

RESEARCH ARTICLE

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Polyphenol Mediated Suppression of Hepatocellular Carcinoma (HepG2) Cell Proliferation by *Clerodendrum infortunatum* L. Root

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Abstract

Objective: *Clerodendrum infortunatum* L. has long been used in traditional medicine in Sri Lanka for tumours, cancer, and certain skin diseases. The present study aimed to assess the anticancer properties of the aqueous extract of *C. infortunatum* L. root (AECIR) through the activation of the apoptotic pathway on hepatocellular carcinoma (HepG2) and thus give it a scientific validation. Further, the contribution of polyphenols in antioxidant activity and cell cytotoxicity was investigated. **Methods:** Powdered plant material was boiled with water (100°C) to obtain AECIR. The DPPH assay was used to determine the antioxidant potential. The activity of AECIR on HepG2 and normal rat fibroblast (CC1) cell growth was determined using MTT assay. The morphological changes related to apoptotic pathway were examined by Ethidium Bromide/Acridine Orange (EB/AO), Rhodamine 123 (Rh123) and DNA fragmentation assay. **Results:** The AECIR demonstrated antioxidant potential with an EC_{50} of 350.2 ± 1.5 μ g/mL for DPPH assay. The $HO\cdot$, H_2O_2 and $\cdot NO$ free radical scavenging activity was observed with EC_{50} of 19.7 ± 2.3 , 11.7 ± 0.1 and 273.1 ± 0.9 μ g/mL, respectively. The antiproliferative effect of AECIR on HepG2 cells was observed in a time and dose dependent manner with an EC_{50} of 239.1 ± 1.3 μ g/mL while CC1 cells showed a nontoxic effect with an EC_{50} of 1062.7 ± 3.4 μ g/mL after 24hrs treatment. A significant decrease in antioxidant activity ($p < 0.001$) and 90% HepG2 cell viability was observed with polyphenol removed AECIR compared to the polyphenol present AECIR. The EB/AO uptake, depletion of mitochondrial transmembrane potential, and DNA fragmentation assay results revealed that the apoptosis was induced by AECIR. **Conclusion:** The obtained result of the present study demonstrates that the antioxidant potential and antiproliferative activity of AECIR is attributed to the presence of polyphenols. Furthermore, the findings provide the scientific base for anti-cancer potential of AECIR.

Keywords: Antiproliferative activity- Antioxidant activity- Mitochondrial transmembrane potential- DNA fragmentation

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Introduction

Cancer is a complex disease that develops in the body due to various internal and external factors, with uncontrolled cell growth following different multi-step signalling pathways [1-3]. However, chemotherapy and radiotherapy, the two primary therapies currently used, have a risk of inducing undesirable effects and bringing down the quality of life of cancer patients [4, 5]. Hence, this is still a leading health problem worldwide [5]. Nevertheless, many researchers in the field of oncology are intensively working on novel effective therapeutic strategies to achieve positive responses with the least side effects of anticancer therapy [6]. Among the treatment methods in traditional medicine around the world that have existed since ancient times, herbal plant based medicine takes a prominent place [7]. Thus, the long term use of these plants for diseases including cancer has shown fewer

side effects and potential therapeutic effects on people [8]. Hence, traditional knowledge of medicinal uses of natural products has paved the way for most drug discovery research [9]. The majority of the current anticancer drugs, such as vinca alkaloids, podophyllotoxin, taxanes, camptothecins and combretastatins used in therapy are active ingredients derived from natural sources [10, 9]. Epigallocatechin, a main phenolic antioxidant compound in *Camellia sinensis*, can increase apoptotic cell death in various cancer cell types [11]. In addition, curcumin and myricetin polyphenolic phytochemicals have been reported to have anticancer properties [12, 13]. In Sri Lankan traditional medicine, the decoction of *C. Infortunatum* root prepared with water is used in the treatment of cancer. *C. Infortunatum* belongs to the family Verbenaceae and can be found in moist regions of Sri Lanka [14]. Several studies have demonstrated that *C. Infortunatum* possesses a number of therapeutic values

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and presence different phytochemicals such as alkaloids, phenol, tannin, flavonoid, thiamine, riboflavin etc. [15]. Chacko et al. 2015 [16] have reported that the ethanolic extract of *C. infortunatum* root has shown inhibition of cell proliferation in PC-3, A549, and HCT-116 cancer cells and apoptotic effect and caspase activation in murine tumour cells (Dalton's lymphoma ascites cells - DLA) [17]. In another study performed by Chandra et al. 2017 [18] ethanolic, methanolic and chloroform extracts of leaves of the plant have also been shown to have antioxidant activity, wound healing, anti-microbial activity and antiproliferative potential on MCF-7 and Hela Cells. In literature, few studies have investigated the antioxidant activity and antiproliferation of ethanol, ethyl acetate, and hexane extracts of *C. infortunatum* leaves and roots grown in different Asian countries. Since the roots of this plant are used as a decoction of water according to the prescription of traditional medicinal practitioners in Sri Lanka, it is intriguing to investigate the molecular mechanism of anticancer activity of water extract of root.

Currently, no studies have investigated the traditional decoction of the *C. infortunatum* root on HepG2 cancer cells and polyphenolic effects on cell viability by removing polyphenols from the extract to evaluate the direct involvement of polyphenols in apoptotic induction. Hence, the current study was designed to investigate the apoptotic activity on HepG2 cells and CC1 cells and consequently provide scientific validation to the traditional prescription of the plant and establish this as an anticancer therapeutic agent.

Materials and Methods

Chemicals and equipment

The chemicals, cell culture media, and other supplements were purchased from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA) and Fluka (Fluka chemie GmbH, CH-9471 Buchs). Cells were incubated at 37°C in a humidified carbon dioxide incubator (SHEL LAB/ Sheldon Manufacturing Inc. Cornelius, OR 97113, USA), and the cells were observed using Olympus (1X70-S1F2) inverted fluorescence microscope (Olympus Optical Co. Ltd. Japan).

Plant material

The roots of *C. infortunatum* L. were collected from Malabe in Colombo District, Sri Lanka under supervision and permission of licensed traditional medicinal practitioner following the relevant guidelines and regulations. All sampling processes were performed from February to April of the year 2017. The plant materials were identified by the botanist at the National herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka, and a voucher specimen was deposited at the same premises (voucher specimen number: I-01 - *Clerodendrum infortunatum* L. – Lamiaceae).

Preparation of the plant extract

The plant material (30g) was powdered and boiled with 800 mL of deionised water until the total volume was reduced to 200 mL for 2 hrs. The aqueous extract

was filtered through a Whatman filter paper (No 01). The filtrate was centrifuged at 10,000 rpm for 15 minutes and freeze-dried. The lyophilised product was stored at -20°C until further use. Three individual decoctions were prepared, and all experiments were performed in triplicates (n=3).

Determination of total phenolic content and flavonoid content

The plant's total phenolic and flavonoid content was determined by the Folin Ciocalteu method [19, 20] and aluminium chloride colorimetric method respectively, with slight modifications [21]. The total phenolic content was expressed as w/w% gallic acid equivalents (GAE), and flavonoid content was expressed as w/w% (-)-Epigallocatechin gallate equivalents (EGCGE).

Determination of antioxidant activity

DPPH radical scavenging assay described by Blois (1958) was used to determine the antioxidant activity of freshly prepared AECIR (200 – 1000 µg/mL) with slight modifications [22]. Ascorbic acid was used as the standard.

Hydroxyl radical scavenging activity

The HO• scavenging capacity was measured based on the theory described by Halliwell, 1987 [23] with slight modifications [21]. The generated HO• in the reaction mixture degrades 2- deoxy-D-ribose and forms a pink chromogen on heating with thiobarbituric acid at low pH. The pink chromogen produced by the plant extract (25-400 µg/mL) was measured by its absorbance at 532nm. A calibration curve was constructed with gallic acid (25-250 µg/mL).

Nitric oxide radical scavenging activity

The •NO scavenging activity was performed according to the GRIESS-ILOSVAY'S reaction with slight modification [24]. The AECIR (2-10mg/ mL) was prepared and allowed to compete with oxygen leading to reduced production of nitrite ions which may ultimately reduce the production of the azo-dye. The scavenging ability was compared with the L-ascorbic acid as the reference standard (50-100 µg/mL).

Hydrogen peroxide scavenging activity

The H₂O₂ scavenging activity of the AECIR was determined by an enzymatic method described by Fernando, (2015). Different concentrations of AECIR (200 -2000 µg/mL) were analysed with the standard L-ascorbic acid (20- 40 µg/mL).

Determination of ferric ion reducing power

The reducing power of the decoction was performed according to the method described by Oyaizu 1986 with slight modifications [20]. The prepared concentration series (200-2000 µg/mL) of AECIR were compared with the reducing power of the L-ascorbic acid (25-250 µg/mL).

Removal of polyphenols

The removal of polyphenols was carried out according to the method described by Ranatunge, 2017 [25]. The

polyvinyl polypyrrolidone (PVVP) column was prepared using the PVVP powder 1.9g. The AECIR (2mg/ mL) was filtered through the column by centrifuging at 2000 g for 10 min. The collected fractions were analysed for the presence of polyphenols, antioxidant potential and antiproliferative activity.

Cell line

HepG2 (Human, hepatocellular carcinoma) and CC1 (Normal rat liver fibroblast) cell lines were obtained from Dr. Panjwani Center for Molecular Medicine and Drug Research, International Centre for Chemical and Biological Sciences, University of Karachi. The cells were cultured and preserved in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Colombo, Sri Lanka. The ethical approval was obtained by the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka. (Reference No: EC-17-013)

Cell culture

Cells were cultured in Modified Eagle's medium (MEM) growth medium (10%), supplemented with 10% foetal bovine serum (FBS), L-glutamine (3%), 10,000 U/mL penicillin and 10 mg/mL streptomycin, the pH of the growth medium was adjusted to physiological pH 7.4 using 7.7% sodium bicarbonate and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, 1M). Cells were maintained at 37°C in a 5% carbon dioxide (CO₂) atmosphere with 95% humidity. The culture flask, which reached 80% confluent monolayer, was subjected to analysis.

In all experiments, except for fragmented DNA analysis, cells were seeded in 96-well plates at a density of 1×10^5 cells and cultured overnight to get a confluent monolayer. The monolayer was treated with different concentrations (25 - 1000 µg/mL) of AECIR (dissolved in culture media) in triplicates. The treated cells were maintained at 37°C for 24, 48, and 72 hours separately in a humidified CO₂ incubator and were subjected to experiments at the end of the exposure period. A negative control without the AECIR and positive control with cycloheximide (25mM, 50µL) were used in each experiment.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The MTT assay developed by Mosmann (1983) [26] was used to calculate the percentage of cell viability with slight modifications [27]. The cultured HepG2 and CC1 cells were treated with different concentrations (25 - 1000 µg/mL) of AECIR and incubated for 24, 48 and 72 hrs as mentioned above. The utilised culture medium was carefully removed, and MTT (5mg/mL in phosphate buffer saline (PBS); 50µL) was added. The cells were incubated at 37°C for 3 hours, and the MTT solution was carefully removed without disturbing formazan crystals. The crystals were dissolved in acidified isopropyl alcohol (0.05 HCl in IPA; 100µL) by constant agitation on a reciprocating orbital shaker. The absorbance of the resulting solution was immediately measured at 570nm.

Sulforhodamine B (SRB) assay

SRB assay was used to investigate cell proliferation as a measurement of cellular protein content [28]. SRB is an amino xanthene dye that binds to cellular proteins under mildly acidic conditions and extracts under basic conditions in the quantification of proteins [29]. This measures the cell number, which reflects cell viability. The AECIR-treated cells were washed with sterile PBS, and trichloroacetic acid (10% TCA; 100µL) was added. After incubation at 4°C for 1 hour, cells were washed five times with deionised water. The plate was air-dried, and SRB (0.06%, 50µL) was added and kept for 15 minutes at room temperature. SRB was removed and washed with acetic acid (1%, 200µL) to remove unbound dye. The Tris base (10mM, pH 9, 200µL) was added to the air-dried plate and kept on the shaker for 1 hour. The absorbance was then measured at 540nm [27].

Nitric oxide production in cells

The supernatant was collected from the spent medium used for MTT assay and 100 µL was incubated with GRIESS reagent (100 µL) at room temperature for 10 minutes [24]. The absorbance was then measured at 540 nm. The standard curve was constructed using sodium nitrite (0.25-5 µg/mL).

Ethidium Bromide/Acridine Orange (EB/AO) stain

EB/AO stain was used to visualise nuclear changes and apoptotic body formation [30]. Cells were exposed to different concentrations (100 - 1000 µg/mL) of AECIR for 24 hours. The spent medium was removed, and 6 µL of dye mix (100 µg/mL EB and 100 µg/mL AO in PBS) was added to each well prior to observation. The stained cells were observed under a fluorescent microscope using 200X magnification.

Giemsa stain

Giemsa stain was used as a further observation of apoptotic cells [27]. The culture medium was removed from cells exposed to AECIR (100 - 1000 µg/mL), and Giemsa (diluted with PBS 1:4, 100 µL) was added to the cells. After 30 minutes, cells were washed with PBS and observed under a light microscope using 200X magnification.

Rhodamine 123 stain

Rhodamine 123 stain (Rh123) was used to analyse mitochondrial transmembrane potential ($\Delta\Psi_m$). It detects the depletion of $\Delta\Psi_m$, which is associated with mitochondrial apoptosis [31]. The loss of $\Delta\Psi_m$ will result in a loss of Rh123 fluorescence. The AECIR-treated cells were stained with 10µL of Rh123 dye (10 µg/mL Rh123 in PBS). The plate was then incubated for 30 minutes at 37°C, and the dye was removed by washing off with PBS prior to observation at 200X magnification under a fluorescent microscope.

DNA fragmentation

The assay was carried out to detect the DNA fragmentation caused by the AECIR [32]. The cells were seeded in a 12-well plate with a density of 2

x106. The confluent monolayer was treated at different concentrations (125, 250, 500, 750 µg/mL) of AECIR in triplicates. After 48 hours, cells were trypsinised and centrifuged at 2000 rpm for 5 minutes at 4°C in pre-cooled sterile micro-centrifuge tubes. TES lysis buffer (20 µL) was added to the cell pellet and mixed without disturbing the DNA. RNase (500U/mL, 20 µL) was then added to the cell suspension and incubated for 4 hours at 37°C. After the incubation period, proteinase K (1mg/mL, 20 µL) was added to the mixture and incubated at 50°C overnight. The 6X DNA loading buffer (5 µL) was added to the above DNA sample (25 µL) and loaded into wells of a 1.2% agarose gel. A 100bp ladder was used for size reference. The gel was run in Tris-borate-EDTA buffer (TBE) at a low voltage (50V) for 1 hour. The gel was finally visualised by a UV light source and was documented by photography.

Statistics and calculations

The results were presented as mean ± standard deviation (Mean ± SD). Experiments were carried out in triplicates for three independent extracts in all experiments. The dose response curves (plotted using five concentrations of the plant extract) were used to determine the EC₅₀ values and calibration curves of the standards were considered linear if R² > 0.99. The significance of the difference between means was statistically analysed by applying a t-test using Microsoft Excel 365. The value of p<0.05 was considered to indicate statistical significance. Pearson correlation values were obtained from Microsoft Excel 365 to evaluate the correlation between NO concentration and cell viability.

Results

Phenolic and flavonoid content

The extraction yield obtained for three independent decoctions of *C. infortunatum* L. is depicted in Table 1 as a percentage of the dry weight of each sample used. The percentage of total phenolic content and total flavonoid content is expressed in gallic acid equivalents (GAE) and (-)-Epigallocatechin gallate equivalents (EGCGE) per dry weight of the decoctions (Table 1).

Antioxidant and radical scavenging capacity

The effective concentration of the AECIR required to scavenge free radicals by 50% (EC₅₀) is depicted in Table 2. As lower EC₅₀ indicates a higher scavenging activity of a substance, (Table 2) shows that AECIR possesses higher OH radical scavenging activity (p<0.005) compared to gallic acid, whereas scavenging of H₂O₂ is similar to ascorbic acid. However, scavenging of DPPH or NO radicals was very low compared to standard antioxidants, gallic acid, and ascorbic acid.

Ferric ion-reducing antioxidant power (FRAP) assay

The ability of the plant extract to quench free radicals by hydrogen donation was observed with the reduction reaction of Fe³⁺ ions into Fe²⁺. The potential of AECIR to reduce Fe³⁺ ions into Fe²⁺ was low compared to the reducing power of the standard L-ascorbic acid (Figure 1).

Antiproliferative activity

The antiproliferative activity of AECIR on HepG2 and CC1 cells was assessed by MTT assay. The AECIR inhibited the proliferation of HepG2 cells by 50% at a concentration of 239.1 ± 1.3 µg/mL after 24 hours of treatment. Further, it demonstrated a significant dose dependent activity (p<0.005) with an EC₅₀ of 64.6 ± 2.6 µg/mL and 20.1 ± 3.9 µg/mL after 48 and 72 hours of exposure, respectively (Figure 2a). In contrast, the EC₅₀ values; 1062.7 ± 3.4, 939 ± 0.4, 759.1 ± 3.5 µg/mL, were significantly high (p<0.01) showed with CC1 for 24, 48 and 72 hours respectively (Figure 2b). The EC₅₀ values obtained for cell proliferation by SRB assay was also consistent with that of MTT assay and revealed a dose and time dependent activity after exposure of AECIR to both HepG2 and CC1 cells. The morphological changes of HepG2 and CC1 cells after AECIR treatment for 24hrs are presented in supplementary Figure 1.

In addition to the activity of AECIR in free radical scavenging and cell growth, the influence of polyphenolic compounds on antioxidant activity and cell growth was investigated by removing polyphenols from the AECIR. The AECIR, in the absence of polyphenols, showed a significant decrease (p<0.001) in DPPH scavenging activity and exhibited cell viability over 90% along with the concentration range (25 to 500 µg/mL) in HepG2

Table 1. Extraction Yield, Polyphenolic and Flavonoid Content of AECIR

Plant	Extraction Yield (g) w/w% of dry weight	Polyphenolic content w/w% of GAE	Flavonoid content w/w% of EGCGE
AECIR	5.6 ± 0.2	2.0 ± 0.6	2.3 ± 0.5
Polyphenol removed AECIR	--	0.2 ± 0.1	--

Table 2 Antioxidant Activity and radical Scavenging Activities of AECIR

Sample	DPPH scavenging	OH scavenging	H2O2 scavenging	NO scavenging
		EC50 (µg/mL)		
AECIR	350.2 ± 1.5	19.7 ± 2.3*	11.7 ± 0.1	273.1 ± 0.9
Ascorbic Acid	5.0 ± 1.7	--	12.2 ± 1.9	56.0 ± 5.6
Gallic Acid	2.4 ± 0.3	25.5 ± 1.7	--	--

*, Significantly lower (p < 0.005) than gallic acid

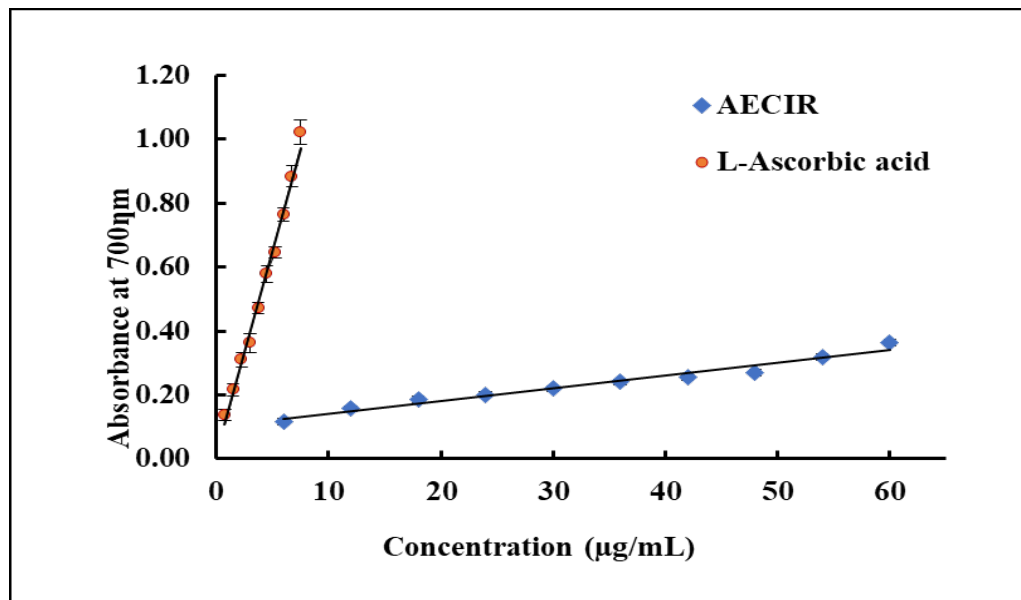


Figure 1. Ferric Ion Reducing Power of AECIR in Comparison with L-ascorbic acid. Data represent mean \pm SD (n=3).

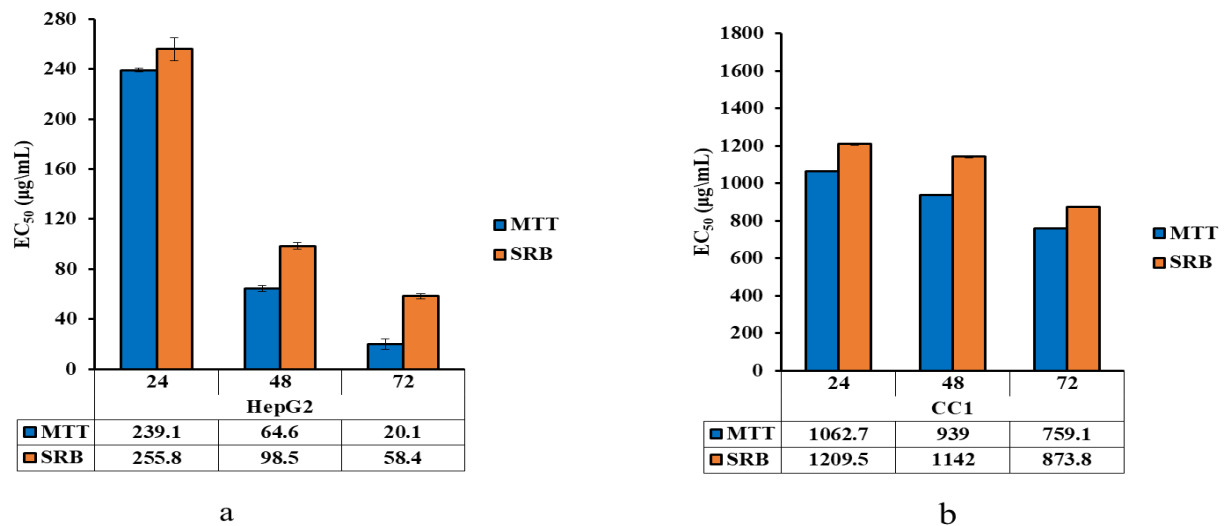


Figure 2. The Mean EC_{50} (\pm SD) Values of MTT and SRB Assay. (a) HepG2 cells (b) CC1 cells; after 24, 48, and 72 hours of exposure to a series of concentrations (25 - 1000 μ g/mL) of AECIR (n=3).

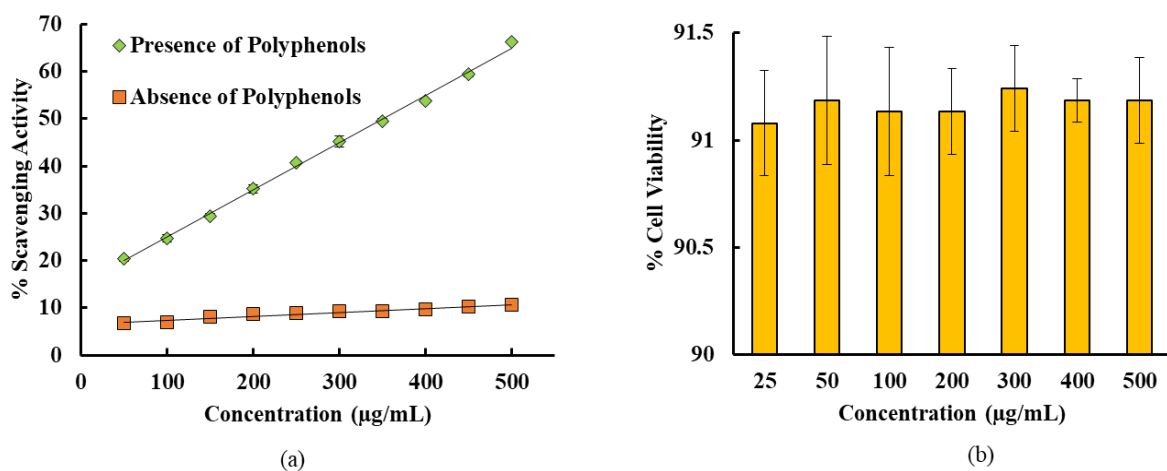


Figure 3. Percentage DPPH Scavenging Activity and Percentage Cell Viability of MTT Assay. (a) AECIR in the presence and absence of polyphenols (n=3); mean (\pm SD). (b) HepG2 cell viability after 24 hours of treatment of polyphenol removed AECIR.

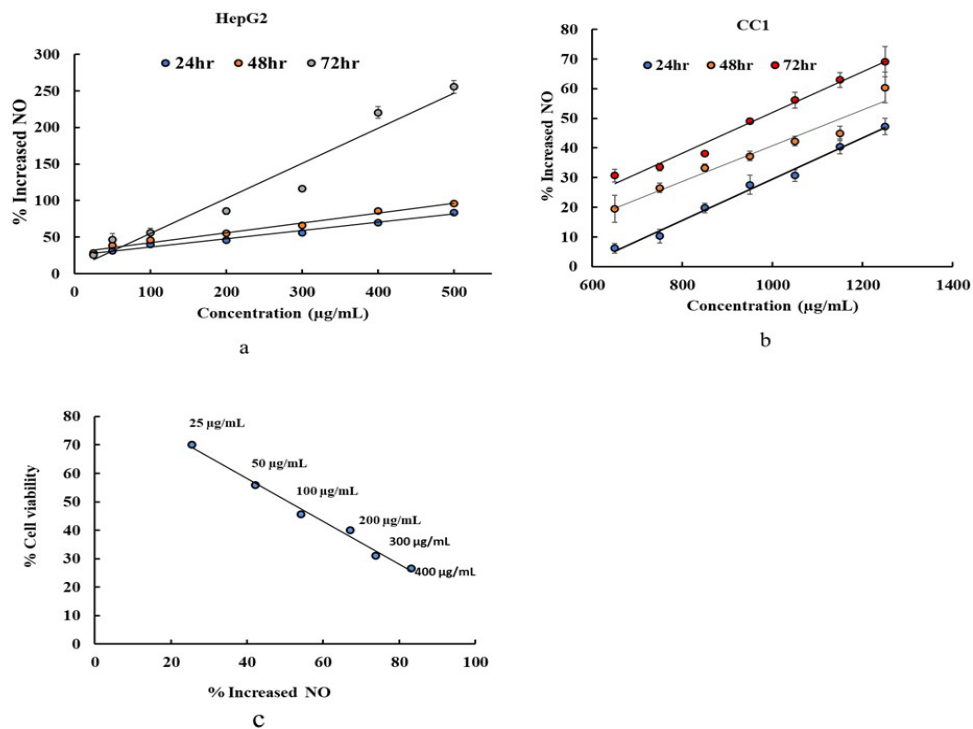


Figure 4. The Percentage of Increased NO Content Relative to Negative Control of the Respective Cell Type after 24, 48, and 72 hours of Exposure to AECIR (n=3). (a) HepG2 cells, (b) CC1 cells, and (c) the correlation of NO oxide levels against percentage cell viability for HepG2 cells. Concentrations relevant to percentage increased NO and percentage cell viability are indicated at each data point.

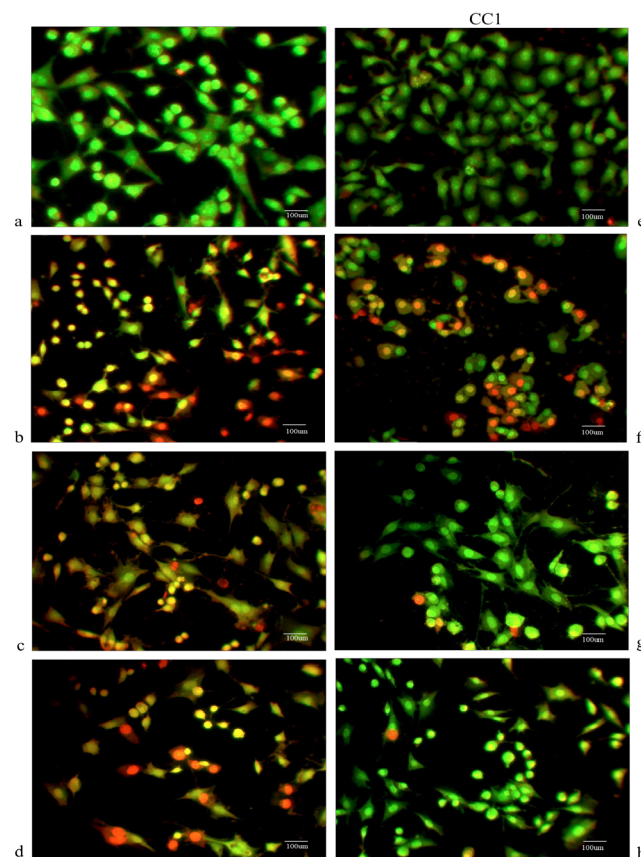


Figure 5. Representative Fluorescent Micrograph of HepG2 and CC1 Stained with EB/AO Double Stain after 24 hours of Treatment with AECIR (n=3) at Different Concentrations (200x). HepG2 cells (a) negative, (b) positive, (c) 125 µg/mL and (d) 375 µg/mL. CC1 cells (e) negative, (f) positive, (g) 500 µg/mL and (h) 1000 µg/mL. Green fluorescence indicates viable cells; bright green condensed chromatin indicates early apoptotic cells, and yellow/orange fluorescence indicates late apoptotic cells. Negative control with untreated cells; Positive control with Cycloheximide (25mM, 50µL).

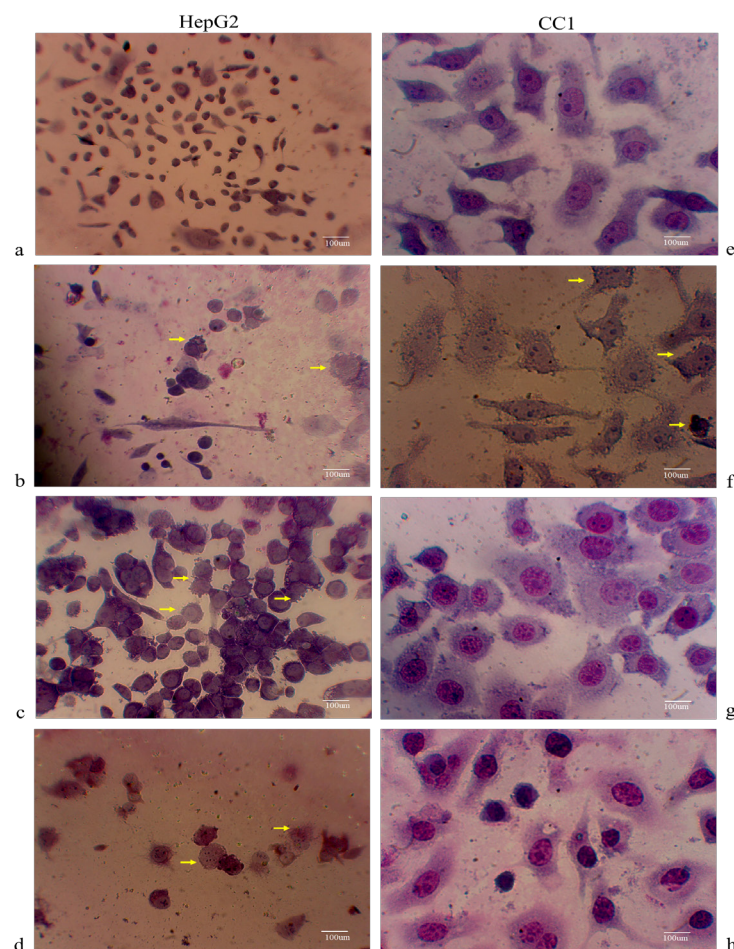


Figure 6. Morphological Changes of Apoptotic Cell Death Caused by AECIR in HepG2 and CC1 Cells after 24 hours of Treatment (200x). HepG2 cells (a) negative, (b) positive, (c) 125 µg/mL and (d) 375 µg/mL. CC1 cells (e) negative, (f) positive, (g) 500 µg/mL and (h) 1000 µg/mL. Negative control with untreated cells; Positive control with Cycloheximide (25mM, 50µL). Each image represents a single Giemsa stain light micrograph of three independent experiments. Yellow arrow indicates apoptotic cell death.

cells (Figure 3).

Nitric oxide production in cells

As shown in Figure 4, AECIR-treated HepG2 cells exhibited a significant increase in NO production in a dose and time dependent manner compared to the negative control. An increase of NO production in HepG2 cells by 50% relative to the untreated cells was observed at 221.2 ± 14.6 , 154.9 ± 2.4 , and 88.6 ± 7.8 µg/mL following treatment with AECIR over 24, 48, and 72 hours respectively. In contrast, the values for CC1 cells were observed at concentrations greater than or around 1mg/mL (1297, 1151.5, and 971.9 µg/mL, respectively) after AECIR exposure at the same time intervals (Figure 4b).

Furthermore, the correlation of NO oxide levels against percentage cell viability (MTT assay) was examined, and p values obtained by Pearson correlation were 0.9639, 0.9618, and 0.8807 for 24, 48, and 72 hours (Figure 4c).

EB/AO staining

A series of morphological events in apoptosis was observed after 24 hours of treatment of AECIR in HepG2 cells, as depicted in Figure 5. The negative control of HepG2 cells appeared with uniformly bright

green nuclei. The early apoptotic HepG2 cells appeared at a concentration of 125 µg/mL with greenish-yellow fluorescence and bright green dots in the nuclei. Late apoptotic cells with orange to red nuclei with highly fragmented chromatin and apoptotic bodies-stained orange were observed in a dose dependent manner. The CC1 cells showed a uniformly green appearance even at the high concentration examined (1000 µg/mL) (Figure 5).

Giemsa stain

The morphological changes characteristic to apoptosis, such as shrinkage of the cell, membrane blebbing, chromatin condensation, and membrane bound apoptotic bodies, were further observed in a dose dependent manner in AECIR treated HepG2 cells through the Giemsa staining, as depicted in Figure 6. Morphological features identical to the apoptotic pathway were not observed with AECIR treated CC1 cells (Figure 6).

Rhodamine 123 stain

Changes in $\Delta\Psi$ resulted in a fluorescence intensity decrease in a remarkable dose dependent manner compared to the negative control (Figure 7). CC1 cells stained with Rho 123 were observed to have dose dependent uniform fluorescence intensity as they are unaffected by $\Delta\Psi$

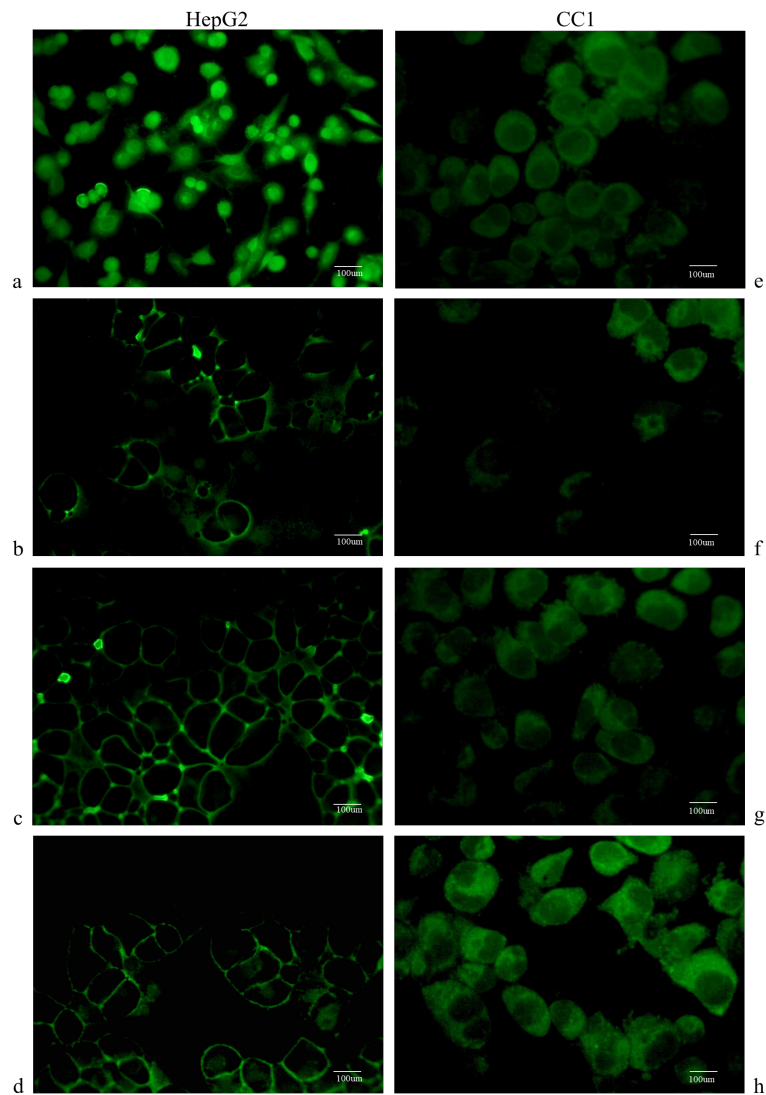


Figure 7. Mitochondrial Transmembrane Potential Changes Caused by AECIR in HepG2 and CC1 Cells. Cells were treated for 24 hours at different concentrations with AECIR and stained with Rho123 stain for fluorescent microscopy (200x). HepG2 cells (a) negative, (b) positive, (c) 125 µg/mL and (d) 375 µg/mL. CC1 cells (e) negative, (f) positive, (g) 500 µg/mL and (h) 1000 µg/mL. Negative control with untreated cells; Positive control with Cycloheximide (25mM, 50µL). Each image represents a single fluorescent micrograph of three independent experiments

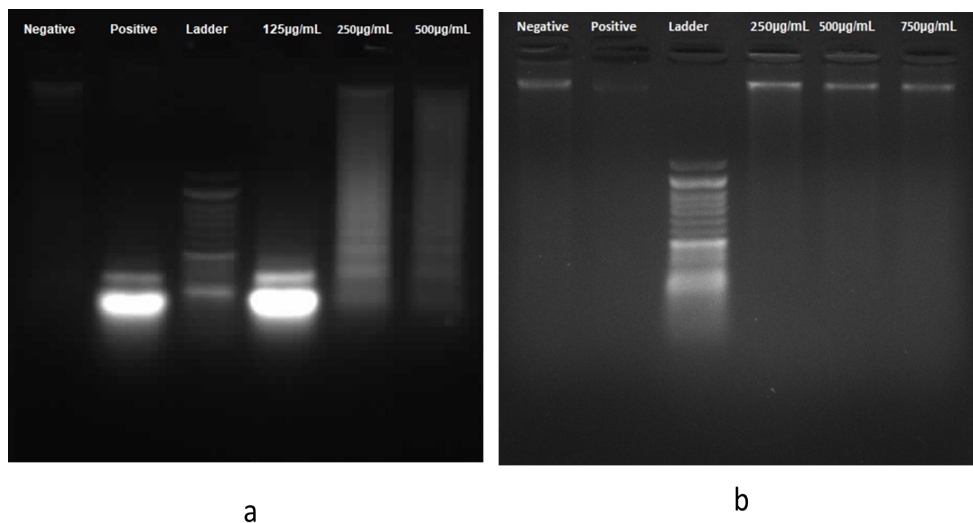


Figure 8. AECIR Induce Apoptotic DNA Fragmentation Analysis. Gel electrophoresis was carried out with DNA extracted from (a) HepG2 cells and (b) CC1 cells which were treated with AECIR for 48 hours with the indicated concentration of EC₂₅, EC₅₀, and EC100.

changes (Figure 7). These results indicate that AECIR can induce apoptosis in HepG2 cells.

DNA fragmentation

HepG2 cells treated with AECIR showed fragmented DNA in a dose dependent manner (Figure 8a). Intact DNA bands were observed with CC1 cells even at a concentration of 750 µg/mL (Figure 8b).

Discussion

Many countries around the world predominantly use plant based medicines for the treatment of ailments, following their own traditional medicinal methods [33]. Similarly, in Sri Lanka, more than 2000 different species of plants have been used as a single whole plant, a single part of a plant or a mixture of plant parts over 3000 years for different diseases [34]. Traditional medicinal practitioners in Sri Lanka provide treatment for the whole body [34]. They do not target different parts of the body to be treated separately [34]. Also, the herbal preparation is considered as a whole product, not as single chemical constituent [9]. Hence, these herbal plant based preparations have given more effective results without side effects and improved the quality of the patients' life over many years [33].

At present, in spite of many attempts to develop new drugs for cancer, about 30% of clinical trials fail [35]. The main reason for this is the side effects that damage other organs of the patients, and the medicine is not effective enough to cure the disease [4, 36]. Natural products can have advantages in the drug discovery process compared to synthetic drugs [37]. Therefore, it is important to present scientifically validated results of the plant-based drugs used in different countries for the attention of the scientific community working in the oncology field.

Considering the valuable health benefits of herbal plants and given the importance of natural antioxidants in anticancer activity, this study was carried out to evaluate antioxidant activity, antiproliferative activity, and induction of apoptosis by the water extract of *Clerodendrum infortunatum* root.

Methanolic and ethanolic extracts of *C. infortunatum* leaves have been reported to have scavenging activity towards hydroxyl, superoxide anion, and nitric oxide [38]. Apart from antioxidant activity, ethyl acetate and chloroform extracts of the root, leaf, and stem of *C. infortunatum* have shown effective antibacterial, antifungal, antimalarial, antitumor, and antiproliferative activity [39]. Numerous in vitro studies indicate that these properties can be attributed to its secondary metabolites such as phenolic compounds and flavonoids [40].

Many studies have shown that plant polyphenols are used in the prevention or as a treatment for oxidative stress related diseases such as cancer, neurodegenerative diseases, diabetes mellitus, etc [41]. The disease preventive ability of plant polyphenols is considered mainly through their antioxidant property [42]. In addition, polyphenols have been shown to exert anticancer properties in vitro and in vivo studies. This includes inhibition of cell proliferation, cell cycle arrest, and induction of apoptosis [43].

A series of complementary free radical scavenging test systems were used to provide an overview of the antioxidant capacity of polyphenolic compounds in the aqueous extract of *Clerodendrum infortunatum* L. root. The data obtained for the DPPH assay, which demonstrates the antioxidant potential, showed that the AECIR has a moderate level of antioxidant activity (Table 1) compared with methanolic and ethanolic extracts of *C. infortunatum* L root of other cultivars from Asia [15, 44, 45]. Methanolic extracts of *C. infortunatum* L root collected from India have shown 99.2% inhibition at 110.40 µg/mL [46]. Thus, the level of antioxidant activity and the proton donating potential of the compounds present in the AECIR was confirmed along with their reactivity towards the free radicals. In order to understand the contribution of polyphenols among the compounds present in the AECIR towards the antioxidant activity, we performed the DPPH assay using the polyphenol removed AECIR. The results revealed a significant reduction in the scavenging of DPPH radical ($p < 0.001$) in the absence of polyphenols (Fig. 3) which facilitates the understanding of the beneficial effect of polyphenols on antioxidant activity.

In biological systems, H₂O₂ has both reducing and oxidizing properties [47]. Though it is moderately reactive, the Fenton reaction, which drives the formation of highly reactive HO•, develops a threat to cellular components triggering oxidative damage to DNA, RNA, and other cellular components [48]. Lipid peroxidation is the most lethal process which is initiated by HO• abstracting an H atom from polyunsaturated fatty acid [49-51]. The initiation step follows several reactions resulting in the production of lipid peroxides contributing to oxidative stress [49, 51]. It is known that cancer cells have an increased level of ROS including HO• and H₂O₂ compared to normal cells [52]. Many anticancer therapies are involved in oxidative stress and ROS by their mechanism [43]. Shendge et al. 2020 [53] have found that solvent fractions of *Clerodendrum viscosum* leaf hexane, ethyl acetate, butanol, chloroform and water have shown dose dependent HO• radical activity better than the standard mannitol. Interestingly, AECIR has also shown a higher scavenging activity with HO• and H₂O₂ (Table 2). The HO• scavenging activity was higher in comparison to the reference standard gallic acid.

In cancer research, •NO has become a molecule of interest as it is linked to cancer promoting and cancer inhibiting activity [54]. Several studies have observed that nitric oxide increases toxicity within the cell when it reacts with certain proteins and mostly with superoxide radicals [55]. Thus, nitric oxide has a direct effect as a radical and an indirect effect with its byproducts [56]. Hence, we focused on determining the scavenging nitric oxide by the herbal extract. In the reaction mixture, oxygen reacts with nitric oxide generated from sodium nitroprusside to form nitrite [57]. The antioxidants present in the AECIR compete with oxygen and donate protons to the nitrite radical. Thus, the number of nitrite ions, that diazotize with sulphanilamide acid, coupled with naphthyl ethylenediamine. Table 2 illustrates that the AECIR scavenged nitrite radical in a dose dependent manner as a function of the combination of antioxidants

with active functional groups such as the methoxy and hydroxyl groups. Similar results were found for ethyl acetate and water extracts of *Clerodendrum viscosum* leaf with IC50 values of 232.89 ± 10.71 and 224.19 ± 5.36 respectively [58].

Furthermore, the results indicated a linear increase in ferric ion-reducing antioxidant power (FRAP) over the concentrations investigated (Fig. 1). The determination of reducing power enables us to understand the formation of stable ferric metal complexes by polyphenolic compounds with comparison to the standard ascorbic acid. The carbonyl moiety and multiple OH binding sites present in plant flavonoids actively react with metal ions to form stable metal complexes [59]. Water extracts of *Clerodendrum viscosum* leaf have shown better activity in ferric ion reduction compared to Hexane, ethyl acetate and butanol extracts [58].

Furthermore, our findings indicate that AICER promotes anticancer activity by inducing apoptotic pathways. Unrestricted cell proliferation is a hallmark of cancer [60]. The MTT assay suggested that the AECIR reduced the proliferation of HepG2 cells by more than 50% at 88.6 ± 7.8 $\mu\text{g/mL}$ after a 72-hour treatment. Antiproliferative properties were observed with EC50 values in a dose and time dependent manner (Fig. 2). In contrast, the CC1 cells responded differently to plant extract, signifying its nontoxic effects towards CC1. SRB assay, which indicates the protein levels further confirms that AECIR induces cell death with increasing concentration. The data from previous studies have explained the dose dependent cytotoxic potential of different solvent extracts of *C. infortunatum* L. grown in different countries. Sannigrahi et al. have revealed that the presence of two bioactive terpenoids, Oleanolic acid and Clerodinin A from methanolic extract of *C. infortunatum* fresh leaves [61]. Preclinical assessment of these two compounds has exhibited anticancer activity on Ehrlich's ascites carcinoma (EAC) bearing Swiss albino mice via inducing the activity of the enzymatic defence system and inhibition of lipid peroxidation [61].

Clerodendrum L. exerts selective cytotoxic activity with phenolic and flavonoids present in the plant extract (leaves, seeds) where they interfere with multiple signal transduction pathways in cells such as TRAIL-induced cytotoxicity, increased expression of p53 and p21, and by increased concentration of cytochrome c [53, 62]. Therefore, we investigated dose dependent HepG2 cell growth inhibition using AECIR after removing polyphenols from the AECIR. The removal of polyphenols from the plant extract exhibited a higher EC50 value compared to the plant extract before removing its polyphenols ($p < 0.005$), suggesting that the plant extract has lost its ability to prevent cell growth signifying its contribution to cell death (Fig. 3).

Cell shrinkage, cell blebbing, apoptotic body formation, nuclear condensation, and fragmented nuclei are morphological changes in the cellular process in response to the apoptotic signalling pathway [33]. Based on this concept, the capacity of AECIR to induce apoptosis in HepG2 cells was tested. The EB/AO stain and Giemsa stain analysis, further reveal that AECIR induces cell

death via an apoptotic pathway in HepG2 cells in a dose dependent manner (Fig. 5 and 6).

The molecular mechanisms of apoptosis in HepG2 cells were further studied by investigating NO production, mitochondrial membrane potential, and DNA fragmentation. As evidenced by the present results, the plant extract increased the production of NO in HepG2 cells in a dose dependent manner and the values were lower in CC1 than that of HepG2 (Fig. 4c). Furthermore, the values are several folds higher after 72-hour exposure of AECIR at higher concentrations. The production of NO from L-arginine by nitric oxide synthase is responsible for diverse activities including cell signalling, cell cycle controlling mechanisms, and other physiological parameters [63]. In a number of studies, it has been documented that the enhanced production of NO contributes, via activating downstream signalling in the p53 pathway, to DNA repair, cell cycle arrest, and apoptosis [64]. The increased level of NO could further upregulate the p21 expression in vivo independent of p53 and cGMP leading to G1 cell cycle arrest [58]. Our data showed that AECIR regulated the production of NO, which could lead to cell cycle arrest either by activating the p53 pathway or p21 gene expression and also an alternative mechanism could be involved such as NOS expression as described in several research studies [65]. Furthermore, NO generates reactive nitrogen species, enhancing cellular oxidative stress leading to apoptotic cell death [66]. The percentage increased in NO levels with that of cell viability correlated significantly with the HepG2 cells exposed to the plant extract at 24 hours (Fig 4c). Similar results were observed for 48 and 72 hours proving the role of NO leading to apoptotic cell death via oxidative stress.

Mitochondria are known to play a central role in the apoptotic process and are likely to activate the intrinsic pathway [67]. The opening of the mitochondrial permeability transition pore leads to depolarization of the transmembrane potential ($\Delta\Psi\text{m}$) and biochemical changes in the apoptotic cascade have resulted from the subsequent release of apoptogenic factors including cytochrome c [68, 69]. The lipophilic cationic fluorescence Rh123 dye, which is sensitive to mitochondrial membrane potential (MMP), accumulates in live cells [70] and the reduction of fluorescence intensity indicates a loss of MMP. Interestingly, the present results as evidenced by a smaller number of cells with accumulated Rh123 dye or increased numbers of apoptotic cells are further explained by a dose dependent mitochondrial membrane potential depolarization in HepG2 cells (Figure. 7).

A series of morphological and biochemical changes result from key mediator caspases at execution phase of apoptosis [71]. Caspases act as key mediators of DNA fragmentation [71]. Wang et al. [13] have reported that when caspase 3 is activated, DNA fragmentation factor (DFF) responds by cleaving chromosomal DNA into high relative molecular mass DNA fragments (50-300kb) and these large DNA fragments serve as precursors for the smaller, multiples of 180bp, DNA fragments [72, 13]. The present study demonstrated that AECIR induced the apoptotic pathway consequently resulting characteristic

apoptotic DNA fragmentation in HepG2 cells (Figure 8a).

The present study deciphered the induction of apoptosis and dose dependant antiproliferative on HepG2 cells through the polyphenols presents in AECIR. Though AECIR represented polyphenol mediated anticancer activity in 2D cell culture system, further evaluation of its activity in 3D cell culture system (organoids) will strengthen the obtained results as it mimics the natural physiological environment of the cancer.

The MIRDA standard reporting recommendation was followed in this manuscript [73]. In conclusion, the present findings collectively suggest that the combination of phenolic compounds and flavonoids present in AECIR enhances antioxidant capacity and selectively demonstrates antiproliferative activity on HepG2 cells via induction of apoptosis compared to CC1 cells. Accordingly, current study provides scientific evidence for using of *Clerodendrum* L. root as a potential therapeutic candidate to treat and prevent liver cancer in future.

Author Contribution Statement

Imali Ranatunge carried out the study data interpretation and prepared the manuscript. Preethi Soysa contributed through designing of the study and revising the manuscript for intellectual content.

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Ethical declaration

Ethical approval was obtained by the Ethics Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka. (Reference No: EC-17-013)

Availability of data and materials

The datasets that were used and analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of interests

The authors declare that there are no conflicts of interest.

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