Chinnasamy *et al.*
Future Journal of Pharmaceutical Sciences (2024) 10:141
https://doi.org/10.1186/s43094-024-00715-0

Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Effect of crude ethanolic seed extract from *Mucuna pruriens* on proliferation, apoptosis, and cell cycle arrest in gastric adenocarcinoma (AGS) cells

Arulvasu Chinnasamy[1*], Vennila Jayaprakash[1], Deepakrajasekar Padmanaban[1], Niranjni Sekar[1], Rajasekar Valayapathi[1], Aarthi Azhagudurai[1] and Sumathi Ethiraj[2]

Abstract

Background Gastric cancer is a prevalent form of malignancy among many common carcinoma cases globally. This study was designed to assess the anticancer potential of the crude ethanolic seed extract from *Mucuna pruriens* against the gastric cancer cell line (AGS). Various assays were employed to assess the anticancer properties, including examinations of cell viability, nuclear morphology, apoptosis using AO/EB staining, changes in mitochondrial membrane potential, lactate dehydrogenase activity, DNA fragmentation, and cell cycle arrest.

Results The crude extract exhibited significant anticancer activity against the human gastric cancer cell line (AGS), as determined by the MTT assay, with an inhibition concentration (IC_{50}) of 600 µg/mL at 24 h. Distinct cellular and nuclear morphological changes were observed with different concentrations of crude ethanolic seed extract. The LDH release assay reveals cell death in AGS cells, as evidenced by a significant increase in the release of LDH enzyme. DNA fragmentation analysis and flow cytometry results indicate that the extract induces chromatin condensation, apoptotic cell death, and cell cycle arrest at the G0/G1 phase in the AGS cancer cell line. These results highlight the potential therapeutic advantages of *Mucuna pruriens* seed extract against gastric cancer cells.

Conclusion This study could pave the way for identifying diverse natural bioactive compounds sourced from *Mucuna pruriens* seed, leading to the development of novel drug with potential anticancer properties.

Keywords *Mucuna pruriens*, Gastric cancer, Apoptosis, AGS cells, Flow cytometry

Background

During the preceding decades, cancer has risen to prominence as a significant concern within the realm of public health. Normal cells undergo a series of multistep changes leading to their transformation into cancer cells, influenced by factors such as inadequate nutrition, obesity, alcohol consumption, genetic mutations, urbanization, an unhealthy lifestyle, and exposure to air pollution. Based on the Global Cancer Observatory (GLOBOCAN) 2020 report from the International Agency for Research on Cancer (IARC), estimates for new cancer cases and mortality rates were provided for 38 cancer types across 185 countries. Across the globe, 19.3 million individuals were newly diagnosed with cancer, and 10.0 million succumbed to the disease. The top-ranking cancers worldwide include breast cancer in

*Correspondence:
Arulvasu Chinnasamy
carulvasu@gmail.com
[1] Department of Zoology, University of Madras, Guindy Campus, Chennai, Tamil Nadu 600 025, India
[2] Department of Biotechnology, University of Madras, Guindy Campus, Chennai, Tamil Nadu 600 025, India

females, lung cancer, and prostate cancer due to their widespread occurrence. Among the leading factors that lead to cancer-related mortality internationally are lung cancer, liver cancer, and stomach cancer [1, 2].

Stomach cancer, also known as gastric cancer, stands as the third most common cause of cancer-related deaths globally, with over 1 million new cases resulting in approximately 769,000 deaths. This complex and aggressive disease is characterized by its multifaceted nature, high prevalence, polygenic origins, and severity [3]. The World Health Organization (WHO) highlights stomach cancer as one of the top five most prevalent cancer diagnoses [4]. Gastric cancer exhibits a higher prevalence among males compared to females and is predominantly found in regions such as China, Japan, and Korea. While there has been a general decrease in cancer-related deaths in recent years, the incidence of new cases of gastric cancer has risen [5]. The progression of gastric cancer spans from the cellular to the molecular level, displaying a diverse range of developments. Moreover, gastric cancer is characterized by a multistep process that involves concurrent molecular and genetic abnormalities. Consequently, researchers are motivated to explore more effective strategies for the treatment of gastric cancer [6].

Standard therapies for gastric cancer encompass surgical resection of cancer cells, chemotherapy, radiation therapy, or a combination of chemotherapy and radiotherapy (chemoradiotherapy). Despite the significant advantages of these conventional treatments, the prevalence of the disease continues to rise, and late-stage diagnosis complicates treatment. As a result, there is an urgent need to investigate alternative ways to conventional chemotherapy, which may have drawbacks such as suspension of bone marrow function, nausea, vomiting, and antibiotic resistance [7].

Natural chemotherapy has emerged as a promising avenue for treating gastric cancer. In many cases, radiation therapy is combined with chemotherapy and surgery to address the complexities of gastric cancer treatment. Successful cancer therapies should effectively halt the growth of cancer cells while minimizing harm to healthy cells. Recognizing and refining such therapies can reduce or eliminate adverse effects on the immune system. One approach involves inducing apoptosis in cancer cells, aiming to achieve a state where these cells undergo programmed cell death [8]. Nowadays, numerous phytochemical agents have shown their valuable characteristics, such as anticancer and pharmacological properties [9]. These phytochemicals are mostly found in vegetables, fruits, spices, and soy, offer numerous health benefits and are known for being non-toxic and readily available. A multitude of studies have explored the essential role of phytochemicals in the prevention and treatment of gastric cancer [10].

Plant extracts comprise a blend of metabolites distinguished by varying chemical properties and structures. Drugs derived from these extracts are recognized as highly effective chemotherapeutic drugs, drawing from natural origin. Therefore, researchers are actively seeking new compounds that exhibit lower toxicity towards non-cancerous cells [5]. The velvet bean, scientifically known as *Mucuna pruriens*, is a plant renowned for its regenerative properties in pharmaceutical and therapeutic applications. Rich in essential phytoconstituents such as tannins, flavonoids, alkaloids, and phenolic compounds, it exhibits promising pharmacological properties. It is widely consumed as a food source in Asia, America, Africa, and the Pacific Islands. The seeds of this plant contain abundant fiber, carbohydrates, and proteins, as well as important minerals, essential amino acids, and low levels of fats. *Mucuna pruriens* demonstrates diverse pharmacological activities, including antioxidant, anticancer, anticholesterolemic, anti-Parkinson's, antidiabetic, sexually enhancing, anti-inflammatory, antibacterial, and antivenom properties [11]. Different extracts derived from *M. pruriens* seeds, *Asteracantha longifolia* seeds, and *Sphaeranthus indicus* stems displayed cytotoxic effects against MCF7 and A549 cell lines, highlighting the potential of plants as a valuable reservoir of anticancer properties [12]. In another investigation, it was reported that the combination of doxorubicin with the aqueous leaf extract of *M. pruriens* displayed significant potential against several carcinoma cell lines [13]. Therefore, in this present study, we investigated the anticancer capabilities of the ethanolic seed extract derived from *Mucuna pruriens* on the human gastric cancer cell line (AGS).

Methods

Collection of *Mucuna* pruriens

The plant specimens of *M. pruriens* were collected from Mahadevan hills in Kanguppam in Vellore district of Tamil Nadu State, India (12.934968°N, 79.14688°E) during May – October 2023. The mature and desiccated pods were collected and threshed manually by placing them in a bag and gently beating or rubbing it to release the seeds. The separated seeds were cleaned with tap water to eliminate extraneous impurities, oven-dried at 50 °C until dry weight stability was observed, and subsequently pulverized using an electric grinder. Three days were spent macerating 500 g of powdered seed materials and then used in the further extraction process. The taxonomic identification and authentication of plants were carried out by the Senior Professors Centre for Advanced Studies in Botany, University of Madras.

Preparation of seed extract

About 500 g of *M. pruriens* seed powder was dissolved in 2000 mL of solvent, ethanol, extracted using a Soxhlet apparatus until the solvent became colorless. The ethanol extract was then concentrated in a rotary vacuum evaporator at 40 °C under lowered pressure and temperature. Following complete evaporation, the yield of the 28 g extract was quantified and stored in a sealed container for further use. Then, the dried seed extract of *M. pruriens* was dissolved using Dimethyl sulfoxide (DMSO) and filtered using a 0.2 µm syringe filter, and finally made into a stock solution of 100 mg/mL and stored at − 20 °C for further use [14].

Cell line and chemicals

The normal (Vero) and cancer cell lines (AGS) were obtained from the National Center for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Trypsin–EDTA, 3-(4, 5-Dimethyl Thiazol-2yl)-2,5-Dimethyltetrazolium Bromide (MTT), Dimethyl Sulfoxide (DMSO), Sodium Bicarbonate, and antibiotic solution were purchased from Hi-Media Laboratories Ltd., Mumbai, India. 96 well plates, 6 well plates, tissue culture flasks (25–75 mm^2), and centrifuge tubes were purchased from Tarsons Products Pvt. Ltd., Kolkata, India. All other chemicals used in the present study were analytical grade.

MTT assay

Cytotoxicity and cell viability were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [15]. The vero and AGS cells were cultured in DMEM media along with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 humidified incubator. Initially, the vero cells (5×10^3 cells/mL) were seeded in 96-well plates and treated with various concentrations ranging from 200, 400, 600, 800, and 1000 µg/mL for 24 h. Similarly, gastric adenocarcinoma (AGS) (5×10^3 cells/mL) was seeded and treated with 200, 400, 600, 800, and 1000 µg/mL of crude extract for 24 h. After the treatment period, 10 µL of 10 mg/mL MTT solution was added to the wells and allowed to incubate for 4 h. Subsequently, the formazan crystals developed were dissolved by adding DMSO (100 µL) and the absorbance was measured at 570 nm using a microplate reader. The percentage of cell viability was calculated using the following formula.

$$\text{Cell viability} = \text{A570 of treated cells} \times 100$$

A570 of control cells.

Analysis of cell morphology

Morphological examinations were conducted using an inverted light microscope (Nikon Eclipse TS100). Gastric cancer cells (5×10^3 cells/mL) were seeded on 6-well plates with DMEM medium supplemented with 10% FBS and incubated at 37 °C under 5% CO_2. Various concentrations of crude ethanolic seed extract minimum (200 µg/mL), IC50 (600 µg/mL), and maximum (1000 µg/mL) were added to the cells and allowed to incubate for 24 h. Then, the cells were observed under an inverted microscope at 20× magnification and photographed [16].

Nuclear morphological examination

Fluorescent staining was conducted to visualize nuclear damage in the cells [17]. AGS cells were plated at a density of 1×10^4 cells/mL in 6-well plates containing DMEM medium with 10% FBS. After 24 h of treatment, the cells were initially washed with phosphate-buffered saline (PBS) and then fixed using methanol-acetic acid (3:1 v/v) for 10 min, and then stained with 10 µg/mL of fluorescent propidium iodide for 20 min. The nuclear morphological changes were examined using a fluorescence microscope at 20× magnification (Nikon Eclipse E200).

Apoptosis analysis by acridine orange/ethidium bromide

Apoptotic changes in cell morphology were evaluated using differential staining with acridine orange and ethidium bromide [18]. Approximately 5×10^3 cells were seeded in six-well plates and cultured in a humidified incubator with 5% CO_2 at 37 °C for 24 h. Following this, the cells were exposed to crude ethanolic seed extract at concentrations of 200 µg/mL, 600 µg/mL, and 1000 µg/mL, and then further incubated for 24 h. After incubation, the cells were stained with AO/EB solution (100 µg/mL) and observed using a fluorescence microscope (Nikon Eclipse E200) at 20× magnification.

Measurement of mitochondrial membrane potential

For observing the mitochondrial membrane potential (MMP), Rhodamine 123 (RH-123) was used [19]. AGS cells (3×10^3 cells/well) were treated with the minimum (200 µg/ mL), IC_{50}(600 µg/ mL), and maximum (1000 µg/mL) concentrations of the crude ethanolic seed extract of *M. pruriens* and incubated for 24 h. After treatment, the cells were washed with PBS pH (7.4) and incubated with 10 µM of Rhodamine-123 for 10 min. The green filter was used to detect the potential of mitochondrial membrane. Images were taken and documented using a fluorescence microscope (Nikon Eclipse E200) at 20× magnification.

LDH leakage assay

Lactate dehydrogenase (LDH) leakage was used to evaluate the cell membrane integrity and cell viability based on the release of LDH from the cytosol into the surrounding media [20]. AGS cells were seeded in 6-well plates at a density of 3×10^4 cells per well in a DMEM culture medium for 24 h. Cells were treated with selected concentrations of the crude ethanolic seed extract, and it was further incubated for 24 h. The control and treated cell medium were added to the tubes containing 1 ml of buffered solution (125mLof glycine buffer containing 7 mL of 0.1 N sodium hydroxide solution, 2.75 g of lithium lactate was dissolved freshly) and then incubated for 15 min at 37 °C. Then, 0.2 ml of NAD + solution was added and further incubated for 20 min. After that, the reaction was stopped by adding 1 mL of 2,4-Dinitrophenylhydrazine (DNPH) reagent, and incubated for 15 min at 37 °C. After the incubation time, 7 ml of 0.4 N NaOH solution was added, and the color formation was read spectrophotometrically at 420 nm in a Shimadzu UV-Spectrophotometer.

DNA fragmentation analysis

DNA fragmentation stands as a crucial biochemical indicator of apoptosis in the majority of cells. AGS cells (5×10^3 cells/mL) were seeded and treated with 200 µg/mL, 600 µg/mL, and 1000 µg/mL concentrations of crude seed extract. After a 24-h incubation period, the cells were harvested, centrifuged for 5 min at 4 °C, and then washed twice with PBS at pH 7.2. Following this, cell lysis was carried out using a buffer solution containing Tris–HCl (0.05 M, pH 8.0), EDTA (0.01 M), NaCl (0.1 M), and SDS (0.5%). DNA extraction was performed using a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1), followed by precipitation with 7.5 M ammonium acetate and ethanol. The resulting DNA was then reconstituted in TE (Tris–EDTA) buffer. Subsequently, the DNA samples were subjected to electrophoresis on a 0.8% agarose gel, and visualization was achieved using a gel documentation system (GELSTAN) [21].

Cell cycle analysis by flow cytometry

Cell cycle changes were evaluated using flow cytometry to determine the proportion of cells in each phase. Cell cycle perturbations caused by the crude ethanolic seed extract were analyzed using propidium iodide DNA labeling. 2×10^5 cells per well were plated in six-well plates, allowed to attach, and then incubated for 24 h. Then, the cells were treated with 200 µg/mL, 600 µg/mL, and 1000 µg/mL of crude ethanolic seed extract for 24 h. After incubation, the cells were collected, fixed in ice-cold 70% ethanol for 4 h, and kept at 4℃. The cells suspended in ethanol were centrifuged at 1000 rpm for 5 min, then rinsed twice in PBS to remove any remaining ethanol. Pellets were suspended in 1 mL of PI/RNase A reagent and incubated at 37 °C for 30 min. Flow cytometry analysis was conducted using a 575-nm band filter (Beckman-Coulter) [22].

Statistical analysis

The results were presented as means ± standard deviation (SD) and analyzed for one-way ANOVA following Dunnett's test to determine the significant differences between control and treated groups using SPSS version 20.0 software (Chicago, IL). $p<0.05$, 0.01, or 0.001 was considered to be statistically significant.

Results

Cytotoxicity and cell viability

The crude ethanolic seed extract derived from *Mucuna pruriens* was tested on normal cells (Vero) at concentrations reaching up to 1000 µg/mL, maintaining 90% cell viability even after 24 h of treatment. As a result, concentrations lower than 1000 µg/mL, specifically 200, 400, 600, 800, and 1000 µg/mL, were fixed ($^*p<0.05$; Fig. 1). Upon treating the gastric cancer cell line (AGS) with the crude ethanolic seed extract at increasing concentrations for 24 h, a consistent decrease in the percentage of viable cells was observed. The cells treated with crude ethanolic seed extract show a reduction of 50% in viability with 600 µg/mL ($^*p<0.05$; Fig. 2). The effect of ethanolic seed extract with three different concentrations (minimum (200 µg/mL), IC50 (600 µg/mL), and maximum (1000 µg/mL) was taken forward for further analysis.

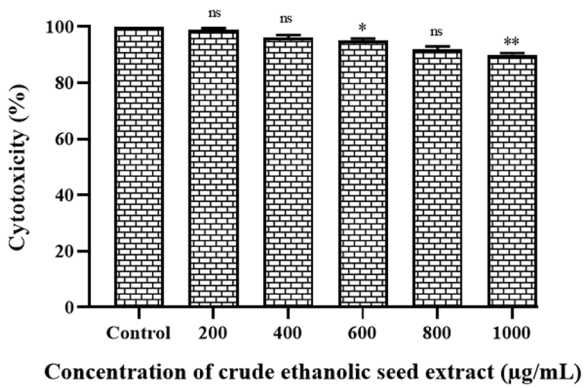

Fig. 1 Cytotoxic effect of crude ethanolic seed extract from *M. pruriens* on Vero cells at different concentrations over 24 h. All experiments were done in triplicates (n = 3), and data was represented as Mean ± SD

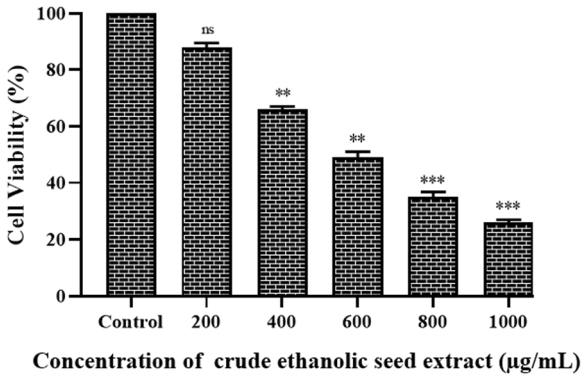

Fig. 2 Effect of crude ethanolic seed extract from *M. pruriens* on the cell viability of human gastric adenocarcinoma cell line (AGS) at 24 h. All experiments were done in triplicates (n = 3), and data was represented as Mean ± SD

Cytomorphology observation

The morphological changes between the control and treated AGS cells were observed. The control cells showed polygonal shape morphology with an even monolayer, whereas the treated cells showed cell shrinkage and changes in their original morphology (Fig. 3) which include disruptions in the even monolayer, and the cells were found to be aggregated due to the effect of the crude ethanolic seed extract of *M. pruriens*. These results suggested that the toxicity of the crude ethanolic seed extract is particular to treated AGS cancer cells with non-toxicity to control cells at a specific concentration.

Nuclear morphology assessment

The changes in the nuclear morphology of control and treated cells with crude ethanolic seed extract (200 µg/mL as the minimum, 600 µg/mL as the IC50, and 1000 µg/mL as the maximum) were observed using the fluorescent stain propidium iodide. Propidium Iodide is a nuclear-specific dye which is impermeable to the nuclear membrane unless the nuclear membrane is damaged. Hence PI is permeable to both viable and non-viable cells, it binds to the intercalating region of the DNA and emits red fluorescence. Cells with damaged nuclear membranes due to the action of compound emit a higher degree of fluorescence than the normal cells. The nuclear damage of treated AGS cells induced by the crude ethanolic seed extract exhibited distinct characteristics in IC50 and maximum concentrations showed reduced nuclear size, intense bright red fluorescence indicating condensed nuclear chromatin, and the formation of membrane blebs. As the concentration of the extract increases, there is a proportional rise in the extent of nuclear damage within the cells. In contrast, control cells absorbed dye primarily in the cytoplasm and maintained intact nuclei (Fig. 4).

Dual staining

Early and late apoptosis were identified through AO/EB staining after treating the cells with crude ethanolic seed extract at different concentrations. This method utilizes fluorescent dyes to detect specific changes in the cell membrane associated with apoptosis. The ability to induce apoptosis by *M.pruriens* ethanolic seed extract was assessed using AO-EB dual staining on AGS cells. AO can penetrate live cells easily, while EB only enters dead cells with ruptured membranes, on treatment with different concentration of compound, the cell membrane integrity if lost, so that EB gets access to the cells and emit bright red fluorescence. AO penetrate both viable and non-viable cells and emit green fluorescence. On merging both the images, the early apoptotic cells appear as dark green to orange in color and late apoptotic cells appear red in color. Control cells displayed uniform green fluorescence and normal characteristics, whereas cells treated with the minimum concentration (200 µg/mL) of *M.pruriens* seed extract exhibited bright green staining indicative of early apoptosis. At the IC50 (600 µg/mL)

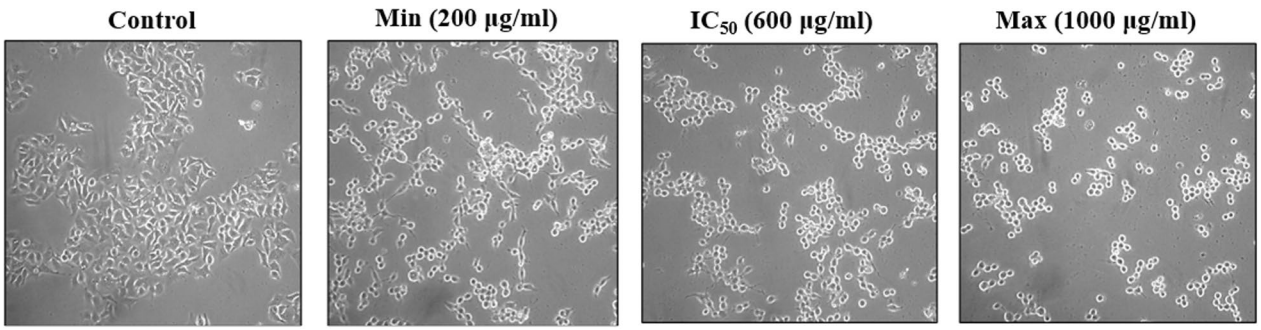

Fig. 3 Cellular morphological observation of human gastric adenocarcinoma (AGS) cells treated with crude ethanolic seed extract of *M. pruriens* at 24 h (20× magnification)

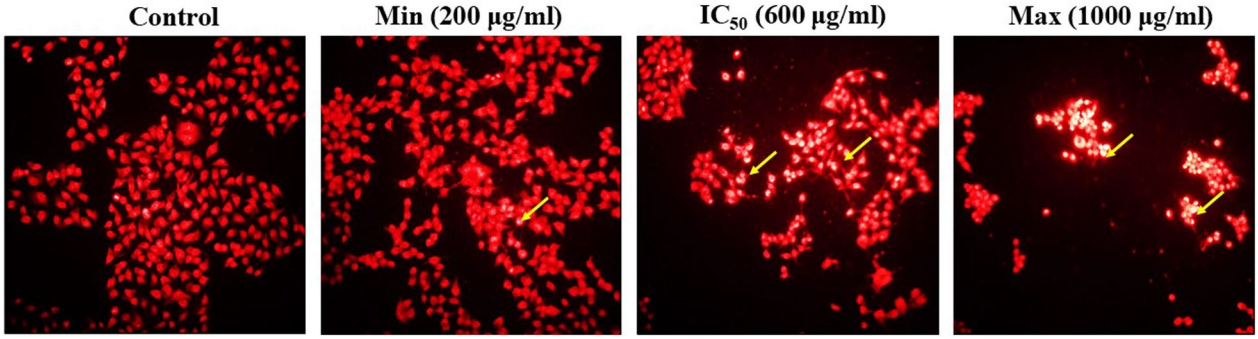

Fig. 4 Nuclear morphological observation of crude ethanolic seed extract of *M. pruriens* on human gastric adenocarcinoma (AGS) cells at 24 h (20 × magnification; arrow indicates nuclear damage)

and maximum concentration (1000 µg/mL) orange and red fluorescence were observed, indicating both early and late apoptosis (Fig. 5).

Rhodamine 123 staining

Rhodamine123 is a fluorescent stain that accumulates within mitochondria due to the transmembrane potential and is retained in living cell organelles. When a cell loses its integrity, the mitochondrial membrane potential is lost, and the organelle does not retain the stain.

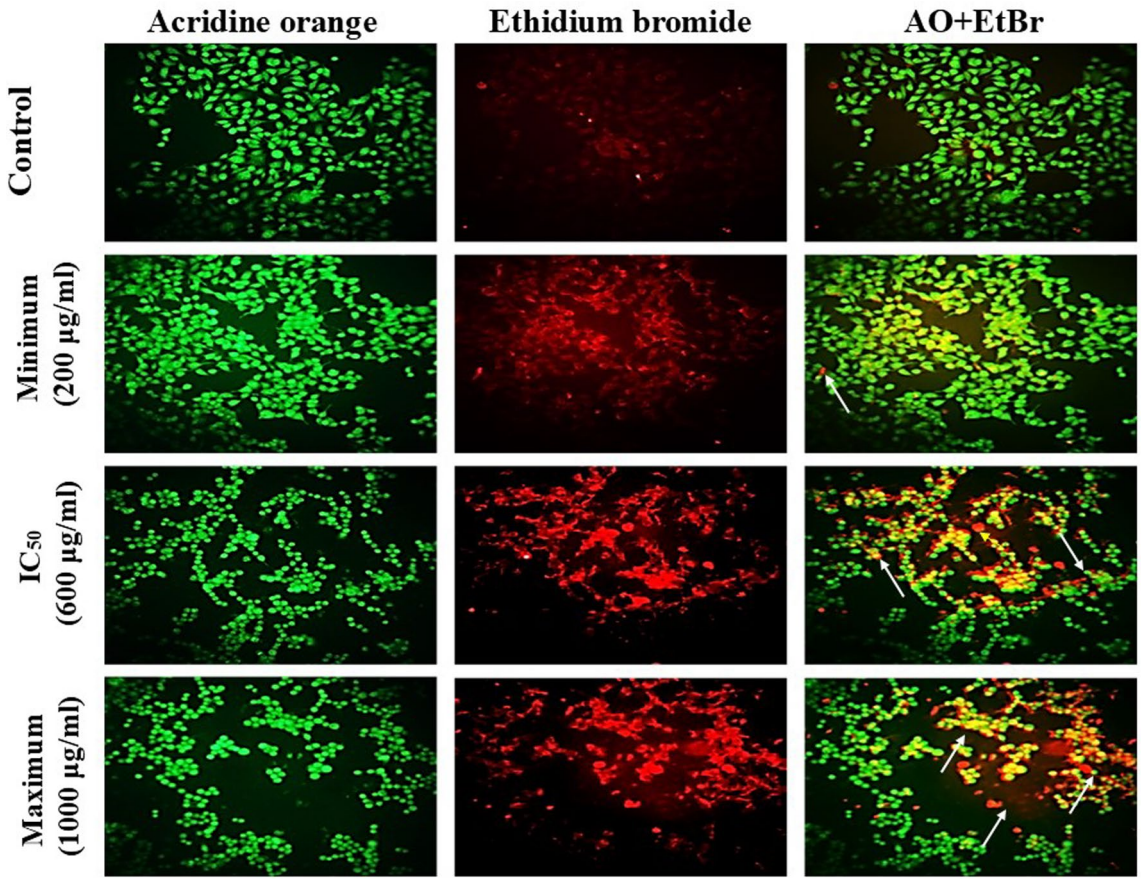

Fig. 5 Dual staining by AO/EtBr on gastric adenocarcinoma cell line (AGS) treated with a crude ethanolic seed extract of *M. pruriens* at 24 h (20 × magnification; arrow indicates early and late apoptotic cells)

Alteration in the mitochondrial membrane leads to the release of mitochondrial content. AGS cells exposed to crude ethanolic seed extracts (at concentrations of 200, 600, and 1000 µg/mL) in a dose-dependent manner exhibited reduced fluorescence levels, suggesting the presence of apoptotic cells with diminished mitochondrial potential. In contrast, untreated control cells displayed high mitochondrial potential, evident from their healthy state with green fluorescence resulting from electrophoretic accumulation in the mitochondria (Fig. 6).

LDH analysis

The release of LDH, an internal cytoplasmic enzyme found in live cells, was measured in treated AGS cells as a sign of compromised membrane integrity and damage. When the plasma membrane is disrupted, LDH enzyme will rapidly release into the cell culture medium, which is a fundamental aspect of cells suffering apoptosis and other forms of cellular destruction or cell injury. Cell membrane integrity was assessed by measuring the LDH level in the cell cultured medium using NAD and DNPH. The percentage of LDH release increased significantly after treatment with different doses of crude ethanolic seed extract, suggesting apoptosis. At doses of 200, 600, and 1000 µg/mL, LDH enzyme levels increased significantly compared to the control group. The LDH release test revealed that the crude ethanolic seed extract of *M. pruriens* caused increased damage in AGS cells in response to dosage concentrations. The enzyme leakage percentages were 25.72%, 49.70%, and 73.63% in treated cells when compared to the control percentage of 14.66% (Fig. 7).

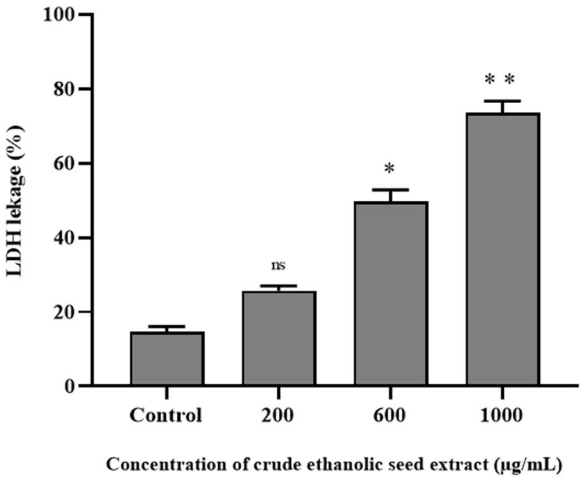

Fig. 7 LDH leakage in a gastric cancer cell line treated with ethanolic seed extract from *M. pruriens* at 24 h. All experiments were done in triplicates (n = 3), and data was represented as Mean ± SD

DNA fragmentation assay

DNA fragmentation or ladder assay is used for the detection of apoptosis by the binding of ethidium bromide to DNA. DNA fragmentation, a sign of apoptosis, was examined using agarose gel electrophoresis. Apoptosis causes nuclear DNA to be cleaved into internucleosomal fragments by caspase-3 activated DNAase. AGS cells treated with crude ethanolic seed extract showed concentration-dependent apoptosis over 24 h. The whole genomic DNA of both control and treated cells were isolated and resolved in an agarose gel. The DNA from control cells appeared intact in nature as a single band in the control lane whereas the cells treated with 200, 600, and 1000 µg/mL showed substantial streaking patterns, indicating DNA damage (Fig. 8). DNA isolated from treated cells exhibited a sequential progression of fragmentation, which was confirmed through DNA fragmentation analysis using ethidium bromide and visualized as a characteristic ladder pattern on agarose gel electrophoresis.

Cell cycle analysis

Cell cycle progression strongly correlates with cell proliferation. The untreated and treated AGS cells were analyzed using RNase and PI staining in combination using flow cytometry. Cell cycle arrest has the potential to manifest across various phases of the cell cycle. The

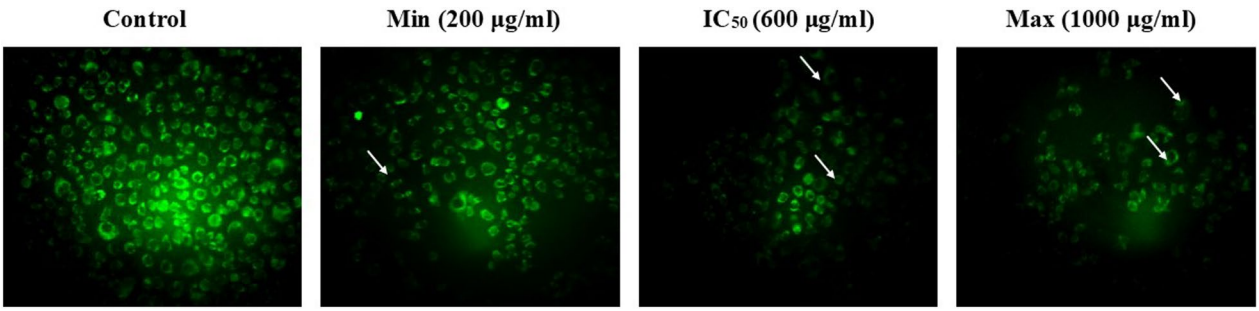

Fig. 6 Analysis of the mitochondrial membrane potential by Rhodamine 123 staining on human gastric adenocarcinoma cells with a crude ethanolic seed extract of *M. pruriens* at 24 h (20 × magnification; arrow indicates loss of mitochondrial membrane potential)

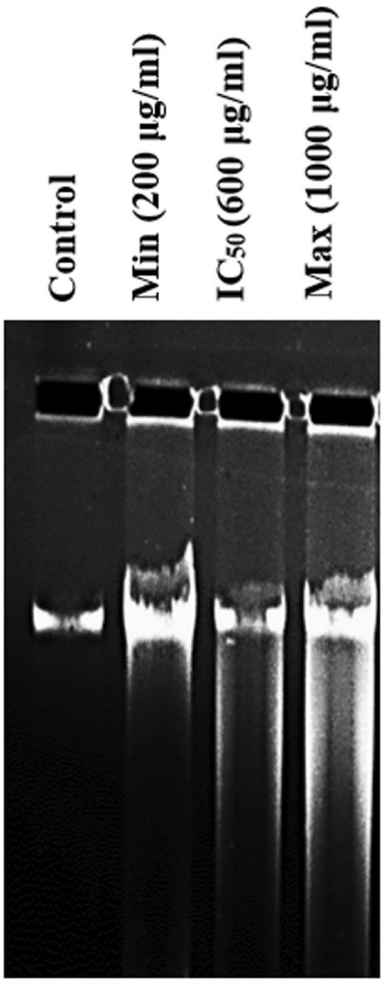

Fig. 8 Agarose gel electrophoresis of genomic DNA from AGS cells treated with different concentrations of *M. pruriens* seed extract

univariate analysis of cellular DNA content following staining with propidium iodide in both treated and control cells showed distribution of three major phases of the cells (G0-G1/S/G2/M) and makes it possible to detect cell cycle arrest relation to the amount of DNA in each phase. In our result, the accumulation of cells were higher in the G0/G1 phase, it indicates our compound arrest the cancer cell at this phase of the cell cycle. The results showed that upon treatment with extract, the AGS cells started accumulating at the G0/G1 phase of the cell cycle in a concentration-dependent manner. This accumulation coincided with a reduction in cells within the S and G2/M phases of the cell cycle. Specifically, the percentage of cells in the G0/G1 phase increased from 78.09% to 85.21%, while the proportions in S phase decreased from 9.84% to 7.50%, and in the G2/M phase from 11.17 to 6.37%. In contrast, the control group exhibited cell populations of 74.78, 12.52%, and 11.44% in the G0/G1, S, and G2/M phases, respectively. These results suggest that *M.prurenis* induces G0/G1 arrest and impedes progressive cell growth (Fig. 9a and b).

Discussion

Plants possess a wide range of bioactive substances that exhibit antioxidant, anti-inflammatory, and anti-tumor properties. Several herbal extracts have demonstrated promising results in combating cancer cells when compared to traditional chemotherapy or hormonal medications [23]. *Mucuna pruriens* is widely recognized for its valuable therapeutic effects throughout India. The seeds, flowers, and leaves of this plant are known for their medicinal properties. Moreover, the seeds of this plant display remarkable properties that can combat cancer [24] [25].

Our research on *M.pruriens* seed extract showed non-toxicity toward Vero cells, with no significant cell mortality observed at 1000 µg/mL and gastric cancer cells showed a 50% reduction in cell viability at 600 µg/mL. Likewise, Fenugreek seed extract exhibited inhibitory effects on the MCF-7 cell line at a concentration of 400 µg/ml after 24 h [26]. Previous studies on the anticancer activity of *Moringa peregrina* seed ethanolic extract (MPSE) on breast cancer cells (AU565) revealed an inhibitory concentration of 201.7 µg/mL [27].

The cytomorphological observation of AGS cells was sensitive to the inhibitory action of crude ethanolic seed extract. Earlier studies on A549 and HCT 15 cells treated with sardine oil emulsion revealed morphological changes in treated and untreated cells [28]. Similarly, observations of treated A431 cells with grape seed extract indicated cell detachment, shrinkage, nuclear condensation, and fragmentation [29].

Apoptosis is a biological process of cell destruction that plays an important role in morphological and biochemical changes in the nucleus and cytoplasm. Apoptotic cells exhibit distinct features such as condensation, cell shrinkage, and the presence of condensed and cleaved chromatin [30]. Additionally, during apoptosis, cells become more permeable to propidium iodide (PI), a fluorescent DNA stain that cannot penetrate the membranes of live cells. Morphological examination of AGS cells using PI staining revealed nuclear shrinkage and red fluorescence emission, indicating the initiation of apoptosis by crude ethanolic seed extracts. Control cells show red fluorescence in the cytoplasm with an intact nucleus. The nuclear damage was reported in *Swietenia macrophylla* seed extracts on HCT116 colorectal carcinoma cells and *Vitis vinifera* seed extracts in A431 skin cancer cells [29, 31, 32]

The AO/EtBr dual staining assay serves as an in vitro method for detecting apoptosis. It involves the use of acridine orange and ethidium bromide staining to visualize

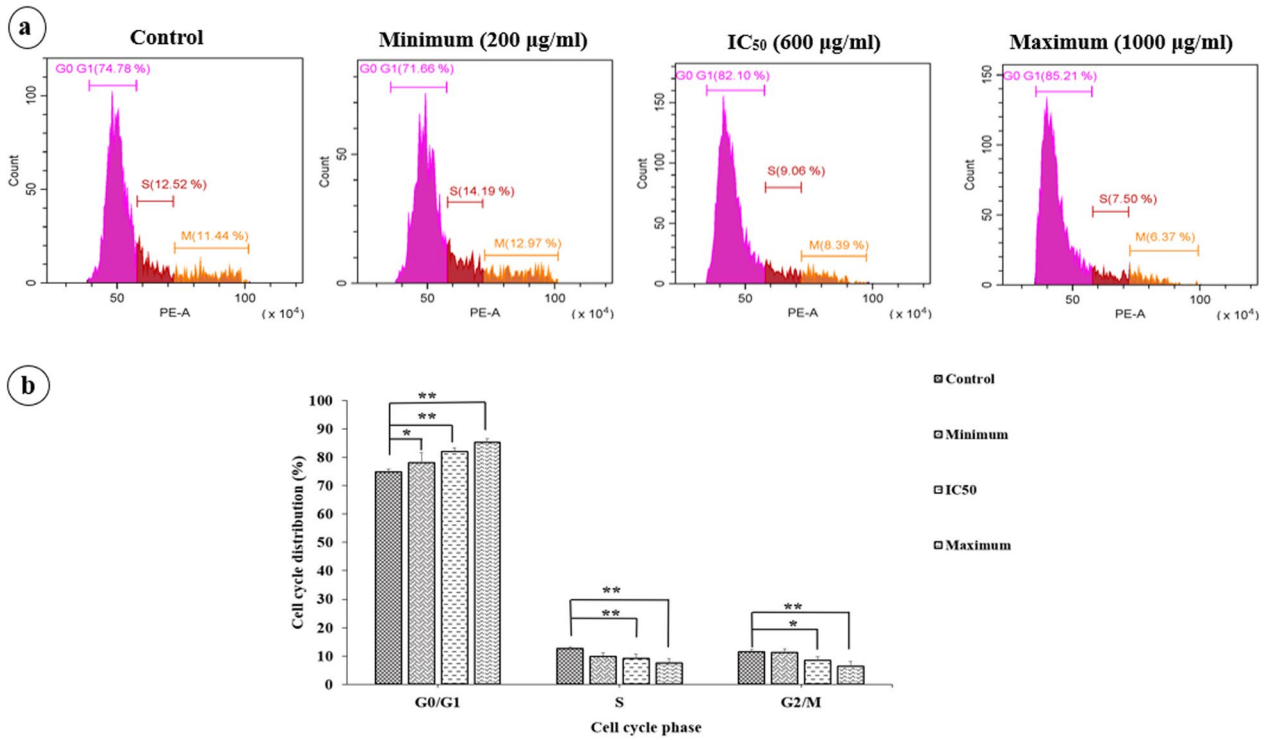

Fig. 9 **a** Flow cytometry image of human gastric adenocarcinoma (AGS) cells treated with *M. pruriens* ethanolic seed extract **b** Cell cycle distribution graph of AGS cells treated with *M.pruriens* ethanolic seed extract at 24 h. All experiments were done in triplicates (n = 3), and data was represented as Mean ± SD

various stages of apoptotic alterations, including early and late apoptosis. AO, being a membrane-permeable dye, binds to the nucleic acids of viable cells. On the other hand, EtBr, impermeable to membranes, readily enters non-viable cells and binds to DNA. Viable cells exhibit normal green-colored nuclei, while early apoptotic cells with fragmented chromatin display bright green nuclei. Chromatin condensation manifests as bright green patches, and late apoptotic cells exhibit orange to red nuclei [33]. The apoptotic, late apoptotic and necrotic cells observed in hexane seed extracts from *Cuminum cyminum* on bone cancer cells [34].

The most important feature of the induction of the intrinsic apoptotic pathway is the loss of mitochondrial membrane potential. Mitochondria is the primary energy source essential for sustaining life in aerobic conditions, also serve as sources of cell signals that induce apoptosis. During apoptosis, mitochondrial membrane becomes permeable leading to release of apoptogenic factors into the cytosol [35]. The reduction in mitochondrial membrane potential was evaluated using Rhodamine 123, a positively charged fluorescent dye. In this study, the fluorescence intensity decreases which indicates the cell death by *M.pruriens* seed extract in concentration-dependent manner. A significant reduction in mitochondrial membrane potential in Squamous Cell carcinoma—25 (SCC-25) oral cancer cells treated with *Annona muricata* crude extract [36].

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is found in all cells. Also, it serves as an ideal indicator for cytotoxicity studies due to plasma membrane damage, which is a hallmark of various cellular processes such as apoptosis, necrosis, and other forms of cellular injury [37]. These enzymes facilitate the conversion of pyruvate into lactate when NADH is present. Our findings revealed that elevated concentrations led to increased cell damage, suggesting the release of high levels of LDH into the surrounding medium. This observation shows that the occurrence of apoptosis-induced cell death triggered by the crude ethanolic seed extract of *M.pruriens*. Previously, the hexane extract from cumin seed showed increased release of LDH enzyme from MG63 cells after 24 h of incubation [34]. However, there was no reported dose-dependent effect of LDH in HepG2 cells when exposed to aqueous pomegranate seed extract (PSE). The percentage of LDH release ranged from 9.7 to 9.2% across concentrations of 1–100 µg/mL in HepG2 cells, suggesting a lack of cytotoxicity even at high concentrations [38]. The cytotoxic impact of *Ferula hermonis* root hexane extract (FHRH) on MDA-MB-231 and LoVo

cells was assessed by monitoring LDH leakage in the culture media [39].

Another key aspect of triggering apoptosis is the occurrence of nuclear fragmentation, as validated through agarose gel electrophoresis. Apoptotic cell nuclear DNA exhibits a distinct ladder-like pattern of oligonucleosomal fragments, widely recognized as the hallmark of apoptosis [40]. In this study, AGS cells treated with *M.puriens* seed extract showed a DNA streaking pattern in time and concentration-dependent manner. Subsequent staining with ethidium bromide on agarose gel revealed observable DNA fragmentation. *Mucuna pruriens* extract induces apoptosis through DNA fragmentation and decreases the mitochondrial potential of the cancer cells after treatment, thereby it induces apoptosis by the mitochondrial pathway. Similarly, DNA fragmentation was evident in oral squamous cell carcinoma following exposure to 250 µg/mL of grape seed extract [41]. Likewise, results for induction of apoptosis have been reported for the seed and leaf extracts and saponins of *Tribulus terrestris* inducing DNA fragmentation in MCF-7 cells [42].

Cancer is identified as a disorder resulting from abnormalities in the cell cycle [43]. Quantitative analysis of the cell cycle plays a crucial role in investigating the molecular mechanisms underlying cell death and cell cycle advancement. Within the cellular division cycle, failure at checkpoints results in increased proliferation rates and the onset of cancer. Nevertheless, the blockage of the cell cycle at these checkpoints can be hindered by activating signaling pathways or utilizing specific inhibitors, thus impeding further proliferation of cancer cells. This method is beneficial for precisely assessing the distribution of cells across different phases of the cell cycle [44]. The present study demonstrated changes in cell cycle progression by flow cytometry. We observed that *M.prureins* ethanolic seed extract arrests the AGS cell cycle at the growth-static G1 phase which explains the anti-proliferation of AGS cells by crude ethanolic seed extract. The number of cells in the G0 phase of treated AGS cells increased in a dose-dependent manner compared to the control, which is in close agreement with the previous results [45] where ionic liquid-graviola fruit extract impeded cell cycle arrest at G0/G1 phase in MCF-7 cells. Similarly, the G1 phase of cell cycle arrest was reported in T47D and MCF-7 breast cancer cells treated with *M.pruriens* ethanolic seed extract [46].

Conclusion

The present study revealed the anticancer activity of the ethanolic seed extract obtained from *Mucuna pruriens* on human gastric cancer cells The extract exhibited notable cytotoxic effects, inducing cell death, apoptosis, DNA fragmentation, and cell cycle arrest at the G0/G1 phase. These findings underscore the therapeutic promise of *Mucuna pruriens* seed extract as a source of natural bioactive compounds. Future research could focus on these compounds, potentially leading to the development of novel anticancer drugs. Subsequent investigations should prioritize the isolation and identification of the precise bioactive chemicals accountable for these effects. Further mechanistic investigations are required to clarify the mechanisms by which these chemicals trigger cell death and apoptosis. Furthermore, it is necessary to carry out in vivo experiments to assess the effectiveness and safety of the extract and its separated chemicals in living biological systems. The investigation of formulation creation and possible administration systems has the potential to augment the therapeutic capabilities of the extract, ultimately resulting in clinical trials to evaluate its efficacy and safety in the treatment of cancer.

Abbreviations

AGS	Human gastric adenocarcinoma cell line
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
AO	Acridine orange/
EB	Ethidium bromide
LDH	Lactate dehydrogenase
DMSO	Dimethyl sulfoxide
NCCS	National center for cell science
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
DNA	Deoxyribonucleic acid
UV	Ultraviolet
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
R-123	Rhodamine 123
RNase A	Ribonuclease A
IC50	Inhibitory concentration
NAD +	Nicotinamide Adenine Dinucleotide
pH	Potential of hydrogen
ANOVA	Analysis of variance
SPSS	Statistical package for social sciences

Acknowledgements

The authors express their gratitude for the invaluable support, infrastructure, and opportunities provided by the department, established under the DST-FIST program, which enabled the successful execution of the current research work. The authors further acknowledge the GNR Instrumentation Centre at the University of Madras, Guindy Campus, Chennai, for providing an instrumentation facility to carry out the present research work.

Author contributions

AC and SE were involved in conceiving and designing the study. DP conducted material preparation and data collection. Analysis was carried out by DP, VJ, NS, RV, and AA. The initial manuscript draft was written by VJ, DP and NS. AC reviewed and provided feedback on the earlier version of the manuscript. All authors participated in reading and approving the final manuscript.

Funding

The authors are highly obliged to Rashtriya Uchchatar Shiksha Abhiyan (RUSA 2.0), State Project Directorate, Dote Campus, Chennai, Tamil Nadu, for financial support for this work.

Availability of data and materials

The datasets utilized and analyzed in the current study are accessible from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted in strict accordance with local, national, and international guidelines and legislation, with all necessary permits and licenses obtained from the relevant authorities to ensure ethical and legal compliance.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 15 June 2024 Accepted: 25 September 2024
Published online: 08 October 2024

References

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, Bray F (2021) Cancer statistics for the year 2020: an overview. Int J Cancer 149(4):778–789. https://doi.org/10.1002/ijc.33588
2. Siegel RL, Miller KD, Wagle NS, Jemal A (2023) Cancer statistics, 2023. Ca Cancer J Clin 73(1):17–48. https://doi.org/10.3322/caac.21763
3. Sitarz R, Skierucha M, Mielko J, Offerhaus GJA, Maciejewski R, Polkowski WP (2018) Gastric cancer: epidemiology, prevention, classification, and treatment. Cancer Manag Res 10:239–248. https://doi.org/10.2147/CMAR.S149619
4. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer J Clin 71(3):209–249. https://doi.org/10.3322/caac.21660
5. Nakonieczna S, Grabarska A, Kukula-Koch W (2020) The potential anticancer activity of phytoconstituents against gastric cancer—A review on in vitro, in vivo, and clinical studies. Int J Mol Sci 21(21):8307. https://doi.org/10.3390/ijms21218307
6. Hashemi SF, Tasharrofi N, Saber MM (2020) Green synthesis of silver nanoparticles using *Teucrium polium* leaf extract and assessment of their antitumor effects against MNK45 human gastric cancer cell line. J Mol Struct 1208:127889. https://doi.org/10.1016/j.molstruc.2020.127889
7. Liang Z, Xu Y, Zhang Y, Zhang X, Song J, Jin J, Qian H (2023) Anticancer applications of phytochemicals in gastric cancer: effects and molecular mechanism. Front Pharmacol 13:1078090. https://doi.org/10.3389/fphar.2022.1078090
8. Tang Q, Xia H, Liang W, Huo X, Wei X (2020) Synthesis and characterization of zinc oxide nanoparticles from Morus nigra and its anticancer activity of AGS gastric cancer cells. J Photochem Photobiol B 202:111698. https://doi.org/10.1016/j.jphotobiol.2019.111698
9. Cheshomi H, Bahrami AR, Rafatpanah H, Matin MM (2022) The effects of ellagic acid and other pomegranate (Punica granatum L) derivatives on human gastric cancer AGS cells. Hum Exp Toxicol. https://doi.org/10.1177/09603271211064534
10. Mao Q, Xu X, Shang A, Gan R, Wu D, Atanasov AG, Li H (2019) Phytochemicals for the prevention and treatment of gastric cancer: effects and mechanisms. Int J Mol Sci 21(2):570. https://doi.org/10.3390/ijms21020570
11. Sathiyanarayanan L, Arulmozhi S (2007) *Mucuna pruriens* Linn.-A comprehensive review. PHCOG REV 1(1)
12. Gupta PS, Patel S (2020) In vitro antimitotic and cytotoxic potential of plant extracts: a comparative study of *Mucuna pruriens*, *Asteracantha longifolia* and *Sphaeranthus indicus*. Futur J Pharm Sci 6(1):115. https://doi.org/10.1186/s43094-020-00137-8
13. Tumbas-Saponjac V, Akpoveso OO, Oyeniran O, Desancic J, Cetojevic-Simin D (2020) Antioxidant activity and enhanced cytotoxicity of aqueous Mucuna pruriens L. Leaf extract by doxorubicin on different human cancer cell lines. Phcog Mag. 16(68):224–228. https://doi.org/10.4103/pm.pm_413_19
14. Gupta S, Gopinath SM, Shareef IM, Gupta A (2017) In-vitro evaluation of cytotoxic and apoptogenic properties of *Mucuna Pruriens* on Mcf7 cell lines. World J Pharm Pharm Sci 6(9):924–932. https://doi.org/10.20959/wjpps20179-9898
15. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65(1–2):55–63. https://doi.org/10.1016/0022-1759(83)90303-4
16. Arulvasu C, Selvamathi S, Babu G, Dhanasekaran G (2012) Effect of crude and partially purified epidermal mucus proteins of marine catfish *Tachysurus dussumieri* on human cancer cell line. J Acad Indus Res 1(4):164–169
17. Keum YS, Kim J, Lee KH, Park KK, Surh YJ, Lee JM, Yook JI (2002) Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells. Cancer lett 177(1):41–47. https://doi.org/10.1016/S0304-3835(01)00781-9
18. Sahayanathan GJ, Padmanaban D, Raja K, Chinnasamy A (2020) Anticancer effect of purified polysaccharide from marine clam *Donax variabilis* on A549 cells. J Food Biochem 44(11):e13486. https://doi.org/10.1111/jfbc.13486
19. Vaikundamoorthy R, Krishnamoorthy V, Vilwanathan R, Rajendran R (2018) Structural characterization and anticancer activity (MCF7 and MDA-MB-231) of polysaccharides fractionated from brown seaweed Sargassum wightii. Int J Biol Macromol 111:1229–1237. https://doi.org/10.1016/j.ijbiomac.2018.01.125
20. King J (1965) The dehydrogenase of oxidoreductase-lactate dehydrogenase. Practical clinical enzymology Nostrand Co., London, pp 83–93
21. Murad H, Ghannam A, Al-Ktaifani M, Abbas A, Hawat M (2015) Algal sulfated carrageenan inhibits proliferation of MDA-MB-231 cells via apoptosis regulatory genes. Mol Med Rep 11(3):2153–2158. https://doi.org/10.3892/mmr.2014.2915
22. Rohit Singh T, Ezhilarasan D (2020) Ethanolic extract of Lagerstroemia Speciosa (L.) Pers., induces apoptosis and cell cycle arrest in HepG2 cells. Nutr Cancer 72(1):146–156. https://doi.org/10.1080/01635581.2019.1616780
23. Divya BJ, Suman B, Venkataswamy M, ThyagaRaju K (2017) The traditional uses and pharmacological activities of *Mucuna pruriens* (L) DC: a comprehensive review. Indo Am J Pharm Res 7(01):7516–7525. https://doi.org/10.1007/s13205-020-02253-x
24. Pathania R, Chawla P, Khan H, Kaushik RK, M A, (2020) An assessment of potential nutritive and medicinal properties of *Mucuna pruriens*: a natural food legume. 3 Biotech 10(6):261. https://doi.org/10.1007/s13205-020-02253-x
25. Rajeshwar Y, Gupta M, Mazumder UK (2005) Antitumor activity and in vivo antioxidant status of *Mucuna pruriens* (Fabaceae) seeds against Ehrlich ascites carcinoma in swiss albino mice. Iran J Pharmacol Ther 4(1):46–53
26. Al-Timimi LAN (2019) Antibacterial and anticancer activities of fenugreek seed extract. Asian Pac J Cancer Prev 20(12):3771–3776. https://doi.org/10.31557/APJCP.2019.20.12.3771
27. Albaayıt SFA (2020) In vitro evaluation of anticancer activity of *Moringa peregrina* seeds on breast cancer cells. Eurasia Proc Sci Technol Eng Math 11:163–166
28. Arulvasu C, Dinesh D, Manikandan R, Pandi M, Srinivasan P, Radakrishnan N, Sellamuthu S, Prabhu D, Babu G (2010) Evaluation of anti-proliferative effect of sardine oil emulsion on A549 and HCT 15 cancer cell lines. Int J PharmTech Res 2(2):1171–1177
29. Mohansrinivasan V, Devi CS, Deori M, Biswas A, Naine SJ (2015) Exploring the anticancer activity of grape seed extract on skin cancer cell lines A431. Braz Arch Biol Technol 58:540–546. https://doi.org/10.1590/s1516-8913201500076
30. Kadan S, Rayan M, Rayan A (2013) Anticancer activity of anise (*Pimpinella anisum* L.) seed extract. Open Nutr J 6:1–5. https://doi.org/10.2174/1876396001306010001
31. Goh BH, Chan CK, Kamarudin MN, Kadir HA (2014) *Swietenia macrophylla* King induces mitochondrial-mediated apoptosis through p53 upregulation in HCT116 colorectal carcinoma cells. J Ethnopharmacol 153(2):375–385. https://doi.org/10.1016/j.jep.2014.02.036
32. Grace Nirmala J, Evangeline Celsia S, Swaminathan A, Narendhirakannan RT, Chatterjee S (2018) Cytotoxicity and apoptotic cell death induced by *Vitis vinifera* peel and seed extracts in A431 skin cancer cells. Cytotechnology 70:537–554. https://doi.org/10.1007/s10616-017-0125-0
33. Byczkowska A, Kunikowska A, Kaźmierczak A (2013) Determination of ACC-induced cell-programmed death in roots of *Vicia faba* ssp. minor

seedlings by acridine orange and ethidium bromide staining. Protoplasma 250:121–128. https://doi.org/10.1007/s00709-012-0383-9
34. Chandrasekaran R, Krishnan M, Chacko S, Gawade O, Hasan S, Joseph J, George E, Ali N, AlAsmari AF, Patil S, Jiang H (2023) Assessment of anticancer properties of cumin seed (*Cuminum cyminum*) against bone cancer. Front Oncol 13:1322875. https://doi.org/10.3389/fonc.2023.1322875
35. Faddan NH, Sayed D, Al-Sharkawy N (2010) Apoptosis and mitochondrial membrane potential changes of T lymphocytes from children with down's syndrome. Egypt J Pediatr Allergy Immunol 8:35–40. https://doi.org/10.1016/j.apt.2017.02.003
36. Rekha M, Bhuminathan S, Dineshkumar T, Lakshmanan A (2024) deciphering Annona's anticancer potential: comparative analysis of crude extract versus nanoformulation on SCC-25 oral cancer cells through cell viability, apoptosis, cell cycle, ROS, and MMP analysis. J Maxillofac Oral Surg 5:1–8. https://doi.org/10.1007/s12663-024-02184-7
37. Kumar P, Nagarajan A, Uchil PD (2018) (2018) Analysis of cell viability by the lactate dehydrogenase assay. Cold Spring Harb Protoc 6:465–468. https://doi.org/10.1101/pdb.prot095497
38. Navarro M, Amigo-Benavent M, Mesias M, Baeza G, Gökmen V, Bravo L, Morales FJ (2014) An aqueous pomegranate seed extract ameliorates oxidative stress of human hepatoma HepG2 cells. J Sci Food Agric 94(8):1622–1627. https://doi.org/10.1002/jsfa.6469
39. Abutaha N, Nasr FA, Al-Zharani M, Alqahtani AS, Noman OM, Mubarak M, Abdelhabib S, Wadaan MA (2019) Effects of hexane root extract of *Ferula hermonis boiss*. On human breast and colon cancer cells: an in vitro and in vivo study. Biomed Res Int 2019:1–12. https://doi.org/10.1155/2019/3079895
40. Lien HM, Lin HW, Wang YJ, Chen LC, Yang DY, Lai YY, Ho YS (2011) Inhibition of anchorage-independent proliferation and G0/G1 cell-cycle regulation in human colorectal carcinoma cells by 4, 7-dimethoxy-5-methyl-l, 3-benzodioxole isolated from the fruiting body of *Antrodia camphorate*. Evid-Based Complementary Altern Med 2011:984027. https://doi.org/10.1093/ecam/nep020
41. Aghbali A, Hosseini SV, Delazar A, Gharavi NK, Shahneh FZ, Orangi M, Bandehagh A, Baradaran B (2013) Induction of apoptosis by grape seed extract (*Vitis vinifera*) in oral squamous cell carcinoma. Bosn J Basic Med Sci 13(3):186–191. https://doi.org/10.17305/bjbms.2013.2360
42. Patel A, Soni A, Siddiqi NJ, Sharma P (2019) An insight into the anticancer mechanism of *Tribulus terrestris* extracts on human breast cancer cells. 3 Biotech. 9:58. https://doi.org/10.1007/s13205-019-1585-z
43. Mantena SK, Sharma SD, Katiyar SK (2006) Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. Mol Cancer Thera 5(2):296–308. https://doi.org/10.1158/1535-7163.MCT-05-0448
44. Benada J, Macurek L (2015) Targeting the checkpoint to kill cancer cells. Biomolecules 5(3):1912–1937. https://doi.org/10.3390/biom5031912
45. Daddiouaissa D, Amid A, Kabbashi NA, Fuad FA, Elnour AM, Epandy MA (2019) Antiproliferative activity of ionic liquid-graviola fruit extract against human breast cancer (MCF-7) cell lines using flow cytometry techniques. J Ethnopharmacol 236:466–473. https://doi.org/10.1016/j.jep.2019.03.003
46. Sinha S, Sharma S, Vora J, Shah H, Srivastava A, Shrivastava N (2018) *Mucuna pruriens* (L.) DC chemo sensitize human breast cancer cells via downregulation of prolactin-mediated JAK2/STAT5A signaling. J Ethnopharmacol 217:23–35. https://doi.org/10.1016/j.jep.2018.02.006

Publisher's Note