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ORIGINAL ARTICLE

Nyctanthes arbor-tristis alkaloids activates p53 independent cell death receptor and necroptosis pathways in HepG2 cells

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Abstract

Nyctanthes arbor-tristis is a traditional medicinal plant with potential anti-cancer properties. In this study, crude and alkaloid extracts were prepared from different parts of the plant, and their cytotoxicity was evaluated on four different cancer cell lines. The alkaloid extracts from the leaf and fruit showed promising results, with the HepG2 cell line exhibiting significant cytotoxicity. The promising extracts were further studied for their apoptotic potential using various methods, including DNA fragmentation, TUNEL, Caspase-3 activity, Giemsa, and Hoechst staining. Our results indicated that the fruit extract had the highest apoptotic potential, with clear nuclear condensation, fragmentation, and apoptotic bodies observed. We also investigated the alteration of the Bax/Bcl-2 ratio both at the mRNA and protein levels. Our results showed a significant upregulation of the Bax gene and downregulation of the Bcl-2 gene for the fruit alkaloid extract. This indicates that the phenomenon of cell death expression might be following a p53-independent extrinsic pathway and Bax-activated caspase-independent AIF-mediated necroptosis in the HepG2 cancer cell line. Overall, our findings suggest that *Nyctanthes arbor-tristis* has potential as a therapeutic option for cancer treatment. The alkaloid extracts from the leaf and fruit may hold promise as a source of bioactive compounds for further development into anti-cancer agents. Further studies are needed to explore the underlying mechanisms of their cytotoxic and apoptotic effects and to evaluate their safety and efficacy in animal models and clinical trials.

Keywords Cytotoxicity · Apoptosis · Necroptosis · Apoptotic pathways · Caspases · Bax/Bcl-2 ratio

Introduction

Cancer is a significant public health issue and one of the leading causes of death worldwide. According to recent reports, female breast cancer has become the most commonly diagnosed cancer (2.21 million), followed by lung (1.80 million) and liver (0.83 million) cancers (Ferlay et al. 2021). Chemotherapy and radiotherapy treatments for cancer can cause damage to the cardiovascular and renal systems (Wu et al. 2021; Herrmann et al. 2016), leading to dehydration and malnutrition in older patients (le Saux and Falandry 2018). Other reported side effects include gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, hematopoietic system injury, and neurotoxicity (Zhang et al. 2018). To prevent and treat cancer, natural products such as plant crude extracts, bioactive component-enriched fractions, and pure compounds prepared from medicinal plants have been found to be effective.

The Oleaceae family, which comprises 200 species found throughout the world, has been used in traditional medicine in various regions, including Asia, South Africa, Island, European Mediterranean, Italy, and Spain (El-Shiekh et al. 2020; Msomi and Simelane 2017). *Nyctanthes arbor-tristis*, a member of the Oleaceae family, is commonly known as "Harshinghar" and "Parijat" and is well known for its various pharmacological properties (Pundir et al. 2022). Each part of this plant has been documented for its medicinal usage in ancient works of literature. It is used in Ayurveda, Siddha-Ayurveda, and Unani systems of medicine as a laxative, diuretic, anti-venom, digestive, mild bitter tonic, and expectorant, and is also documented in modern pharmacology for its various pharmacological activities (Parekh and Soni 2020). These pharmacological activities of medicinal

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plants are attributed to their secondary metabolites (Jain et al. 2019). Reports have revealed the presence of anticancer bioactive compounds in different genera of this family, such as β-sitosterols, Oleuropein, Hydroxytyrosol, and Hydroxytyrosyl acetate from Olea europaea. Crude extracts of medicinal plants possess compounds effective as therapeutic agents in cancer. Various studies have been conducted to detect the efficacy of crude plant extracts as a source of active components in anticancer therapy (Al-Rimawi et al. 2016; Al-Snafi and Al-Snafi 2016; Syed Najmuddin et al. 2016; Ghagane et al. 2017; Kukula-Koch et al. 2018; Al-Dabbagh et al. 2018, 2019; Nguyen et al. 2020).

Research has demonstrated that *N. arbor* is abundant in iridoid glycosides and alkaloids, both of which have been investigated for their anticancer properties and mechanisms of action. Glycosides of *N. arbor* have been studied for their role in inhibiting the growth of HepG2, MCF-7, and MDA-MB-231 cell lines and their mechanisms of action (Ma et al. 2013). While alkaloids have previously exhibited various pharmacological activities, including anticancer effects, there is a lack of information on the anticancer potential and mechanistic pathways of *N. arbor* alkaloids.

This study aims to evaluate the anticancer potential of crude and alkaloid extracts obtained from different parts of *N. arbor*, namely the stem, leaves, flowers, and fruits. The primary objective was to assess the cytotoxicity of both crude and alkaloid extracts and determine which extract exhibited the highest apoptotic potential. Additionally, the study aimed to analyze the mechanism of action of the identified anticancer agent. MTT and LDH assays were utilized to conduct cytotoxicity studies on four cell lines, namely U937, Jurkat J6, MCF-7, and HepG2. The HepG2 cell line was selected for apoptotic studies, which involved DNA fragmentation, TUNEL, Caspase-3 activity, Giemsa, and Hoechst, RT-PCR and Immunostaining techniques were utilized to examine the up and down regulation of genes responsible for apoptotic pathways in cancer. The study's findings have the potential to provide new opportunities for the use of *N. arbor* alkaloids as combinational chemotherapeutic agents for cancer treatment.

Materials and methods

Collection of plant materials and authentication

Fresh leaves, stems, flowers, and fruits were gathered from Jolva village, located in the South Gujarat zone with the coordinates of 21.1719896, 73.0003919. The collected plant materials underwent botanical authentication (Voucher No-VNSGU/2019/09/TC-01) by Dr. Farzin M. Parabia, an associate professor and botanist at the Department of Bioscience in Veer Narmad South Gujarat University, Surat, Gujarat, India.

Preparation of crude extracts (CE)

To prepare the crude extracts, fresh plant parts were first washed thoroughly with water, then dried in an oven and finely blended into powder form. The resulting samples were then subjected to defatting using n-hexane through the Soxhlet extraction method. Next, the defatted extracts underwent another round of extraction using ethanol. The resulting extracts were then concentrated using a rotary vacuum evaporator (manufactured by Sigma Scientific Glass Pvt. Ltd. in Gujarat, India), and any remaining solvent was evaporated until dryness was achieved. Finally, stock solutions were prepared by dissolving the extracts in a precise quantity of solvents.

Preparation of alkaloid extracts (AE)

In order to isolate the alkaloids, a modified version of the 'acid–base shakeout' method described by Gonzales et al. (2014) was used. Initially, 25 g of powder from each plant part was defatted with petroleum ether, and the remaining marc was then moistened with 95% ethanol and made alkaline with ammonia (NH3). This mixture was macerated overnight and extracted with 95% ethanol using a Soxhlet apparatus. The resulting extract was concentrated to 1/4 of its original volume at 60 °C. Next, the concentrated ethanolic extract was treated with 1N HCl, filtered, and then treated with alkali (25% NH3) until the pH reached 9.0. The mixture was then transferred to a separating funnel and a measured volume of chloroform was added. The mixture was shaken five times and allowed to separate into two layers. The upper aqueous layer was then extracted with chloroform, and this process was repeated several times. The resulting chloroform layers were combined and concentrated at 60 °C to obtain a semi-dry residue. The weight of this residue was recorded and it was dissolved in DMSO. The % yield was calculated using the following formula:

$$\% \text{ Yield} = (\text{Weight of residue}) / (\text{Weight of initial dried powder}) \times 100.$$

Cell cultures and treatment

Four different cell lines, including two suspension cell lines (U937 and Jurkat J6) and two adherent cell lines (MCF-7 and HepG2), were procured from the National Center for Cell Sciences (NCCS) in Pune, Maharashtra, India. These cells were cultured in T-25 vented tissue culture grade flasks,

using RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 20 µg/ml Penicillin, and 100 µg/ml Streptomycin. The cells were maintained in a humidified CO2 incubator at 37 °C with 5% CO2. For experimental treatment, 1 mg/ml stock solutions of each plant extract were prepared in serum-free RPMI medium, filtered through 0.22 µm membrane filters, and used in final concentrations ranging from 0 to 100 µg/ml.

Peripheral blood mononuclear cells (PBMCs) isolation and culture

To evaluate the impact of *Nyctanthes arbor-tristis* extracts on normal human cells, PBMCs were isolated. This was done by using HisepTM LSM (Cat. No: LS001, HiMedia, India) density gradient to centrifuge heparinized blood. The blood was diluted with PBS (pH 7.4) in a 1:2 ratio, and 7.5 ml of diluted blood was gently layered on top of 2.5 ml of Hisep™ LSM. Care was taken to ensure that the layers did not mix, in order to achieve optimal separation. The mixture was then centrifuged at 2400 rpm (400 × g) for 20 min using a swinging bucket rotor.

After centrifugation, a buffy coat containing leukocytes and mononuclear lymphocytes was obtained above the Hisep™ LSM. This buffy coat was carefully aspirated and washed twice with an equal volume of isotonic PBS at 2000 rpm (160–260 × g) for 10 min at room temperature. Once the cells were washed, they were counted and seeded at a concentration of 1 × 104 cells/ml in a 96-well plate. The cells were then treated with plant extracts (6.25–100 µg/ml) for 24 h, after which a MTT cell viability assay was performed.

Cytotoxicity assays for preliminary screening

MTT dye reduction assay

A series of organic extracts were prepared and subjected to the MTT assay to assess their potential inhibition of cell growth. The assay used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction method with slight modifications based on the procedure described by Senthilraja and Kathiresan (2015). The MTT dye is reduced by viable cells in culture through their mitochondrial respiratory function, leading to the formation of a coloured formazan product that is taken up by the cells. The coloured formazan was then measured spectrophotometrically at 490 nm. To perform the assay, 1 × 104 cells/ml were plated and allowed to adhere for 24 h. Then, different concentrations of the test compound were added to the cells and incubated for an additional 24 h. After treatment, 10 µl MTT dye (5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37 °C to obtain coloured formazan products. Finally, 100 µl of Sorrenson's solubilizing buffer was added to each well, and the readings were taken at 490 nm. The % viability was calculated using the formula:

$$\% \text{ Viability} = (\text{Treated-Control}/\text{Untreated-Control} \times 100).$$

Additionally, the % cytotoxicity was calculated using the formula:

$$\% \text{ Cytotoxicity} = 100 - (\text{Treated-Control}/\text{Untreated-Control} \times 100).$$

LDH leakage assay

The LDH assay was used to evaluate the extent of cell membrane damage, which is indicated by the release of LDH into the surrounding media. This method followed the protocol described by Nadumane and Timsina (2014). Initially, cells were seeded at a density of 1×10^4 in 96 well plates and then incubated overnight at 37 °C in 5% CO_2. The plant extracts that exhibited good activity in the MTT assay were selected for the LDH assay. After 24 h of treatment with the plant extracts, the LDH assay was performed according to the instructions provided by the manufacturer of the Ezcount™ LDH cell assay kit (HiMedia). The appropriate assay controls were employed as specified in the protocol to ensure accuracy.

To conduct the LDH assay, 50 µl of the supernatant was taken from each well and transferred to a separate plate. Then, 50 µl of LDH reagent was added to each well, and the plate was placed in the dark for 2 h. Subsequently, 50 µl of solubilizing buffer was added to each well, and the absorbance was measured at 490 nm using a microplate ELISA reader (ELx800, BioTek). The LDH assay is a widely used method for investigating cell membrane damage and can be valuable in examining the effects of different treatments or compounds on cells.

Apoptotic assays for secondary screening

DNA fragmentation assay

The DNA fragmentation assay is commonly performed using an agarose gel. In this study, fragmented DNA was isolated following the protocol provided by the manufacturer of the Apoptotic DNA ladder kit (Roche Diagnostics, GmbH, Germany). HepG2 cells were cultured in RPMI-1640 media at a density of 2×10^6 cells/ml per well and treated with the

selected concentrations of the extracts that were identified in the LDH assay. After treatment, both adherent and floating cells were collected.

To isolate the DNA fragments, 200 µl of Binding/lysis buffer was added to the collected cells and mixed well. The mixture was incubated at 20 °C for 10 min, and then 100 µl of cold isopropanol was added to solubilize the DNA material. The resulting lysate was added to a spin column, which was then combined with a collection tube. The spin column was centrifuged at 8000 rpm for 1 min, and the flow-through was discarded. Next, 500 µl washing buffer was added to the spin column, and it was centrifuged at 8000 rpm for 1 min. This washing step was repeated for 10 s at maximum speed (13,000 × g). Finally, the spin column (filter tube) was placed on a clean collection tube, and 200 µl elution buffer was added. The mixture was centrifuged at 8000 rpm for 1 min to elute the DNA.

From the eluted DNA, 2 µl (250 ng/µl) aliquots were taken and run on a 1% agarose gel. The fragmented DNA was visualized and analysed for fragmentation under UV light after EtBr (Ethidium Bromide) treatment. The DNA fragmentation assay is a reliable method to detect apoptotic cells and is commonly used in research to investigate the mechanism of cell death.

TUNEL assay

The TUNEL assay is an immunohistochemical technique used to detect and quantify apoptosis (programmed cell death) at the single cell level, based on the labelling of DNA strand breaks by terminal transferase enzyme, which can be analysed by light microscopy. This method followed the protocol described by Chen et al. (2015) with slight modifications. The assay was performed according to the protocol of the in situ cell death detection kit, POD, from Roche Diagnostics, GmbH, Germany. Briefly, cells were cultured on coverslips at a density of 0.5×10^6 cells/ml and treated with plant extracts for 24 h. After treatment, cells were fixed with 4% formaldehyde/PBS (pH 7.4) for 60 min at room temperature. The cells were then washed with PBS and incubated with blocking solution (3% H2O2/Methanol) for 10 min. After another wash with PBS, the cells were incubated with permeabilization solution (0.1% Triton X-100 in 0.1% Sodium Citrate) for 2 min at 2 ° –8 °C. Next, cells were incubated with 50 µl of the TUNEL reaction mixture for 60 min at 37 °C in a dark, humidified chamber. After a final wash with PBS, the coverslip was removed, mounted, and analysed under a fluorescence microscope, where apoptotic cells appeared as green fluorescent cells.

The percentage of apoptotic cells was calculated using the following formula:

$$\% \text{ Apoptosis} = (\text{Number of Green Fluorescent cells}) / (\text{Total Number of cells}) \times 100.$$

Caspase-3 activity assay

Caspase 3 plays a crucial role in inducing programmed cell death by cleaving key proteins. The colorimetric-based Caspase-3 activity assay was performed according to the instructions provided by Thermofischer, USA. The experimental procedure involved culturing cells in tissue culture-grade Petri plates at a density of 3.5×106 cells/ml for 24 h. The following day, the cells were treated with plant extracts for an additional 24 h. After the treatment period, the cells were harvested using trypsin and transferred to 2 ml tubes, which were then centrifuged at 1000 rpm for 10 min. The spent media was removed, and the cell pellet was resuspended in 50 µl of chilled cell lysis buffer for 10 min. The cells were then centrifuged at 10,000 rpm for 1 min, and the supernatant was transferred to a 96-well plate and kept on ice. The protein concentration for each well was measured using the micro-Lowry assay (Peterson 1977), and protein concentrations greater than 200 µg/ml were diluted. Following the dilution, a reaction mix was prepared by mixing 1 M DTT (10 µl per ml of 2X reaction buffer). Subsequently, 50 µl of the reaction mix was added to each well, followed by the addition of 50 µl of 4 mM DEVD pNA substrate to achieve a final concentration of 200 µM. The plate was then incubated in the dark at 37 °C for 2 h, and the absorbance was measured at 405 nm using an ELISA Reader.

Morphological methods to detect apoptosis

Hoechst staining

Upon treatment with plant extracts, cells may undergo apoptosis, which could result in nuclear damage such as changes in nuclear morphology, DNA condensation, nuclear shrinkage, and nuclear fragmentation. Hoechst is a DNA staining dye that stains chromatin material. By using the method described by Li et al. (2017), condensed and fragmented chromatin can be visualized. To do this, cells were incubated for 24 h on a coverslip in a 6-well plate at a density of 3×10^5 cells/ml, followed by treatment with plant extracts for 24 h. After a PBS wash, cells were fixed with Carnoy's fixative (Methanol: Glacial Acetic acid-3:1) for 10–15 min. After rinsing the cells with PBS, they were stained with a working solution of Hoechst [1:10,000 in PBS] for 10–15 min in the dark at room temperature. After another PBS wash, the coverslip was removed, mounted with mounting agent (Glycerol: PBS- 9:1), and visualized under a fluorescence microscope using a DAPI filter.

Giemsa staining

Giemsa is a staining dye that can be used to stain cell membranes and cell nuclei, making it useful for staining apoptotic cellular morphology. The method used to stain HepG2 cells was described by Wu et al. (2011). Cells were seeded at a density of 3×10^5 cells/ml in a 6-well plate on a coverslip and treated with plant extracts for 24 h. After treatment, the cells were fixed with Carnoy's fixative for 5–10 min. Following a PBS wash, the fixed cells were trypsinized, centrifuged, and resuspended in fresh media. The resuspended cells were then dropped onto a clean slide and spread using another slide. After drying, the cells were stained with 5% Giemsa for 2 min at room temperature. Following staining, the slide was rinsed with a small amount of distilled water, air dried, and mounted using mountant (Glycerol: water-3:1). The slides were observed under a light microscope at 100X.

Molecular Studies of apoptotic pathways genes and Protein profiling

mRNA Profiling

The expression of well-known apoptotic and anti-apoptotic genes in the context of an anticancer drug (plant extracts) was investigated through a differential mRNA expression study using the Real Time PCR technique. mRNA isolation was carried out in accordance with the manufacturer's protocol of HiMedia (Cat. No: MB602, HiMedia, India). To induce apoptosis, cells were seeded at a density of 1×107 cells/ml and treated with selected plant extracts for 24 h. After the treatment, the cells were trypsinized and pelleted down. mRNA isolation was performed following the manufacturer's protocol of HiMedia (Cat. No: MB602, HiMedia, India). The isolated mRNAs were then ready to be used for cDNA synthesis. The content remains the same, but the language has been improved.

cDNA Synthesis

To study the differential mRNA expression via PCR technique, cDNA synthesis was carried out according to the manufacturer's protocol using TAKARA cDNA synthesis kit (Cat. No: 6110A, Takara Bio, India).

Real time PCR

In this study, intrinsic and extrinsic apoptotic pathway genes, including Bax, Bcl-2, p53, Caspase 3, Caspase 8, FADD (Fas-associated protein with Death Domain), AIF (Apoptotic inducing factor), and cell cycle analysis genes, such as Cyclin D, Cyclin E, CDK-4, were used in gradient PCR to optimize the annealing temperatures (Kim et al. 2011). The housekeeping gene β-actin was also used in the study. The RT-PCR was performed at the optimized annealing temperature, and the C_T (Cycle threshold) values were used to calculate the fold increase/decrease expressions of apoptotic genes compared to the control.

Immunocytochemistry

The expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 was analyzed through immunocytochemistry. The procedure followed the methodology outlined in Patel et al. (2019). Initially, HepG2 cells were cultured on sterile coverslips in a 6 well plate and treated with plant extracts for 24 h, with each treatment performed in triplicate. After 24 h, cells on coverslips were washed three times with PBS for 5 min each and fixed with 4% paraformaldehyde for 20 min. The cells were washed again with PBS and permeabilized with 0.1% TritonX-100 in 0.1% Sodium citrate for 15 min at room temperature. Following removal of the permeabilization solution, the coverslips were rinsed thrice and immersed in 10% FBS for 60 min at room temperature. Subsequently, the wells were incubated overnight at 4 °C with anti-Bax and anti-Bcl-2 Antibodies (Thermofischer Scientific, Rockford, USA) simultaneously at 1:100 concentrations. The next day, primary antibody was removed, and the wells were incubated with Goat anti-Mouse IgG (H + L) Secondary antibody tagged with FITC (Fluorescein Isothiocyanate, Thermofischer Scientific, Rockford USA) at 1:100 concentrations for 2 h at 37 °C in the dark. After washing with PBS, cells were stained with 1 µg/ml Hoechst for 30 min and incubated at 37˚C in the dark. Following incubation, cells were washed 2–3 times with PBS and mounted with mountant and observed under a fluorescence microscope (Nikon Eclipse e200). Images were captured using DAPI, FITC, and merged filters. Further analysis of images was carried out using image visualization software (AxioVision 4.7.2).

Table 1 Yield of plant extracts from different parts of *Nyctanthes arbor-tristis*s

Sr. no	Extracts	Concentration (mg/ml)
1	Leaf ethanol (LCE)	358
2	Stem ethanol (SCE)	210
3	Flower ethanol (FCE)	362
4	Fruit ethanol (FrCE)	40
5	Leaf alkaloid (LAE)	140
6	Stem alkaloid (SAE)	40
7	Flower alkaloid (FAE)	860
8	Fruit alkaloid (FrAE)	300

Table 2 Cytotoxicity of *N. arbor-tristis* extracts against different cell lines by LDH Assay

Extracts	Conc (µg/ml)	Cytotoxicity (%)			
		U937	Jurkat J6	MCF-7	HepG2
LCE	6.25	–	–	13.26 ± 0.005 ns	34.97 ± 0.009**
	12.5	–	–	–	35.45 ± 0.004**
	25	–	–	–	–
SCE	6.25	–	–	–	–
	12.5	–	–	–	–
	25	–	–	–	56.27 ± 0.044**
FCE	6.25	–	–	–	–
	12.5	–	–	–	39.26 ± 0.010**
	25	–	–	–	–
FrCE	6.25	–	8.18 ± 0.003 ns	–	51.98 ± 0.004**
	12.5	–	–	28.31 ± 0.008**	60.73 ± 0.049**
	25	–	–	–	–
LAE	6.25	–	–	27.60 ± 0.008**	51.57 ± 0.014**
	12.5	–	–	25.44 ± 0.003**	53.35 ± 0.015**
	25	–	–	–	–
SAE	6.25	–	–	–	–
	12.5	–	–	–	–
	25	–	28.46 ± 0.023**	–	59.93 ± 0.008**
FAE	6.25	–	–	–	–
	12.5	–	–	–	63.11 ± 0.028**
	25	–	–	–	–
FrAE	6.25	–	26.33 ± 0.006**	33.08 ± 0.084**	61.20 ± 0.016**
	12.5	–	–	45.51 ± 0.031**	68.51 ± 0.030**
	25	–	–	–	–

(*LCE* Leaf crude extract, *SCE* Stem crude extract, *FCE* Flower crude extract, *FrCE* Fruit crude extract, *LAE* Leaf alkaloid extract, *SAE* Stem alkaloid extract, *FAE* Flower alkaloid extract, *FrAE* Fruit alkaloid extract)

The values represent means ± SD of three individual experiments. The values were significant at *$p<0.05$, **$p<0.01$ with respect to untreated control using one−way ANOVA. *ns* non significant

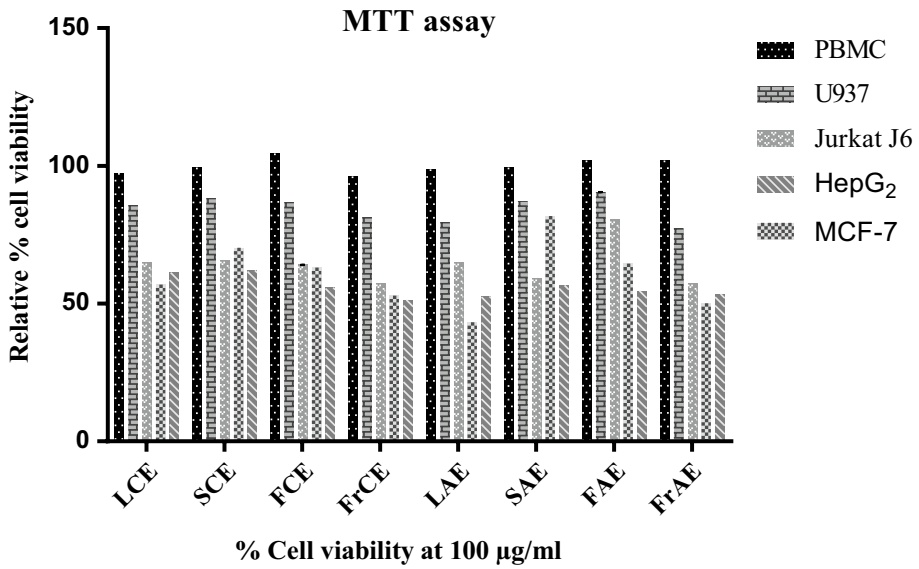

Fig. 1 The graphical representation of MTT assay showing % cell viability (at 100 µg/ml concentration) of different cancer cells and PBMCs treated with 100 µg/ml CE and AE of *N. arbor* from different parts. (*LCE* Leaf crude extract, *SCE* Stem crude extract, *FCE* Flower crude extract, *FrCE* Fruit crude extract, *LAE* Leaf alkaloid extract, *SAE* Stem alkaloid extract, *FAE* Flower alkaloid extract, *FrAE* Fruit alkaloid extract)

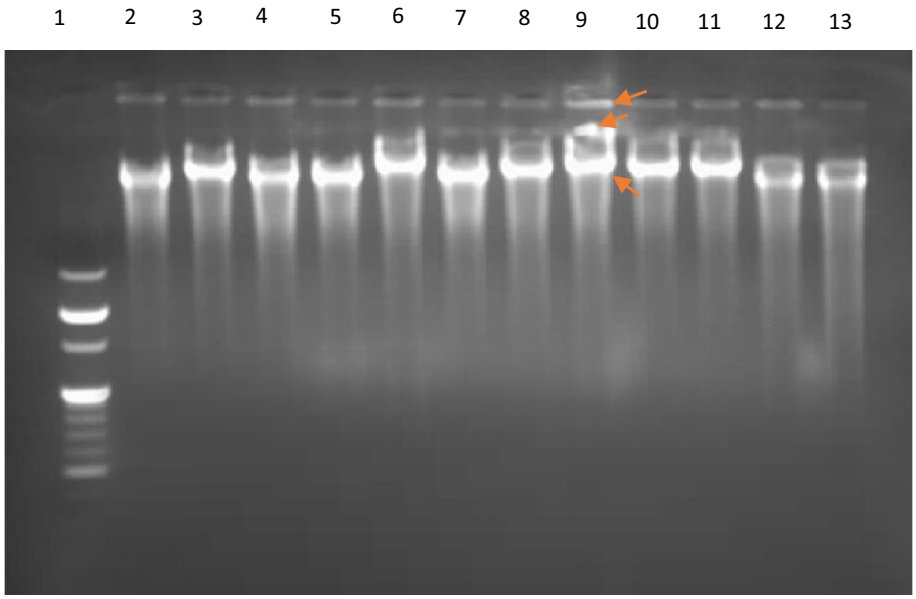

Fig. 2 An image of 1.5% agarose gel electrophoresis showing DNA fragmentation. (1) 100 bp DNA ladder, DNA isolated from HepG2 cells treated with extracts (2) LCE- 6.25 µg/ml (3) SCE- 25 µg/ml (4) LAE- 6.25 µg/ml (5) LAE—12.5 µg/ml conc. (6) FrCE—6.25 µg/ml (7) FrCE—12.5 µg/ml (8) FrAE- 6.25 µg/ml (9) FrAE- 12.5 µg/ml (10) SAE- 25 µg/ml (11) FCE- 12.5 µg/ml (12) FAE- 12.5 µg/ml (13) (UC- Untreated control, *LCE* Leaf crude extract, *SCE* Stem crude extract, *FCE* Flower crude extract, *FrCE* Fruit crude extract, *LAE* Leaf alkaloid extract, *SAE* Stem alkaloid extract, *FAE* Flower alkaloid extract, *FrAE* Fruit alkaloid extract)

Statistical analysis

To ensure statistical significance, all experiments were performed in triplicates, and the results were expressed as mean ± standard deviation (SD) using Excel. The normal distribution of the data was confirmed using the Shapiro–Wilk test. Values were considered significant at $*p < 0.05$ and $**p < 0.01$ compared to the control using one-way ANOVA followed by Tukey's post hoc analysis. Graphical representation with standard deviation bars was created using GraphPad Prism 9.0 software.

Results

Extracts were obtained from the leaf (LCE and LAE), stem (SCE and SAE), flower (FCE and FAE), and fruit (FrCE and FrAE) of *N. arbor* using Soxhlet extraction and acid–base shakeout methods, resulting in a total of eight extracts. The concentrations of each extract are presented in Table 1.

Effect of *N. arbor* extracts on cell proliferation

To assess the potential cytotoxicity of the extracts, a preliminary MTT dye reduction assay was conducted. The study evaluated the effect of CE and AE on four cell lines, namely U937, Jurkat J6, MCF-7, and HepG2. It was found that the extracts were effective in concentrations ranging from 6.25 to 25 µg/ml, and dose-dependent activity was not observed. A plateau phase was reached where increased dosage did not significantly affect the percentage inhibition of cancer cells. Supplementary Tables S1 and S2 show the results for cytotoxicity of CE and AE against different cell lines as measured by MTT assay.

The LDH leakage assay is a useful tool for determining cytotoxicity based on the loss of membrane integrity in apoptotic and necrotic cells (Ma et al. 2013). To confirm the

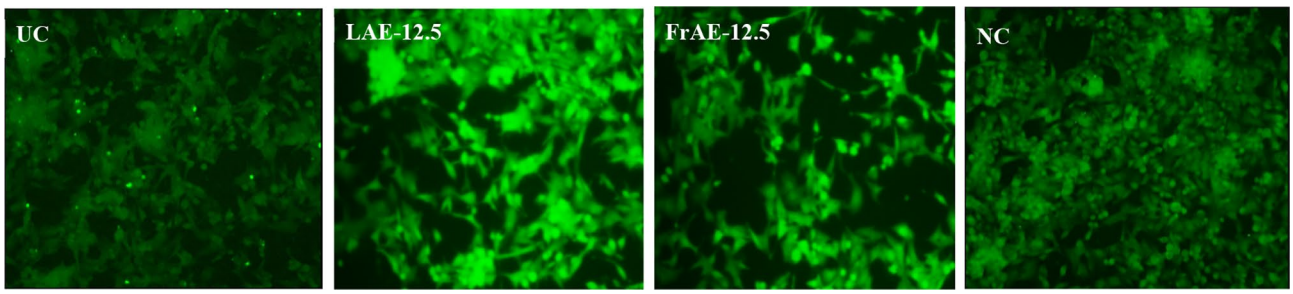

Fig. 3 Fluorescence micrographs of HepG2 cells treated with *N. arbor* extracts for 24 h. Viable cells did not show green fluorescence, whereas apoptotic cells emitted green fluorescence under fluorescence microscope at 10X magnification. *LAE* Leaf alkaloid extract, *FrAE* Fruit alkaloid extract, *UC* Untreated control, *NC* Negative control

results of the proliferation assay, we used lower concentrations with over 40% cytotoxicity for the LDH assay. Both CE and AE were found to be effective against the HepG2 cell line. In the case of CE, all four extracts showed cytotoxicity ranging from 34.97 to 60.73%, whereas in the case of AE, it was 51.57 to 68.515%. FAE and FrAE showed the best results with 63.11% and 68.51% cytotoxicity, respectively, at a concentration of 12.5 µg/ml. The relative trend in percentage cytotoxicity shown by different extracts in the case of CE was FrCE > SCE > FCE > LCE, whereas in the case of AE, it was FrAE > FAE > SAE > LAE against the HepG2 cell line. The results for the cytotoxicity of CE and AE against different cell lines by LDH are shown in Table 2. The cytotoxicity of CE and AE on PBMCs taken as normal cells using the MTT assay was also performed, which was found to be very negligible. The results are shown in supplementary Table S3. The relative percentage cell viability of different CE and AE against all cell lines used in the present study using the MTT assay clearly indicated that these extracts have no toxic effect on PBMCs, whereas they were found to be effective against the HepG2 cell line. Therefore, for further apoptotic studies, the HepG2 cell line was selected. The results are shown in Fig. 1.

Table 3 Percentage of apoptotic cells treated with *N. arbor* extracts against HepG2

Sr. no	Extracts	Conc. (µg/ml)	% Apoptosis
1	LCE	6.25	27.50 ± 0.97**
2		12.5	35.56 ± 1.31**
3	SCE	25	35.64 ± 1.78**
4	FCE	12.5	32.34 ± 1.61**
5	FrCE	6.25	39.72 ± 0.82**
6		12.5	31.83 ± 2.34**
7	LAE	6.25	38.33 ± 1.17**
8		12.5	48.54 ± 0.65**
9	SAE	25	31.90 ± 1.78**
10	FAE	12.5	31.15 ± 0.92**
11	FrAE	6.25	39.37 ± 1.37**
12		12.5	59.16 ± 0.38**
13	Untreated control	–	9.84 ± 2.15
14	Negative control	–	0.00 ns

(*LCE* Leaf crude extract, *SCE* Stem crude extract, *FCE* Flower crude extract, *FrCE* Fruit crude extract, *LAE* Leaf alkaloid extract, *SAE* Stem alkaloid extract, *FAE* Flower alkaloid extract, *FrAE* Fruit alkaloid extract)

The values represent means ± SD of three individual experiments. The values were significant at *$p<0.05$, **$p<0.01$ with respect to untreated control using one−way ANOVA

Apoptotic studies

DNA laddering assay

DNA laddering is a distinctive biochemical feature of apoptosis that is triggered by caspase-activated DNase (CAD). The activation of the caspase cascade leads to the cleavage of DNA at intra-nucleosomal linker sites, resulting in the formation of fragments that are approximately 180–200 base pairs in length, which are commonly referred to as DNA fragments. To explore whether *N. arbor* extracts can induce apoptosis in HepG2 cells, we conducted a DNA laddering assay. Although the majority of CE and AE did not exhibit a typical DNA laddering pattern, we observed a ladder-like pattern in the case of FrAE at a concentration of 12.5 µg/ml, as highlighted by the red arrows in Fig. 2. This finding suggests that the fruit alkaloids extract may contain phytoconstituents that facilitate apoptosis.

Quantification of apoptosis by TUNEL assay

To confirm and quantify the induction of apoptosis by *N. arbor* extracts in HepG2 cells, we conducted a TUNEL assay. This assay detects the presence of apoptotic bodies by measuring the degree of DNA fragmentation/breaks, which results in fluorescent cells. As depicted in Fig. 3, after exposure to selected concentrations of both CE and AE for 24 h, apoptotic bodies were formed. FrAE (12.5 µg/ml) and LAE (12.5 µg/ml) exhibited the highest number of apoptotic bodies. We determined the percentage of apoptosis by directly counting the number of green fluorescent cells. At a concentration of 12.5 µg/ml, a relatively high percentage of apoptotic cells, i.e., 59.16% and 48.54%, were observed in FrAE and LAE of N. arbor, respectively, as shown in Table 3.

Morphological studies of apoptotic cells

We utilized Hoechst stain to examine the morphological characteristics of apoptotic cells, which typically exhibit condensed and fragmented nuclei, and Giemsa stain to observe cytoplasm blebbing and dead cells. Our analysis revealed no significant morphological alterations in the majority of CE and AE extracts. However, LAE and FrAE at a concentration of 12.5 µg/ml displayed noticeable changes in morphology, as depicted by the coloured arrows in Fig. 4. Therefore, we selected these two extracts, LAE and FrAE, for additional molecular investigations of the apoptotic pathway.

Caspase-3 activity assay

We observed no substantial elevation in Caspase-3 activity in the majority of the selected CE and AE at different

concentrations, except for SCE and FrAE at concentrations of 25 and 12.5 µg/ml. Caspase-3 activity is considered a critical marker of apoptosis among several Caspases. Our findings are illustrated in Fig. 5.

Molecular studies

At mRNA level using RT-PCR

A detailed molecular study was conducted to determine the probable apoptotic pathway utilized by *N. arbor* extracts in HepG2 cells at the mRNA level using Real Time (RT) PCR. Two extracts, LAE and FrAE, were used for the study, and treatment was given for 24 h followed by mRNA isolation and cDNA synthesis. Primers for Bax, Bcl-2, Caspase-3, p53, FADD, Caspase-8, and AIF genes were used in PCR with β-Actin as a reference (Table S4). The relative expression of all target genes with reference was determined using the $2^{-\Delta\Delta CT}$ (Livak) method. The levels of AIF, FADD, and Caspase-8 were found to be 8402.8, 642.11, and 5454.8 folds higher, respectively, in FrAE-treated HepG2 cells compared to untreated cells, whereas no CT values were obtained for p53 and Caspase-3 genes (Fig. 6). In our study, Bax, FADD, AIF, and Caspase-8 genes were found to be upregulated, indicating that apoptosis was triggered through the Caspase-dependent extrinsic pathway. The Bax/Bcl-2 ratio was also altered, as the gene expression of Bax was upregulated while that of Bcl-2 was downregulated upon treatment with LAE and FrAE of *N. arbor* (Fig. 6). This suggests that the fate of the apoptotic stimuli is determined by the ratio of concentrations of the Bax and Bcl-2 family proteins, as reported by Kale et al. (2018) and Senichkin et al. (2020).

At protein level using Immunostaining

An immunocytochemistry assay was conducted to investigate the specific alteration of Bax/Bcl-2 ratio at the protein level. The results revealed that FrAE of *N. arbor* induced a higher fluorescence signal of Bax protein, indicating an

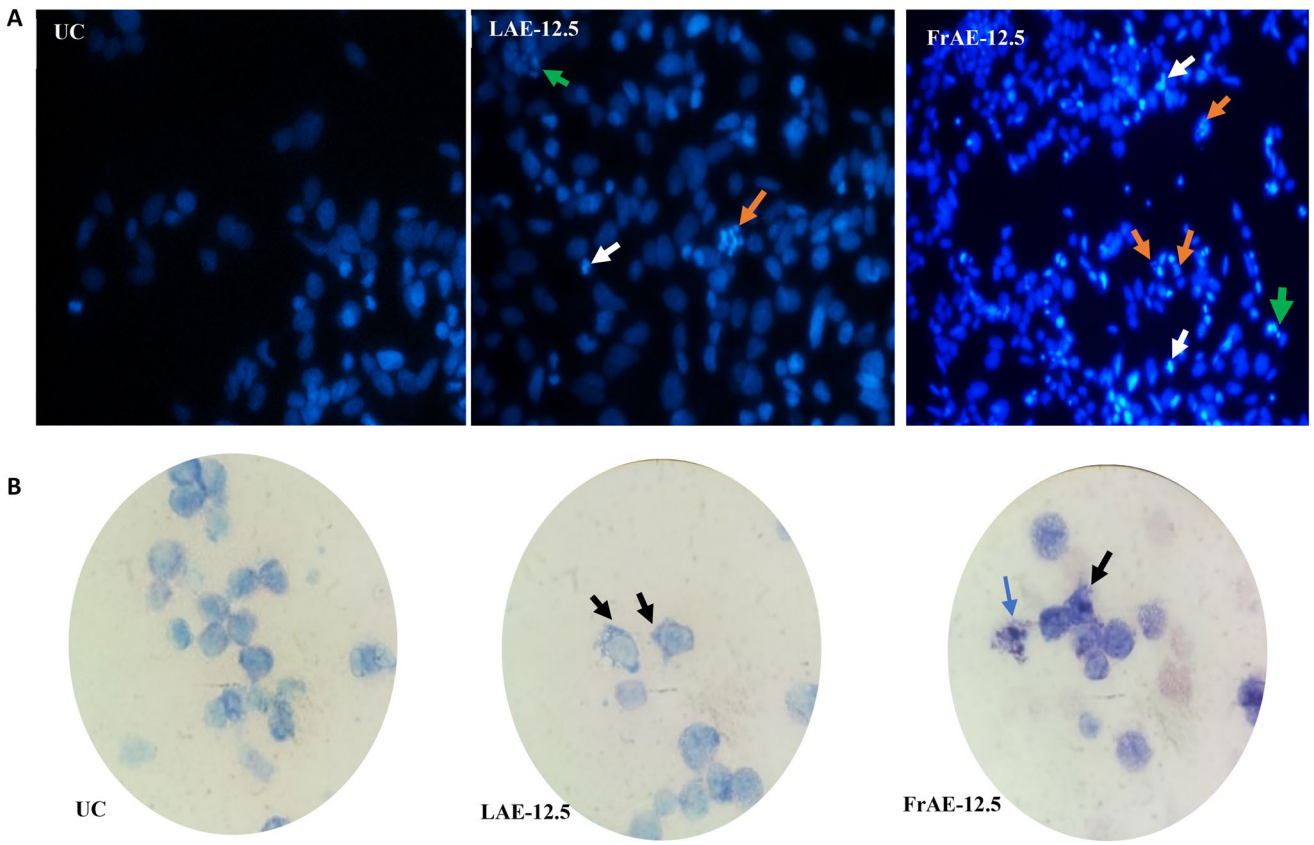

Fig. 4 **A** Induction of apoptosis in HepG2 cells treated with LAE and FrAE at 12.5 µg/ml concentration observed using Hoechst 33,258 stain under fluorescence microscope (10X). Where control cells with normal blue colour nuclei without nuclear condensation, fragmentation. In contrast to control cells, nuclear condensation (White arrow), nuclear fragmentation (Red arrow) and apoptotic bodies (Green arrow) were observed in treated HepG2 cells. **B** When HepG2 cells stained with Giemsa stain, normal cells appeared with blue colour nuclei whereas treated cells appeared with blebbing cytoplasm (Black arrow) and dead cells (blue arrow) observed under light microscope (400X). *UC* Untreated control, *LAE* Leaf alkaloid extract, *FrAE* Fruit alkaloid extract

increase in its expression, while a decreased fluorescence signal of Bcl-2 was observed, suggesting a decrease in its expression. This altered expression resulted in an increased Bax/Bcl-2 protein ratio, as depicted in Fig. 7.

Cell cycle arrest analysis

Cyclin D and CDK-4 are linked to the G1 phase of the cell cycle, while Cyclin E is connected with the transition from G1 to S phase. Our analysis revealed negligible CT values for cyclin E, indicating that HepG2 cells treated with LAE and FrAE for 24 h were arrested at the G1 phase and unable to progress into the S phase of the cell cycle. These outcomes are depicted in Fig. 8.

Discussion

Alkaloid extracts of *N. arbor* from different parts of the plant were isolated and tested for their cytotoxicity on four cell lines. The crude and alkaloid extracts of different plant parts did not show any significant activity against U937 and Jurkat J6 cell lines, while the MCF7 and HepG2 cell lines were found to be responsive. The MTT and LDH assays showed significant cytotoxicity in LAE and FrAE. To ensure that these extracts do not have any cytotoxic effect on normal cells, PBMC cells were used and no cytotoxicity was observed.

Morphological and biochemical changes can be used to assess apoptotic induction, such as nuclear and cellular

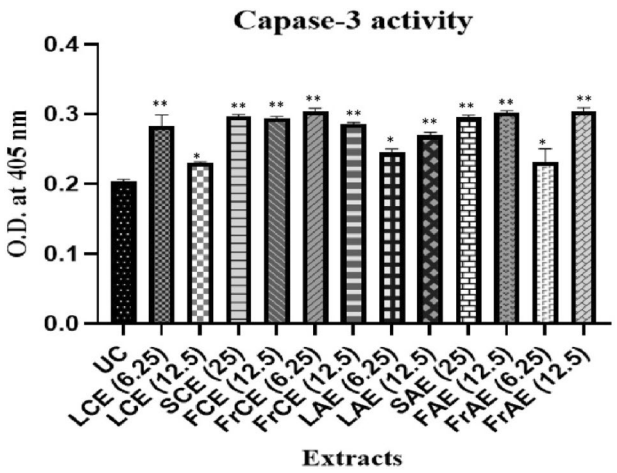

Fig. 5 Caspase-3 activity in HepG2 cells treated with *N. arbor* extracts for 24 h. The values represent means ± SD of three individual experiments. The values were significant at $*p < 0.05$, $**p < 0.01$ with respect to untreated control using one-way ANOVA. (*UC* Untreated control, *LCE* Leaf crude extract, *SCE*- Stem crude extract, *FCE* Flower crude extract, *FrCE* Fruit crude extract, *LAE* Leaf alkaloid extract, *SAE* Stem alkaloid extract, *FAE* Flower alkaloid extract, *FrAE* Fruit alkaloid extract)

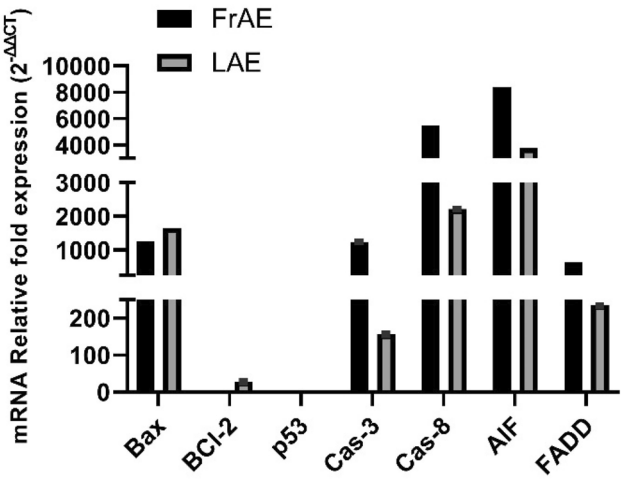

Fig. 6 Comparative study of fold expressions of apoptotic pathways genes in Fruit and leaf alkaloid treated HepG2 cells via RT-PCR technique. The values represent the means ± SD of three individual experiments. Abbreviations: *FrAE* Fruit alkaloid extract, *LAE* Leaf alkaloid extract

changes, DNA fragmentation, cytoplasmic condensation, cytoplasmic blebbing, and membrane shrinkage (Majtnerova and Rousar 2018). In our study, we did not observe typical DNA laddering, but DNA fragments were visible in FrAE at a lower concentration of 12.5ug/ml. The TUNEL assay showed that these extracts induced 59.16 ± 0.38% and 48.54 ± 0.65% apoptosis. The results obtained from both the LDH and apoptotic assays were consistent with each other, indicating that the leaf and fruit alkaloid extracts were most effective against HepG2 cells. These two extracts were further investigated to understand the mechanism of apoptosis via RT-PCR studies.

There are two pathways for activating apoptosis in cancer cells, namely, the intrinsic and extrinsic pathways (Fig. 9A). The p53 protein targets Bcl-2 family proteins, which are composed of pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl-2, Bcl-XL, Bcl-W, MCL-1BFL-1/A1) proteins (Kale et al. 2018). Upregulation of Bax and downregulation of Bcl-2 lead to the activation of Caspases (Mbazima et al. 2008). AIF (apoptosis-inducing factor), which is released by mitochondria upon Bax upregulation, leads to Necroptosis, a programmed form of necrosis (Ma et al. 2013) (Fig. 9B). In our study, we found that the Bax/Bcl-2 ratio was 7451.59-fold higher when treated with FrAE and 58.62-fold higher when treated with LAE compared to the untreated control in HepG2 cells. Interestingly, we did not find any upregulation of the p53 gene. Thus, it may be a p53-independent induction of mitochondrial-mediated (intrinsic pathway) apoptosis. We also found upregulation of the AIF gene, which is a caspase-independent mode of apoptosis. Minutolo et al. (2012) and Cheng et al. (2005) documented similar results

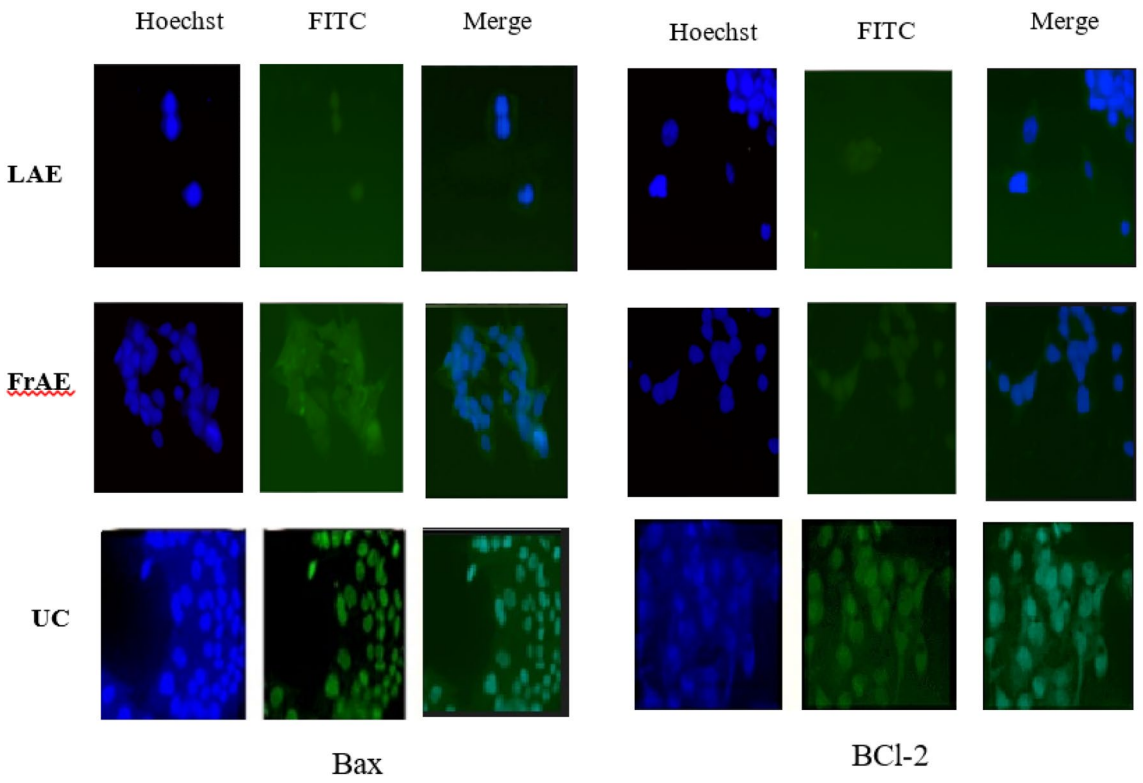

Fig. 7 Immunostaining for Bax and BCl-2 proteins in HepG2 cells treated with *N. arbor* extracts. LAE- Leaf alkaloid extract (12.5 µg/ml), FrAE- Fruit alkaloid extract (12.5 µg/ml)

showing the link between pro-apoptotic Bax activation and the release of AIF in the cytosol. They related this pattern of gene expression to chromatinolysis (DNA fragmentation) and subsequent loss of cell viability.

In the extrinsic pathway, Proapoptotic receptor agonists (PARAs) target death receptors and cause killing of tumor cells that may be resistant to standard chemotherapy. Proapoptotic ligands, such as Apoptosis Ligand 2/Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (Apo2L/TRAIL), bind with specific surface cell death receptors (DRs) and activate them. Active DRs form a Death-Inducing Signaling Complex (DISC) by recruiting Fas-Associated Death Domain (FADD) and procaspase-8 and 10. Further activation of Caspase 8 and 10 leads to apoptosis (Fulda and Debatin 2006). Apo2/TRAIL has significance in cancer therapy and acts via a p53 independent pathway. Bioactive compounds in our extracts downregulated p53 and upregulated FADD and Caspase-8 genes, indicating the probability of a p53 independent extrinsic pathway. The active alkaloids from our extract may act as proapoptotic receptor agonists (PARAs) (Fig. 9C). PARAs have benefits as a combinatorial therapy or a novel option to restore chemotherapeutic sensitivity (Fulda and Debatin 2006; Ashkenazi 2008).

Caspase-3 is an executioner caspase in apoptosis because it plays a role in coordinating the destruction of various cellular structures such as DNA and cytoskeletal proteins (Wang et al. 2019). We observed some upregulation of caspase 3 enzyme with some of the CE and AE of *N. arbor*. Currently, most of the anticancer strategies used in clinical oncology involve the activation of apoptosis signal

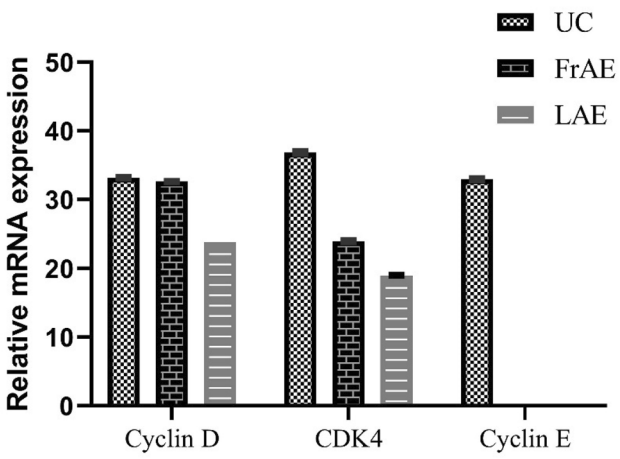

Fig. 8 The elevated G1 arrest in cell cycle by LAE (Leaf alkaloid extract) and FrAE (Fruit alkaloid extract) of *N. arbor* was assessed by relative mRNA levels using RT-PCR

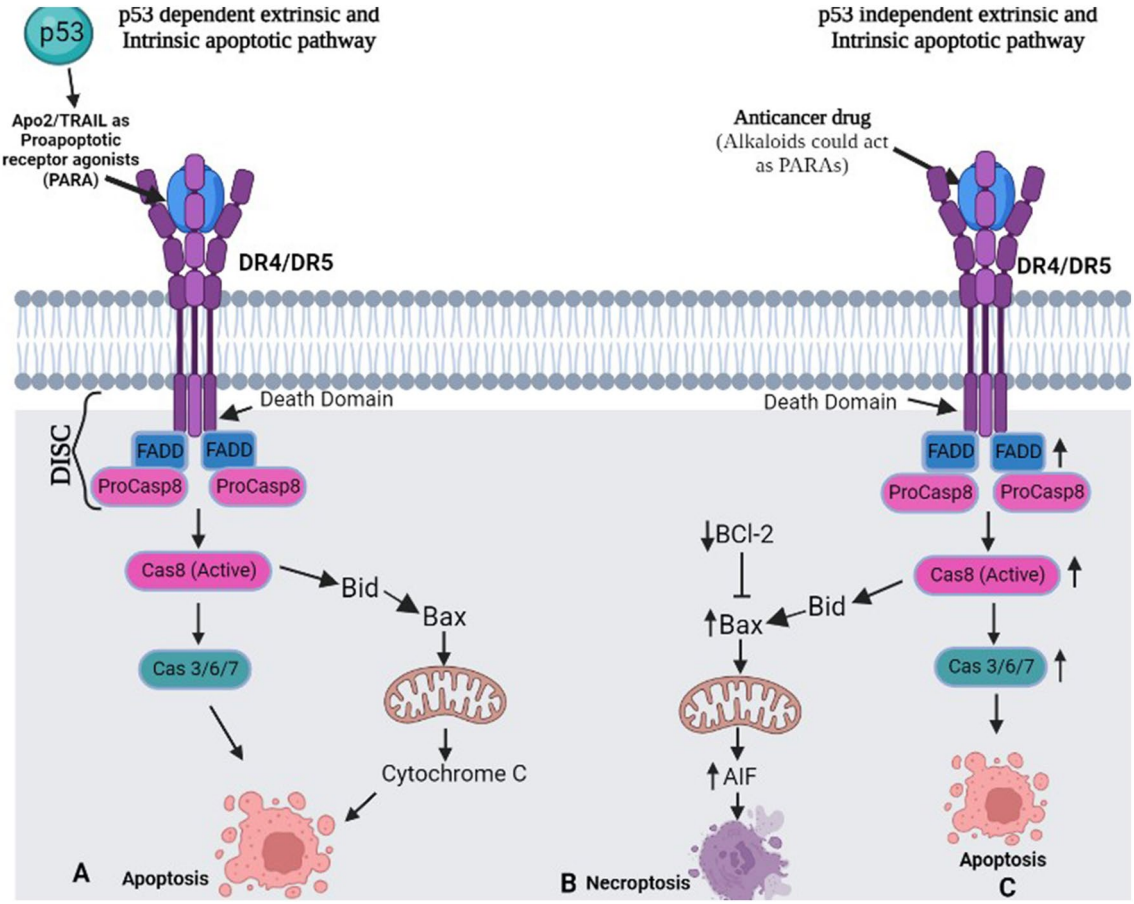

Fig. 9 Mechanism of p53 independent extrinsic and intrinsic apoptotic pathways

transduction pathways such as intrinsic and extrinsic in cancer cells (Ashkenazi 2008).

Upon cellular stress, there are several biochemical pathways that restrain cell cycle transition and induce cell death, which are called cell cycle checkpoints (Wenzel and Singh 2018). In line with Ma et al. (2013), we observed cell cycle arrest in our study. All these observations suggest that the LAE and FrAE of *N. arbor* induce a p53 independent apoptotic pathway in HepG2 cells.

Conclusion

The study provides promising evidence for the potential use of *N. arbor* alkaloids in combination with conventional chemotherapeutic agents for the treatment of cancer. This combination therapy could help overcome the common problem of chemotherapeutic resistance and target the proliferation of cancer cells, leading to cell cycle arrest. The effectiveness of this approach is supported by the findings of the gene-level apoptotic studies. However, further validation through p53-deficient cancer cells and in vivo studies is necessary before the clinical use of *N. arbor* alkaloids in cancer therapy can be confirmed.

Declarations

Conflict of interest The authors did not receive support from any organization for the submitted work. The authors have no competing interests to declare that are relevant to the content of this article. There is no conflict of interest.

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