract; HF: Hexane fraction; DF: Dichloromethane fraction; BF: Butanol fraction; WF: Water fraction

cell inhibition obtained from MTT Assay. A curve was plotted using the values in Graph pad Prism and the graph obtained is represented in the Figure: 1-6. The IC50 value of AE, ME, HF, DF, BF and WF was found to 63.23, 45.78, 37.59, 461.9, 620.9 and 350.9 µg ml^{-1} respectively.

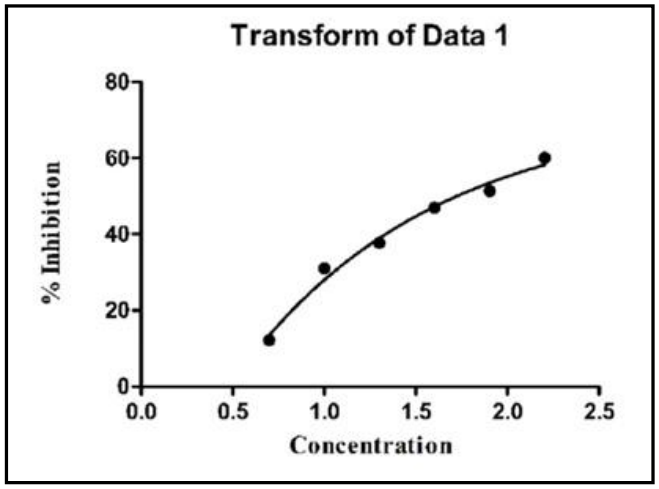

Figure 1: IC_{50} value of AE-63.23 µg ml^{-1}

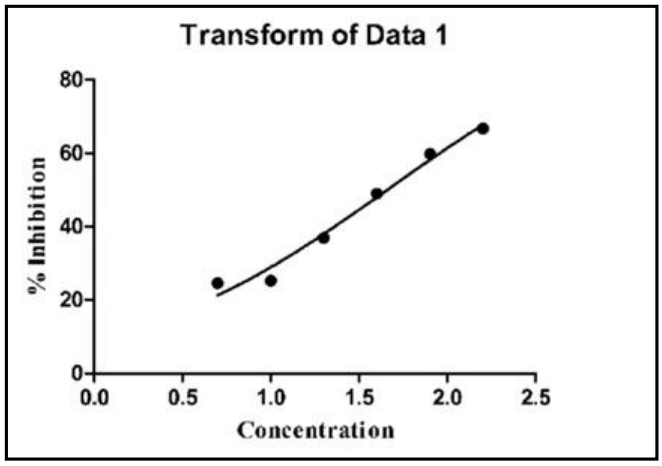

Figure 2: IC_{50} value of ME-45.78 µg ml^{-1}

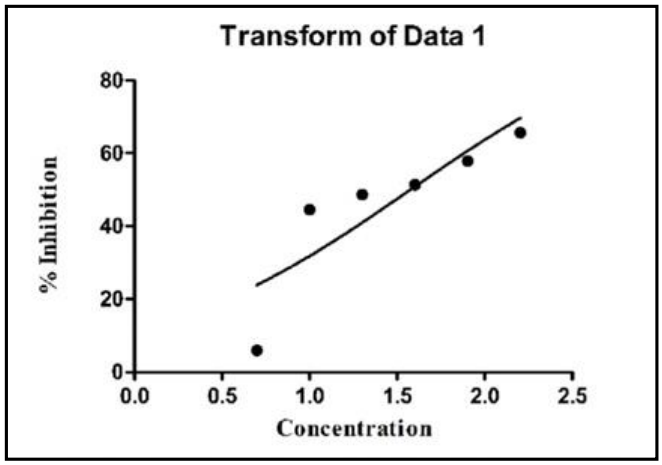

Figure 3: IC_{50} value of HF-37.59 µg ml^{-1}

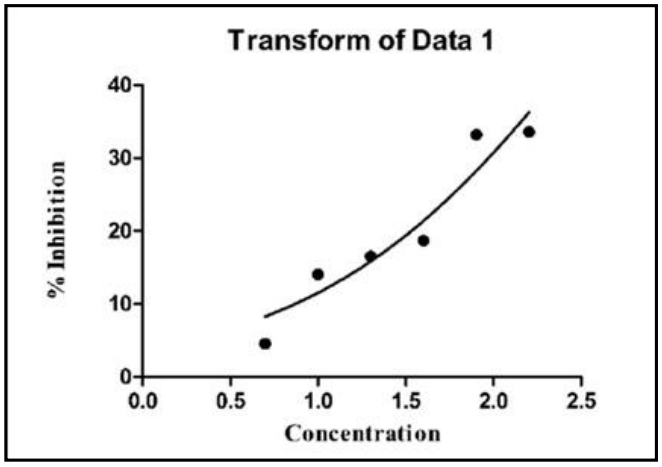

Figure 4: IC_{50} value of DF-461.9 µg ml^{-1}

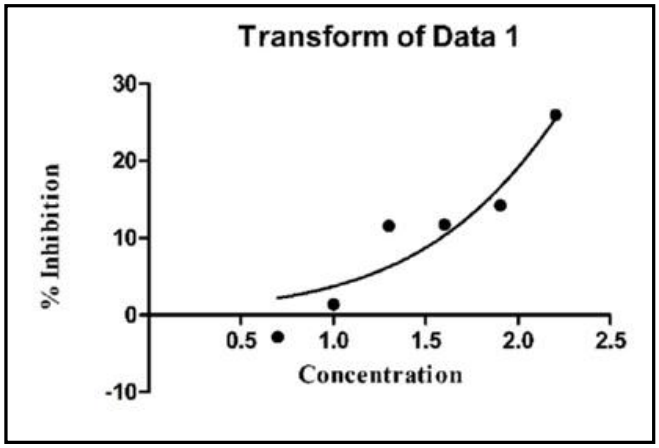

Figure 5: IC_{50} value of BF-620.9 µg ml^{-1}

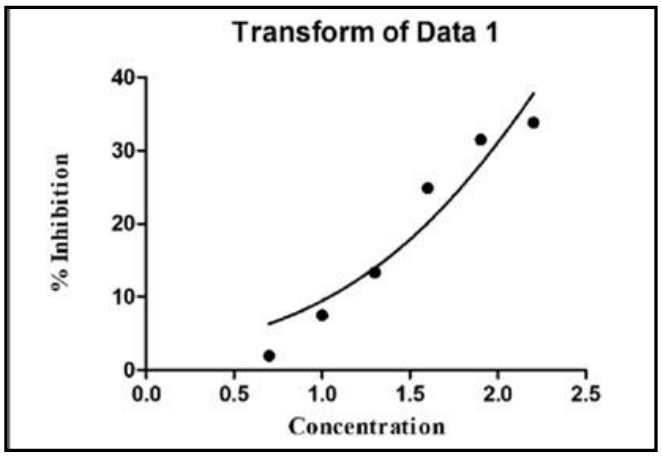

Figure 6: IC_{50} value of WF-350.9 µg ml^{-1}

3.3. Microscopic studies using acridine orange/ethidium bromide (AO/EB) staining

The AO/EB dual staining was done to detect the live, early apoptotic, late apoptotic and necrotic cells after treatment with IC50 concentration of test compounds and control (Figure 7).

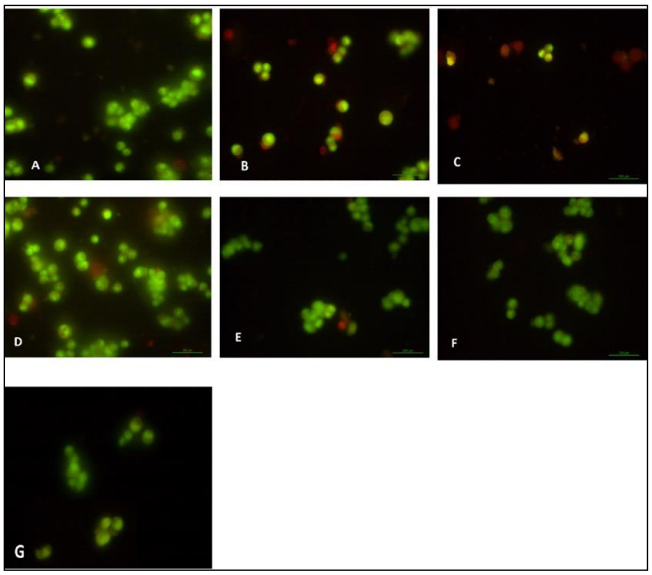

Figure 7: Effects of different extracts and fractions of *C. peltata* on HepG2 cells usingAO/EB staining, 200 X. A: Aqueous extract; B: Methanolic extract, C: Hexane Fraction; D: Dichloromethane fraction; E: Butanol fraction; F: Water fraction; G: Control

In control group, all the cells were live showing greenish fluorescence with circular nucleus uniformly distributed in the centre of the cell. But, cells exposed to aqueous extract, dichloromethane, butanol and water fraction of *C. peltata* showed live cells and cells in early apoptotic stages. In cells exposed to methanolic extract, late apoptotic changes and orange stained nucleus were seen. Majority of the cells exposed to n-hexane fraction showed earlier apoptotic changes and numerous cells were in late apoptotic stage with condensed and asymmetrically localised orange stained nucleus and only few cells were showing green fluorescence.

Fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes (AO/EB staining) is a method of choice for simplicity, rapidity and accuracy. The vital dye acridine orange stain both live and dead cells but ethidium bromide can enter cells that has lost membrane integrity. The live cells exhibited green fluorescence as the dye intercalate into DNA, but EB gives red fluorescence in the nonviable cells that had altered cell membrane. As a result, early apoptotic cells will stain green and have vivid green spots in the nucleus and necrotic cells stain orange with nuclear morphology similar to that of live cells, without condensed chromatin (Kasibhatla et al., 2006).

In this study, the control cells were showing greenish fluorescence indicating healthy cells whereas cells exposed n-hexane fraction of methanolic extract of *C. peltata* showed earlier apoptotic changes and numerous cells were in late apoptotic stages with condensed and asymmetrically localised orange stained nucleus. The cells that are exposed to other extracts and fractions of *C. peltata* showed earlier apoptotic changes with green fluorescence. The cells exposed to positive control showed sever orange to red stained nucleus. Typical characteristics of apoptosis, like nuclear shrinkage, condensation, and the formation of apoptotic body-like vesicles as revealed by fluorescence microscopic analysis after treatment with *Croton crassifolius* essential oil on A549 cells (Liu et al., 2019).

3.4. Microscopic studies using JC-1 staining

The technique of 5,5,6,6'-tetrachloro-1,1',3,3'tetraethylbe nzimidazoylcarbocyanine iodide (JC-1) staining indicates the change in the mitochondrial membrane potential (Δψm) (Figure 8).

A remarkable change in the fluorescence from red to green was observed when cells were exposed to IC_{50} concentrations of test compounds of *C. peltata*. In control cells, JC-1 dye accumulated in the mitochondria as aggregates and was visualized as red fluorescence under fluorescent microscope. In cells exposed aqueous extract, both green and red fluorescence were seen. In dichloromethane, butanol and water fraction, cells changing from red to green fluorescence was observed. In cells exposed to methanolic extract and n-hexane fraction of *C. peltata*, the mitochondrial membrane potential declined, the dye was dispersed throughout the cell as monomers and was observed as green fluorescence.

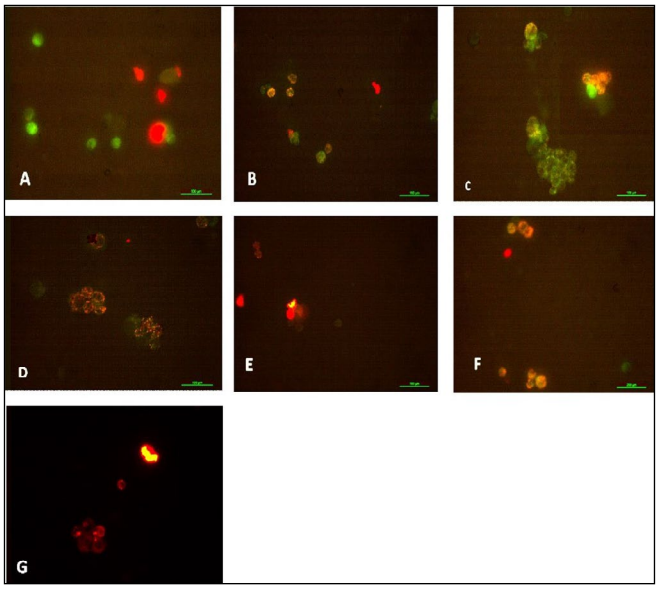

Figure 8: Effect of different extracts and fraction of *C. peltata* on HepG2 cells using JC-I staining, 200 X. A: Aqueous extract; B: Methanolic extract; C: Hesane fraction; D: Dichloromethane fraction; E: Butanol fraction; F: Water fraction; G: Control

The depolarization of the mitochondrial membrane potential is frequently examined by JC staining, which is thought to be one of the earliest stages of apoptosis (Solaiprakash and Devaraj, 2019). In the untreated group, JC-1 exhibited a red fluorescence but when cells got exposed

to n-hexane fraction *C. peltata* for 24 h, JC-1 showed a gradual increase in green with little red fluorescence which implied a decrease in mitochondrial membrane potential. Other *C. peltata* extracts and fractions treated to HepG2 cells exhibited more red fluorescence than the n-hexane fraction, indicating that it has stronger cytotoxic effects. Previous studies also stated a dose dependant reduction in mitochondrial membrane potential from red to green fluorescence following cisplatin treatment (Choi et al., 2015).

4. CONCLUSION

The treatment with aqueous, methanolic and different fractions (especially N-hexane fraction) of methanolic extracts of *Cyclea peltata* were able to produce cytotoxicity against HepG2 cell lines causing apoptosis of the cancer cells as evidenced by AO/EB and JC 1 vital dye staining.

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