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# Cytotoxic and Antioxidant Activities of *Alstonia scholaris*, *Alstonia venenata* and *Moringa oleifera* Plants From India

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## Abstract

**Background:** Cancer is one of the leading causes of human death. The discovery of new generations of low-cost anticancer drugs with high efficacy and low toxicity is necessary, and is only possible by screening medicinal plants with prior knowledge.
**Objectives:** In this study, the bioactivities of three medicinal plants from India, including *Alstonia scholaris*, *Alstonia venenata*, and *Moringa oleifera*, were investigated.
**Materials and Methods:** From each plant, hexane, benzene, isopropanol, methanol, and water extracts were prepared. Cytotoxicity assays were determined by Trypan blue exclusion, MTT, and apoptotic methods. Antioxidant activities were assayed by superoxide scavenging, hydroxyl radical scavenging, and lipid peroxidation.
**Results:** Among the extracts tested for cytotoxicity on DLA cells, the most active extracts from *A. scholaris* and *A. venenata* were selected (extracts from *M. oleifera* did not show 100% cytotoxicity even at a dose of 500 $\mu$g/mL, so it was not considered for determining $EC_{50}$ value), and their $EC_{50}$ values were determined. Among the extracts of *A. scholaris*, hexane extract of stem bark showed an $EC_{50}$ value of 68.75 $\mu$g/mL, while n-hexane extract of the leaves showed a higher $EC_{50}$ value of 118.75 $\mu$g/mL. *A. venenata* showed significant in vitro superoxide scavenging activity, superior to that of quercetin. *A. venenata* showed less superoxide scavenging activity. The IC50 values of hexane extract (*A. scholaris*), isopropanol extract (*A. venenata*), and quercetin were 90.5 $\pm$ 6.2, 7.5 $\pm$ 1.2, and 31.5 $\pm$ 2.5 $\mu$g/mL, respectively.
**Conclusions:** The hexane extract of stem bark from *A. scholaris* and isopropanol extract of leaves from *A. venenata* are candidate materials for the discovery of a new generation of anticancer drugs to combat diseases such as lymphoma and leukemia. These can also be used as antioxidants in dietary supplements.

*Keywords:* Cytotoxicity Assay, Antioxidant Activity, MTT, DLA Cells

## 1. Background

Cancer is one of the leading causes of human death, and its prevalence in the twenty-first century is on the rise. As per the world health organization's report, the global cancer burden doubled in the last three decades of the twentieth century, and it is estimated that it will double again between 2000 and 2020, and the number of new cases each year will nearly triple (to 21.4 million) by 2030. In developing countries such as India, the occurrence of various types of cancer is dramatically increasing for a variety of reasons, such as sedentary lifestyles, frequent intake of fried meat products, low intake of vegetables and fruits, environmental exposure to high levels of radiation and carcinogenic chemicals, and infections with certain viruses (1). Preventing and curing cancer are major worldwide issues due to the economic burden on families and societies caused by this dreadful disease. There are many anticancer drugs in use, but there is no single drug that can cure cancer completely without side effects or toxicity (2). Moreover, there is poor availability of anticancer drugs and treatment strategies (such as surgery, radiation, and chemotherapy), to people of low socioeconomic status. Although most anticancer drugs were first discovered from plant sources, providing them to lower-income populations is less cost effective. The study of the biologically active constituents of medicinal plants has been of continued and immense interest to scientists who are looking for new sources for practical alternatives against disease (3-8). Despite plants being heavily exploited in traditional healing systems, in only some cases have their therapeutic po-

tential been proven in humans (9, 10). The need for herb-based medicines, food supplements, cosmetics, pharmaceuticals, and health products is increasing worldwide because, in some cases, natural products i) are nontoxic or have low toxicity, ii) have low side effects, and iii) are attainable at affordable costs (11).

India is one of the largest mega-biodiverse countries in the world, harboring valuable medicinal plants with various pharmacological effects, including anticancer properties. In various systems of medicine, such as Ayurveda, Sidha, and Yunani, as well as in local health traditions, including tribal knowledge, several medicinal plants are used to treat cancer. Among these, some are unknown or less-known to mainstream populations, and the systematical scientific validation of their anticancer properties has not yet been done. The search for effective preventive and anticancer agents from these medicinal plants is very important and is likely to be rewarding. A large number of chemopreventive and therapeutic agents have been discovered from traditional medicinal plants all over the world. These include important anticancer drugs currently in use, such as Taxol, camptothecin, vincristine, vinblastine, and podophyllotoxin. Throughout the world, numerous chemopreventive/anticancer agents have been identified from natural products. The discovery of new generations of low-cost anticancer drugs with high efficacy and low toxicity is necessary, and is only possible by screening medicinal plants with prior knowledge.

## 2. Objectives

Three medicinal plants, *Alstonia scholaris*, *Alstonia venenata*, and *Moringa oleifera*, which are used to treat cancer and inflammatory diseases in the local health traditions of remote villages in Kerala (India), were selected for this study in order to scientifically validate traditional claims made about them.

## 3. Materials and Methods

### 3.1. Reagents and Chemicals

The ordinary chemicals used in this experiment, including dimethyl sulfoxide, sodium chloride, Tween-80, disodium hydrogen phosphate, monosodium hydrogen phosphate, sodium hydroxide, nitroblue tetrazolium, and thiobarbituric acid, were obtained from Sisco Research Laboratories Pvt. Ltd. (Chennai, India). These chemicals were of analytical grade. MTT, penicillin, streptomycin, acridine orange, and ethidium bromide were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cell culture medium RPMI-1640 and fetal calf serum were obtained from Glibco Life Technologies (Eggenstein, Germany).

### 3.2. Plant Materials

The stem bark and leaves from *A. scholaris* and *A. venenata*, and leaves from *M. oleifera* (Figure 1), were collected from the university campus in Kariavattom, Trivandrum (India). They were cleaned and dried in the laboratory at room temperature under a fan, and then powdered. The powdered stem bark and leaves of *A. venenata* and *A. scholaris*, and leaves of *M. oleifera*, were used for extraction. The respective plant powders were extracted with hexane, benzene, isopropanol, methanol, and water, sequentially.

### 3.3. Extraction Procedures

#### 3.3.1. Hexane, Benzene, Isopropanol, and Methanol Extract Preparation

In this study, hexane, benzene, isopropanol, and methanol were used separately for preparation of the various extracts of stem bark and leaves from *A. venenata* and *A. scholaris*, and of leaves from *M. oleifera*. For extraction, 2 g of plant material powders were extracted separately with 100 mL of each solution (hexane, benzene, isopropanol, and methanol) with constant stirring for 4 hours, and then filtered through Whatman No. 1 filter paper. Residues were again extracted as above with each solution, and this process was repeated three times. The respective filtrates from the extractions were combined separately and dried in a rotary evaporator (Superfit, India) under reduced pressure at 40°C. The yields of each extract were determined, and the residual powders were dried in the laboratory.

#### 3.3.2. Water Extract Preparation

The dried residual powders obtained after methanol extraction (of the stem bark and leaves of *A. venenata* and *A. scholaris* and the leaves of *M. oleifera*) were extracted with distilled water (5 g/100 mL) instead of isopropanol. To ensure complete extraction, 5 g of each powder was extracted separately with 100 mL of water with constant stirring for 4 hours, and then filtered through Whatman No. 1 filter paper. Residues were again extracted as above with water, and this process was repeated three times. The respective filtrates were combined separately and freeze-dried in a lyophilizer. The yields of the water extracts were determined. Since the heat sensitivity of the extract with reference to bioactivity was not known, the extraction was carried out at a low temperature without using rigorous extraction procedures.

### 3.4. Cell Line Preparation, Thymocyte Preparation, and Macrophage Collection

The cancer cell lines used for the study were dalton's lymphoma ascitic (DLA) cells, which were originally obtained from Amala Cancer Research Centre, Thrissur, India. They were propagated as transplantable tumors in the


**Figure 1.** A, *A. scholaris*; B, *A. venenata*; and C, *M. oleifera* plants used for extract analyses in this study.

peritoneal cavities of mice caged in the animal house at Jawaharlal Nehru tropical botanic garden and research institute, Palode, Thiruvananthapuram. The thymus glands were carefully removed from the mice, and trimmed off from the adjoining lymph nodes. Single-cell suspensions were prepared in cold RPMI-1640 medium, and viability was assessed with the Trypan blue exclusion method (12). Peritoneal exudate cells (PECs) were collected by injecting 5 mL of chilled RPMI-1640 medium into the peritoneal cavities of the mice. The glass-adherent cell population (macrophages) was separated by adhering the PECs over glass petri dishes at 37°C. The viable cell count was measured using Trypan blue in a Neubauer counting chamber.

### 3.5. Cytotoxicity Assays

#### 3.5.1. Trypan Blue Exclusion Assay

The effects of various extracts from the stem bark and leaves of *A. venenata* and *A. scholaris* and the leaves of *M. oleifera* on short-term cell viability were assessed by incubating $1 \times 10^6$ DLA cells in 1 mL of phosphate-buffered saline (PBS) containing vehicle (1% dimethyl sulfoxide), with different concentrations of the dried extracts (500 $\mu$g/mL of hexane, benzene, isopropanol, methanol, and water), for 3 hours at 37°C in an incubator (Nat Steel Equipment Pvt. Ltd.). The cell viability was assessed with the Trypan blue exclusion method (13). The most-cytotoxic extracts were identified, and their lower doses (250 - 62.5 $\mu$g/mL) were tested on the DLA cells with the Trypan blue exclusion method as described above, to obtain their $EC_{50}$ values.

#### 3.5.2. Comparison of in Vitro Cytotoxicity of the Most-Active Extracts With Normal and Cancer Cells

The most-active cytotoxic extracts (isopropanol extract of *A. venenata* leaves and hexane extract of *A. scholaris* stem bark) were tested for their comparative short-term cytotoxicity on normal and cancer cells in vitro. Briefly, short-term cell viability was assessed by incubating $1 \times 10^6$ DLA cells, peritoneal macrophages, or thymocytes in 1 mL of RPMI 1640 medium containing vehicle (1% dimethyl sulfoxide) or in 125 $\mu$g/mL of isopropanol extract of *A. venenata* leaves or 125 $\mu$g/mL of hexane extract of *A. scholaris* stem bark for 3 hours in the $CO_2$ incubator at 37°C, with 5% $CO_2$, 95% air, and 95% relative humidity. The cell viability was assessed with the Trypan blue exclusion method (13).

#### 3.5.3. MTT Assay

The MTT assay was essentially performed as described earlier (13). Briefly, $1 \times 10^6$ DLA cells were seeded in 1 mL of RPMI 1640 medium without phenol red, then supplemented with 10% fetal calf serum, penicillin (100 units/mL), streptomycin (100 $\mu$g/mL) with vehicle (1% dimethyl sulfoxide), various doses of isopropanol extract of *A. venenata* leaves, hexane extract of *A. scholaris* stem bark, or vincristine (5, 10, 25, 62.5, 125, 250, and 500 $\mu$g/mL). The cells were then placed in the $CO_2$ incubator at 37°C, with 5% $CO_2$, 95% air, and 95% relative humidity for 48 hours. After 48 hours of incubation, the spent medium was removed, and 1 mL of fresh medium (RPMI 1640 without phenol red) and MTT solution (1.2 mg/mL) was added to each well of the culture plates, and the cells were incubated for an additional 4 hours. After the final incubation, the MTT formazan product was dissolved in dimethyl sulfoxide (DMSO), and the optical density was measured at 570 nm using an ELISA plate reader (Qualigen).

#### 3.5.4. Apoptotic Assay

The apoptotic assay was essentially performed as described earlier (13). Briefly, the DLA cells ($1 \times 10^6$/mL) were seeded in RPMI 1640 medium supplemented with

10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 $\mu$g/mL), and treated with vehicle (1% DMSO), isopropanol extract of *A. venenata* leaves, hexane extract *A. scholaris* stem bark (125 $\mu$g/mL), or vincristine (25 $\mu$g/mL) in the $CO_2$ incubator for 48 hours at 37°C, with 5% $CO_2$, 95% air, and 95% relative humidity. The cells were observed under a phase-contrast microscope to assess nuclear condensation and membrane blebbing. Since the active extract-treated cells showed membrane blebbing and nuclear condensation, which are the preliminary signs of apoptosis, a detailed study of the morphological changes was conducted. Briefly, the treated (control, active extract, and vincristine) cells were washed with PBS and mixed with acridine orange and ethidium bromide stain (100 $\mu$g/mL). After staining, the cells were observed for viability and for morphological and nuclear changes under an inverted fluorescent microscope (Olympus) using a blue filter, and were photographed with a digital camera.

### 3.6. Antioxidant Activity

#### 3.6.1. Superoxide Scavenging Activity

The superoxide scavenging activity of the isopropanol extract of *A. venenata* leaves and hexane extract of *A. scholaris* stem bark (both of which showed potent in vitro anticancer activity in the DLA cells) were determined with light-induced superoxide generation by riboflavin and subsequent reduction of nitroblue tetrazolium (NBT), as previously described (14). The reaction mixture contained EDTA (6 $\mu$M) containing 3 $\mu$g of sodium cyanide (NaCN), riboflavin (2 $\mu$M), NBT (50 $\mu$M), various concentrations of the plant extracts, and phosphate buffer, for a final volume of 3 mL. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15 minutes, and the optical density was measured at 530 nm before and after illumination. The percent inhibition of superoxide generation was evaluated by comparing the absorbance values of the control and experimental tubes. Quercetin was used as a reference compound.

#### 3.6.2. Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the isopropanol extract from *A. venenata* leaves and hexane extract from *A. scholaris* stem bark for hydroxyl radicals generated from the $Fe^{3+}$-ascorbate-EDTA-hydrogen peroxide system (the Fenton reaction). The reaction mixture contained deoxyribose (2.8 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), hydrogen peroxide (1.0 mM), ascorbate (0.1 mM), potassium dihydrogen phosphate/potassium hydroxide buffer (20 mM, pH = 7.4), and various concentrations of the isopropanol and hexane extracts for a final volume of 1 mL. Quercetin was used as a positive control. The mixture was incubated for 1 hour at 37°C. Deoxyribose degradation was measured as thiobarbituric acid-reactive substances by the method of Ohkawa et al. (15).

#### 3.6.3. Lipid Peroxidation

To study the effect of isopropanol extract from *A. venenata* leaves and hexane extract from *A. scholaris* stem bark on lipid peroxidation, lipid peroxidation was induced with the $Fe^{2+}$/ascorbate system. The reaction mixture contained liver homogenate (25%, 0.1 mL) in Tris-HCl buffer (0.2 M, pH = 7.0), ascorbic acid (0.3 mM), ferrous ammonium sulfate (0.8 mM), and various concentrations of the isopropanol and hexane extracts for a final volume of 0.5 mL. Quercetin was used as a positive control. The incubation was carried out for 1 hour at 37°C. The lipid peroxide content was measured as thiobarbituric acid-reactive substances by the method of Ohkawa et al. (15). The percent inhibition of lipid peroxide formation was determined by comparing the results of the herbal-drug-treated and untreated samples.

### 3.7. Statistical Analysis

Analysis of variance (ANOVA) was calculated using SPSS v. 11.5 (IBM, NY, USA), and the differences between the treatment means were compared using Duncan's multiple-range test, at a significance level of $P < 0.05$.

## 4. Results

### 4.1. Cytotoxicity Assays

Among the leaf extracts of *A. scholaris*, the hexane extract showed significant cytotoxicity on DLA cells with short-term (3 hours) incubation in PBS; 100% DLA cell death was observed at 500 $\mu$g/mL of the hexane extract, but its lower doses showed reduced cytotoxicity. A lower dose of 62.5 $\mu$g/mL of hexane extract resulted in only 26% cell death. Benzene, isopropanol, and water extracts of *A. scholaris* leaves showed marginal activity at the 500 $\mu$g/mL dose, but the methanol extract lacked any cytotoxicity against the DLA cells (Table 1).

As shown in Table 1, of the *A. scholaris* stem bark extracts, the hexane extract showed 100% cytotoxicity against the DLA cells at 500, 250, and 125 $\mu$g/mL doses after 3 hours of incubation in PBS. A lower dose of the hexane extract (62.5 $\mu$g/mL) resulted in 48% cell death. Except for the benzene extract, all other extracts (isopropanol, methanol, and water) at doses of 500 $\mu$g/mL did not show any cytotoxicity against the DLA cells. The benzene extract

**Table 1.** Cytotoxicity of Leaf and Stem Bark Extracts of *A. scholaris* Against DLA Cells ($1 \times 10^6$ cells/mL) Incubated in PBS at 37°C for 3 Hours[a]

| Treatment | Dose, $\mu$g/mL | Cell Death, % | |
|---|---|---|---|
| | | *A. scholaris* Leaf Extract | *A. scholaris* Stem Bark Extract |
| Dimethyl sulfoxide (control), % | 1 | 0 | 0 |
| Hexane extract | 62.5 | 26 ± 3 | 48 ± 4 |
| Hexane extract | 125 | 52 ± 4 | 100 |
| Hexane extract | 250 | 65 ± 3 | 100 |
| Hexane extract | 500 | 100 | 100 |
| Benzene extract | 500 | 11 ± 1 | 28 ± 6 |
| Isopropanol extract | 500 | 4 ± 2 | 0 |
| Methanol extract | 500 | 0 | 0 |
| Water extract | 500 | 4 ± 2 | 0 |

[a]Values are mean ± SD of three separate determinations.

showed marginal cytotoxicity of 28%. Various extracts obtained from *A. venenata* leaf with short-term (3 hours) cytotoxicity against DLA cells showed varying levels of activity, as shown in Table 2. Among the extracts tested, the isopropanol and benzene extracts showed concentration-dependent increments in rates of cell death; 100% DLA cell death was observed at doses of 125 $\mu$g/mL of isopropanol extract and 500 $\mu$g/mL of benzene extract. Hexane extract at a dose 500 $\mu$g/mL exerted 33% cancer cell death, but the methanol and water extracts lacked any anti-DLA activity at the 500 $\mu$g/mL dose.

As shown in Table 2*A, venenata* stem bark extract showed varying levels of cytotoxicity against DLA cells incubated in PBS for 3 hours. Among the extracts tested, 100% cytotoxicity was observed at doses of 125, 250, and 500 $\mu$g/mL of hexane and 500 $\mu$g/mL of benzene *A. venenata* leaf extracts. Also, doses of 500 $\mu$g/mL hexane and benzene stem bark extracts of *A. venenata* showed 100% cell death, while the methanol and water extracts did not exhibit any cytotoxicity at a dose of 500 $\mu$g/mL.

The various extracts of M. oleifera leaf with short-term (3 hours) cytotoxicity against DLA cells are shown in Table 3. Among the extracts tested, the hexane and benzene extracts at doses of 500 $\mu$g/mL showed 75 ± 7% and 61 ± 8% anti-DLA activity, respectively, while the methanol extract excreted marginal cytotoxicity of 2 ± 1% at the same drug dose. The isopropanol and water extracts did not show any cytotoxicity against DLA cells at the 500 $\mu$g/mL dose.

Among the extracts tested for cytotoxicity against DLA cells, the most active extracts from *A. scholaris* and *A. venenata* were selected (the extracts from *M. oleifera* did not show 100% cytotoxicity even at the 500 g/mL dose, so it was not considered for determining the $EC_{50}$ value), and their $EC_{50}$ values were determined (Table 4). Among the extracts of *A. scholaris*, the hexane extract of stem bark showed an $EC_{50}$ value of 68.75 $\mu$g/mL, while the n-hexane extract of the leaves showed a higher $EC_{50}$ value of 118.75 $\mu$g/mL. In the case of *A. venenata* leaf and stem bark extracts tested for DLA activity, the benzene and isopropanol extracts of the leaves showed $EC_{50}$ values of 141.65 and 66.67 $\mu$g/mL, respectively, while the hexane and benzene extracts of the stem bark showed $EC_{50}$ values of 154.2 and 480.25 $\mu$g/mL, respectively. Among the extracts tested for cytotoxicity against DLA cells, the hexane extract of *A. scholaris* stem bark and isopropanol extract of *A. venenata* leaves showed significant anti-DLA activity at lower doses on short-term (3 hours) cytotoxic evaluations.

The most active extracts from *A. scholaris* (hexane extract of stem bark) and *A. venenata* (isopropanol extract of leaves) were tested for their cytotoxicity against DLA cells, peritoneal macrophages, and thymocytes in RPMI medium for 3 hours under culture conditions, as shown in Table 5. Hexane extract of *A. scholaris* stem bark at a dose of 125 $\mu$g/mL showed 100% cell death in the DLA cells, while marginal cytotoxicity was shown in macrophages (8 ± 2%) and thymocytes (14 ± 2%). The isopropanol extract of *A. venenata* leaves at a dose of 125 $\mu$g/mL showed 100% cell death in the DLA cells, but exhibited marginal cytotoxicity of 6 ± 2% and 15 ± 3% in macrophages and thymocytes, respectively.

As shown in Figure 2, the MTT assays of *A. scholaris* (hexane extract of stem bark) and *A. venenata* (isopropanol extracts of leaf) in various doses along with the standard drug, vincristine, showed concentration-dependent increments in anti-DLA activity. Both the hexane extract of *A. scholaris* and the isopropanol extract of *A. venenata* showed

**Table 2.** Cytotoxicity of Leaf and Stem Bark Extracts of *A. venenata* Against DLA Cells ($1 \times 10^6$ cells/mL) Incubated in PBS at 37°C for 3 Hours[a]

| Treatment and Dose, $\mu$g/mL | Cell Death, % | |
|---|---|---|
| | Leaf Extracts of *A. venenata* | Stem Bark Extracts of *A. venenata* |
| **Dimethyl sulfoxide (control)** | | |
| 1% | 0 | 0 |
| **Hexane extract** | | |
| 125 | 0 | 36 ± 4 |
| 250 | 0 | 91 ± 2 |
| 500 | 33 ± 4 | 100 |
| **Benzene extract** | | |
| 62.5 | 12 ± 2 | 0 |
| 125 | 42 ± 5 | 21 ± 5 |
| 250 | 96 ± 2 | 38 ± 3 |
| 500 | 100 | 100 |
| **Isopropanol extract** | | |
| 62.5 | 49 ± 7 | 0 |
| 125 | 100 | 0 |
| 250 | 100 | 0 |
| 500 | 100 | 2 ± 1 |
| **Methanol extract** | | |
| 500 | 0 | 0 |
| **Water extract** | | |
| 500 | 0 | 0 |

[a]Values are mean ± SD of three separate determinations.

**Table 3.** Cytotoxicity of Leaf Extracts of *M. oleifera* Against DLA Cells ($1 \times 10^6$ cells/mL) Incubated in PBS at 37°C for 3 Hours[a]

| Treatment | Dose, $\mu$g/mL | Cell Death, % |
|---|---|---|
| **Dimethyl sulfoxide (control), %** | 1 | 0 |
| **Hexane extract** | 500 | 75 ± 7 |
| **Benzene extract** | 500 | 61 ± 8 |
| **Isopropanol extract** | 500 | 0 |
| **Methanol extract** | 500 | 2 ± 1 |
| **Water extract** | 500 | 0 |

[a]Values are mean ± SD of three separate determinations.

100% cell death at the 125, 250, and 500 $\mu$g/mL doses. The standard drug, vincristine, showed 100% cell death even at a low dose of 25 $\mu$g/mL.

The cells treated with *A. scholaris* stem bark extract (hexane), *A. venenata* left extract (isopropanol), or vincristine showed membrane blebbing and nuclear condensation on phase-contrast microscopy. The plant extract-treated or vincristine-treated cells stained with acridine orange-ethidium bromide showed membrane blebbing typical of apoptotic morphology on fluorescent microscopy. The dead cells appeared orange-red in color, while the vehicle (DMSO)-treated cells appeared yellowish-green (live), without membrane blebbing or nuclear condensation (Figures 3 and 4).

**Table 4.** $EC_{50}$ of Active Extracts of *A. scholaris* and *A. venenata* on DLA Cells ($1 \times 10^6$ cells/mL) Incubated at 37°C in PBS for 3 Hours[a,b]

| Plant part | Treatment | EC50 value of standard, $\mu$g/mL |
|---|---|---|
| ***A. scholaris* leaf** | Hexane extract | 500 |
| ***A. scholaris* stem bark** | Hexane extract | 500 |
| ***A. venenata* leaf** | Benzene extract | 500 |
| ***A. venenata* leaf** | Isopropanol extract | 500 |
| ***A. venenata* stem bark** | Hexane extract | 500 |
| ***A. venenata* stem bark** | Benzene extract | 500 |

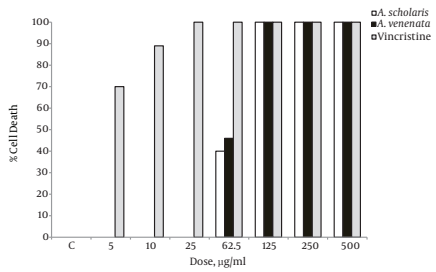
[a]Values are mean ± SD of three separate determinations.
[b]Extracts showing 100% cell death at a dose of 500 $\mu$g/mL were considered for determining their $EC_{50}$ values.

**Table 5.** Comparison of in Vitro Cytotoxicity of *A. scholaris* Bark Extract (hexane) and *A. venenata* Leaf Extract (Isopropanol) on Different Cells ($1 \times 10^6$ cells/mL) Incubated at 37°C in RPMI Medium for 3 Hours in a $CO_2$ Incubator[a]

| Plant part | Treatment | Dose, $\mu$g/mL | Cell Death, % | | |
|---|---|---|---|---|---|
| | | | DLA | Macrophages | Thymocytes |
| | Dimethyl sulfoxide (control) | 1% | 0 | 0 | 0 |
| ***A. scholaris* stem bark** | Hexane extract | 125 | 100 | 8 ± 2 | 14 ± 2 |
| ***A. venenata* leaf** | Isopropanol extract | 125 | 100 | 6 ± 2 | 15 ± 3 |

[a]Values are mean ± SD of three separate determinations.

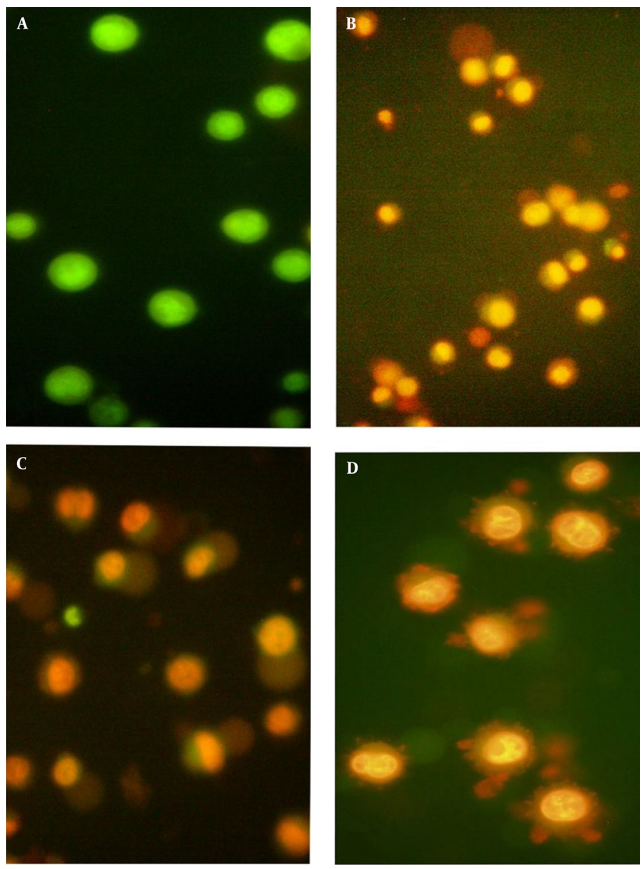
**Figure 2.** In Vitro Cytotoxicity of *A. scholaris* Bark Extract (Hexane) and *A. venenata* Leaf Extract (Isopropanol) on DLA Cells ($1 \times 10^6$ cells/mL)




In vitro cytotoxicity incubated at 37°C in RPMI medium for 48 hours in a $CO_2$ incubator. Values are the mean of three separate determinations. Cytotoxicity was determined by MTT assay. C-control (dimethyl sulfoxide).

This study reports for the first time that the hexane extract of *A. scholaris* stem bark and the isopropanol extract of *A. venenata* leaves showed significant in vitro anticancer activity on DLA cells. Under long-term culture conditions, RPMI medium supplemented with all additives provided optimum conditions for DLA cancer cell growth, as in the animal body, which is an easy way of determining the in vitro anticancer activity of herbal drugs. In short-term (Trypan blue exclusion) and long-term cytotoxic (MTT) assays, various doses of active extracts were tested for efficacy, and 100% cell death was observed at 125 $\mu$g/mL doses. Compared with the standard anticancer drug, vincristine, this dose is higher. However, extracts contain a mixture of compounds, among which the actual anticancer principle is present in lesser quantities, so when it is isolated in a pure form, anticancer efficacy may be obtained at even lower doses than with vincristine. It has been reported that higher doses of herbal extracts and lower doses of their pure compounds show equal efficacy in anticancer activities (16). Many potent anticancer principles now in therapeutic use were isolated from plant sources, such as Taxol from Taxus baccata, vincristine and vinblastine from Vinca rosea, and camptothecin from Ophiorrhiza mungos. On the other hand, toxicity evaluations in animals have shown varying levels of toxicity of these plants (17). In this context, it is important to point out that the active anti-DLA extracts of *A. scholaris* and *A. venenata*, when tested on peritoneal macrophages and thymocytes, showed only marginal cytotoxicity against these normal cells. In contrast, it has been reported that the standard drug, vincristine, showed significant cytotoxicity against cancer cell lines as well as against normal cell lines at doses above 10 $\mu$g/mL (18). DLA cell-specific cytotoxicity is a remarkable property of the active extracts, as the extract-treated DLA cells that were evaluated under phase-contrast and fluorescent microscopy showed significant membrane blebbing,

**Figure 3.** Effect of *A. scholaris* Stem Bark Extract (Hexane) and *A. venenata* Leaf Extract (Isopropanol) on DLA Cells ($1 \times 10^6$ cells/mL)




The effect is incubated at 37°C in RPMI medium for 48 hours in a $CO_2$ incubator, photographed under fluorescent microscopy. A, control, DLA cells treated with 1% DMSO and photographed under fluorescent microscopy with acridine orange-ethidium bromide staining (live cells appear green in color, without membrane blebbing); B, test, DLA cells treated with 125 $\mu$g/mL of isopropanol extract of *A. venenata* leaves, photographed under fluorescent microscopy with acridine orange-ethidium bromide stain (orange-red dead cells show membrane blebbing); C, test, DLA cells treated with 125 $\mu$g/mL of hexane extract of *A. scholaris* stem bark, photographed under fluorescent microscopy with acridine orange-ethidium bromide stain (orange-red dead cells show membrane blebbing); D, Standard drug, vincristine (25 $\mu$g/mL)-treated DLA cells, photographed under fluorescent microscopy with acridine orange-ethidium bromide stain (orange-red dead cells show membrane blebbing).

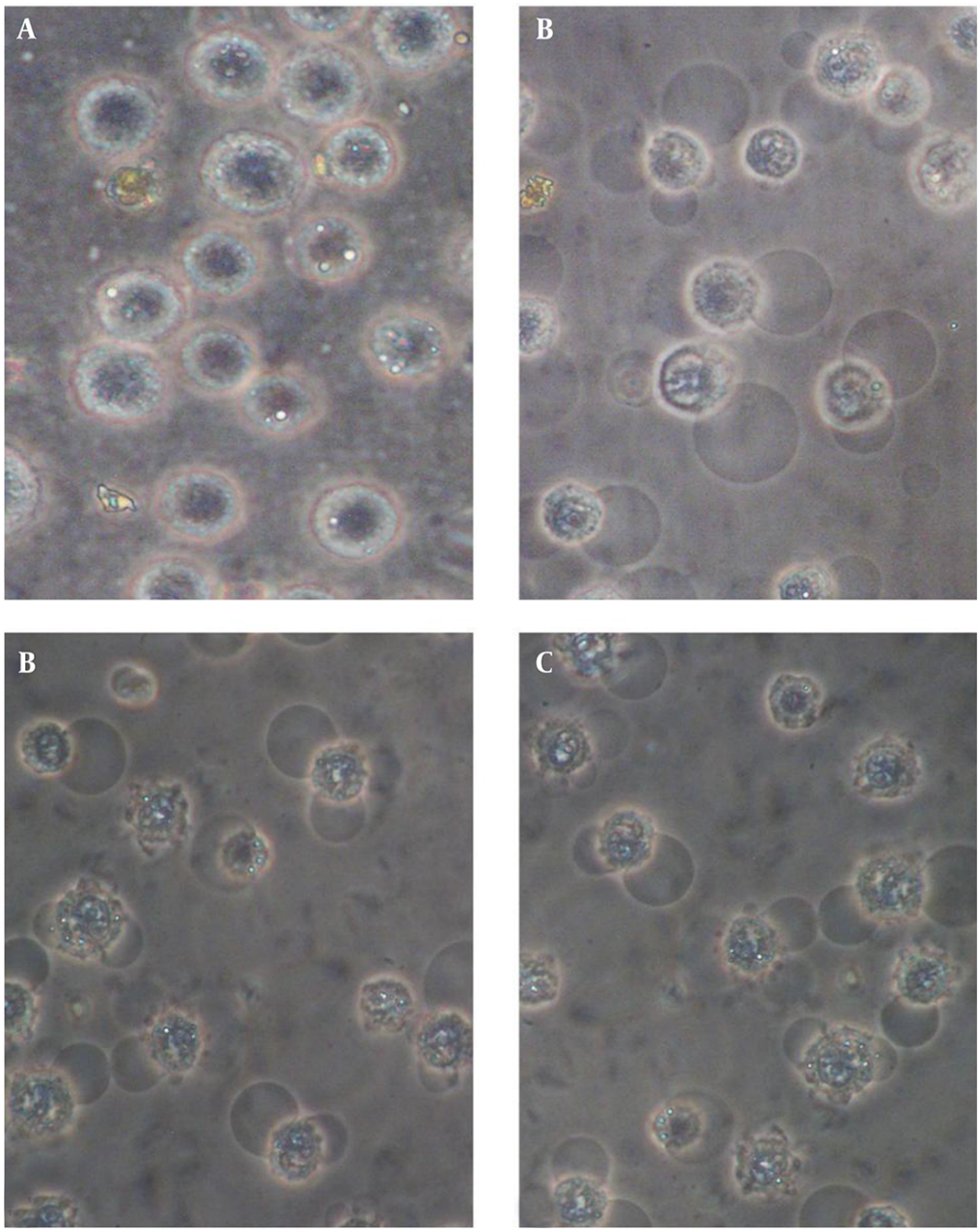
nuclear condensation, and damage, which are hallmarks of apoptosis rather than necrosis. Thus, at least one mechanism of action of these active extracts could be the induction of cell-specific apoptosis. There are several herbal drugs and nutraceuticals known to influence the expression of genes or cell-signaling cascades, preventing carcinogenesis or curing cancer (19-21). Since the active extracts showed significant DLA-cell-specific anticancer activity, isolation of the active compounds and evaluation of their efficacy in in vivo models is warranted.

### 4.2. Antioxidant Activity

Since the hexane extract of *A. scholaris* stem bark and the isopropanol extract of *A. venenata* leaves showed significant DLA-cell-specific cytotoxicity and induced apoptosis, only these extracts were tested for their antioxidant activities. As shown in Figure 5, the in vitro superoxide scavenging activities of various doses of hexane extract (*A. scholaris*) and isopropanol extract (*A. venenata*) were compared with the standard antioxidant compound, quercetin. *A. venenata* showed significant in vitro superoxide scavenging activity, superior to that of quercetin. *A. venenata* showed less superoxide scavenging activity. The $IC_{50}$ values of hexane extract (*A. scholaris*), isopropanol extract (*A. venenata*), and quercetin were 90.5 $\pm$ 6.2, 7.5 $\pm$ 1.2, and 31.5 $\pm$ 2.5 $\mu$g/mL, respectively (Table 6).

The in vitro hydroxyl radical scavenging activities of n-hexane extract (*A. scholaris*), isopropanol extract (*A. venenata*), and quercetin are shown in Figure 6. Both of the extracts showed significant in vitro hydroxyl radical scavenging activity, and the $IC_{50}$ value of isopropanol extract (*A.*

**Figure 4.** Effect of *A. scholaris* Stem Bark Extract (hexane) and *A. venenata* Leaf Extract (Isopropanol) on DLA Cells ($1 \times 10^6$ cells/mL)




The effect is incubated at 37°C in RPMI medium for 48 hours in a $CO_2$ incubator, photographed under fluorescent microscopy. A, control, DLA cells treated with 1% DMSO (live cells without membrane blebbing and nuclear condensation); B, test, DLA cells treated with 125 $\mu$g/mL of isopropanol extract of *A. venenata* leaves, photographed under phase-contrast microscopy (apoptotic cells show membrane blebbing and nuclear condensation); C, test, DLA cells treated with 125 $\mu$g/mL of hexane extract of *A. scholaris* stem bark, photographed under phase-contrast microscopy (apoptotic cells show membrane blebbing and nuclear condensation); D, standard drug, vincristine (25 $\mu$g/mL)-treated DLA cells, photographed under phase-contrast microscopy (apoptotic cells show membrane blebbing and nuclear condensation).

*venenata*) was superior to that of the standard compound, quercetin. The $IC_{50}$ values of hexane extract (*A. scholaris*), isopropanol extract (*A. venenata*), and quercetin were 23 $\pm$ 2.8, 15.0 $\pm$ 2.0, and 21.5 $\pm$ 1.6 $\mu$g/mL, respectively (Table 6).

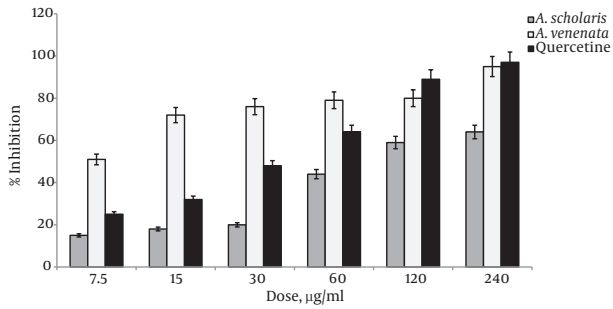
Inhibition of in vitro lipid peroxidation of hexane extract (*A. scholaris*), isopropanol extracts (*A. venenata*), and

**Table 6.** $IC_{50}$ of Active Extracts of *A. scholaris* and *A. venenata* Compared to the Standard, Quercetin, on Various Antioxidant Assays[a]

| Plant Extract and Assay | $IC_{50}$ Value of Extract, $\mu$g/mL | $IC_{50}$ Value of Standard Quercetin, $\mu$g/mL |
|---|---|---|
| ***A. scholaris* stem bark** | | |
| Superoxide radical scavenging | 90.5 ± 6.2 | 31.5 ± 2.5 |
| Hydroxyl radical scavenging | 23.0 ± 2.8 | 21.5 ± 1.6 |
| Inhibition of lipid peroxidation | 182.3 ± 9.5 | 42.5 ± 3.9 |
| ***A. venenata* leaf** | | |
| Superoxide radical scavenging | 7.5 ± 1.2 | 31.5 ± 2.5 |
| Hydroxyl radical scavenging | 15.0 ± 2.0 | 21.5 ± 1.6 |
| Inhibition of lipid peroxidation | 200.0 ± 9.2 | 42.5 ± 3.9 |

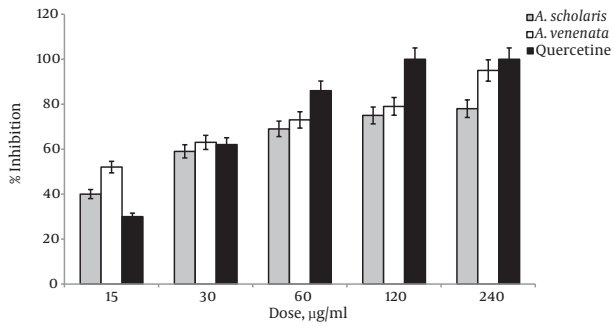
[a]Values are mean ± SD of three separate determinations.

**Figure 5.** In Vitro Superoxide Radical Scavenging Activity of *A. scholaris* Bark Extract (Hexane) and *A. venenata* Leaf Extract (Isopropanol) With Quercetin




Values are mean ± SD of three separate determinations.

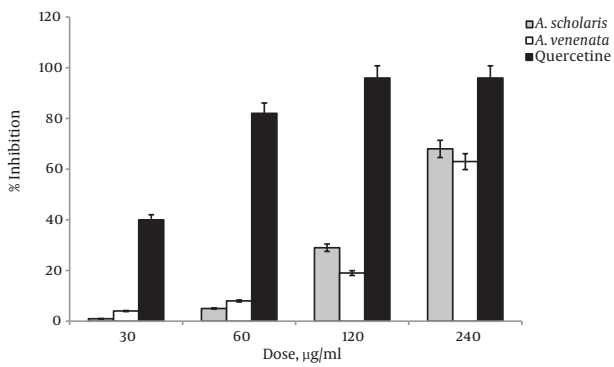
**Figure 6.** In Vitro Hydroxyl Radical Scavenging Activity of *A. scholaris* Bark Extract (Hexane) and *A. venenata* Leaf Extract (Isopropanol) With Quercetin




Values are mean ± SD of three separate determinations.

quercetin is shown in Figure 7. The inhibitory efficacy of both extracts on in vitro lipid peroxidation was less than that of the standard compound, quercetin, and the $IC_{50}$ values of hexane extract (*A. scholaris*), isopropanol extract (*A. venenata*), and quercetin were 182.3 ± 9.5, 200 ± 9.2, and 42.5 ± 3.9 $\mu$g/mL, respectively (Table 6). Many well-known plant principles, including quercetin, curcumin, and resveratrol, have shown significant antioxidant and anticancer activities in in vitro and in vivo conditions. Many plant principles regulate the candidate genes and/or signaling pathways involved in antioxidant activities that internally regulate the inflammatory response, which is crucial in cancer development. The NF-kappa B and pro-inflammatory markers, such as TNF-alpha and cyclooxygenase, are downregulated by herbal materials such as chlorophyll-a, curcumin, resveratrol, and polyphenols, protecting cells from severe oxidation, inflammation, and carcinogenesis (22, 23). The cytotoxic effect of the active extracts of *A. scholaris* and *A. venenata* on DLA cells, and their cytoprotective effect on normal cells, may be due to their higher antioxidant activity.

**Figure 7.** Inhibition of in Vitro Lipid Peroxidation Activity of *A. scholaris* Bark Extract (Hexane) and *A. venenata* Leaf Extract (Isopropanol) Compared to Quercetin




Values are mean ± SD of three separate determinations.

## 5. Discussion

Historically, all medicinal preparations were derived from plants, either in the simple form or in the complex form of crude extracts and mixtures. Today, a substantial number of drugs are developed from plants, which are active against a number of diseases. In India, approximately 20,000 medicinal plant species have been recorded, but more than 500 traditional communities use approximately 800 plant species for curing different diseases. On the basis of the results obtained in the present study, it can be concluded that hexane extract of *A. scholaris* stem bark and isopropanol extract of *A. venenata* leaves have cytotoxic and antioxidant properties. Work is currently being undertaken to isolate the active compound(s) with bioassay-guided fractions from the species that showed high inhibitory activity during screening. This study may serve as a guide for the discovery of a new generation of anticancer drugs to combat cancers such as lymphoma and leukemia.

## Footnote

**Authors' Contribution:** Study idea, design, and protocol: Gholamreza Bagheri; writing of the manuscript: Gholamreza Bagheri, Mehdi Mirzaei, Raheleh Mehrabi, and Javad Sharifi-Rad; Javad Sharifi-Rad critically reviewed the manuscript. All of the authors read and approved the final manuscript.

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