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Novel wild edible mushroom *Astraeus hygrometricus* (Pers.) Morgan induces robust apoptosis on human acute lymphoblastic leukemia cells through a RONS-subsisted mitochondria-dependent pathway

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Abstract— The purpose of this study is to investigate the potential medicinal benefits of the Astraeus hygrometricus (Pers.) Morgan, a newly discovered wild edible fungus, on cells that have developed acute lymphoblastic leukaemia in humans.Methods: Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a battery of tests was conducted to determine the antiproliferative and chemopreventive potential of various extracts from five wild mushrooms: A. hygrometricus, Phallus sp., Lentinus sp., Tricholoma sp., and Serpula sp. The results were evaluated against a panel of six cancer cell lines and normal cells. The following were examined using flow cytometry: cell cycle profiling, apoptosis determination, intracellular reactive oxygen and nitrogen species (ROS and RNS), and mitochondrial membrane potential. We analysed the expression pattern of mitochondrial proteins and used colorimetric methods to quantify caspase activity. A. hygrometricus methanol extract showed the most antiproliferative effect, whereas MOLT-4 cells were the most sensitive, according to the results. Mushroom extract effectively halted cell cycle development at the G0/G1 stage and produced strong selective apoptosis in MOLT-4 cells. In MOLT-4 cells, the extract increased ROS generation and altered the potential of the mitochondrial membrane. By activating the caspase cascade, downregulating Bcl-2 expression, and boosting Bax expression, the methanol extract triggered apoptosis. In conclusion, the new edible wild mushroom may have biomolecules that may be used in the creation of antileukemic medications.

1. Introduction

Among all human diseases, cancer is now the leading killer.American Cancer Society estimates put the number of new cancer cases at 1,918,030 in 2022, with 609,360 deaths attributed to the disease.3 In 91 out of 172 nations, cancer ranks as the leading or second-leading cause of death, indicating a high mortality risk.1 Radiotherapy and chemotherapeutic medications are the principal cancer therapies now available, however they have the potential to damage healthy cells and slow patients' recovery after treatment.5 There is an urgent need to produce a safe anticancer medication from natural sources with decreased side effects in order to eradicate the issues related to the administration of a nonspecific chemotherapeutic agent.

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Numerous bioactive substances, such as phenols, flavonoids, volatile oils, organic acids, polysaccharides, glycosides, carotenoids, and alkaloids, have been discovered in mushrooms, which has led to their inclusion in the quest for alternative natural anticancer medications. Novel anticancer, antibacterial, antioxidant, immunomodulatory, anti-inflammatory, cardiovascular, and antidiabetic medicines may be derived from these chemicals or from alternative natural sources.6e11 – Traditional, all-natural medicine derived from mushrooms has a long history of use in some

Southeast Asian nations. For many centuries, various components originating from mushrooms have been used as nutritional supplements and herbal remedies in traditional Chinese medicine (TCM).12 Unfortunately, only a small fraction of the world's mushroom species have undergone any kind of medicinal screening, and even less have been studied in this area.13 Numerous mushroom species have shown promise as anticancer agents; they include Agaricus, Antrodia, Albatrellus, Cordyceps, Calvatia, Clitocybe, Inocybe, Inonotus, Russula, Schizophyllum, and Trametes.13 This trustworthy alternate supply of anticancer chemicals, however, seems to be understudied. Previous research indicated that methanol extract of Astraeus hygrometricus (Pers.) Morgan (A. hygrometricus) had an antiproliferative action on Jurkat cells with only a small impact on normal peripheral blood mononuclear cells (PBMCs). The five species of mushrooms tested in this study were Phallus sp., A. hygrometricus, A. hygrometricus, Lentinus sp., Tricho-loma sp., and Serpula sp.14 Here, we included six more cancer cell lines in the screening and apoptotic efficacy study: MOLT-4, Reh, and NALM-6, which are leukemic; Hep G2, which is hepatocellular carcinoma; A549., which is lung carcinoma; MCF-7, which is breast cancer; and BEAS-2B, which is normal. Investigating the role of reactive nitrogen species (RNS) and reactive oxygen species (ROS) and their potential effect on mitochondrial membrane potentiality was another extension of our research into the potential mechanism of cancer inhibition. Additionally, we tested the most sensitive cell line for the apoptogenic potential of the strongest extract. To conclude, we sought to determine the molecular mechanism of apoptotic induction by examining how the mushroom extract affected cell cycle progression and the expression levels of apoptotic mediators, caspases, and many important mitochondrial proteins.

2. Materials and methods

2.1. Sample collection and identification

Wild mushroom fruit bodies were collected between 2015 and 2019 from diverse habitats between 22.1203°N, 88.3943°E and 27.0206°N, 88.5650°E, spanning an area from the hinterland of the Bay of Bengal and lateritic region of the western part of West Bengal state to the great Himalayan foothills. The fruit bodies of *A. hygrometricus* (Jamboni, 22.4502°N, 86.8998°E) and *Lentinus* sp. (Sigram, 22.4479°N, 86.8967°E) were collected from the laterite region of Jhargram District; those of *Tricholoma* sp. and *Serpula* sp. (Mathurapur, 22.1203°N, 88.3943°E) were collected from areas with alluvial soil in the South 24-Parganas District; while those of *Phallus* sp. were collected from wood logs associated with moist humus soil (Lolegaon, 27.0206°N, 88.5650°E) in the Kalimpong District of West Bengal, India. Mushroom species were identified using various published standard indentation keys and molecular validation, and voucher specimens were deposited at the Calcutta University Herbarium.[5,14]

2.2. Chemicals

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All chemicals used were of molecular grade. Dulbecco's modi- fied eagle medium (DMEM, Gibco, New York, NY), Roswell Park Memorial Institute Medium (RPMI 1640, Gibco, New York, NY), fetal bovine serum (FBS, Gibco, New York, NY), penicillin-streptomycin (Gibco, New York, NY), amphotericin b (Himedia, Maharashtra, India), 2 mM L-glutamine, phosphate buffer saline (PBS, Gibco, New York, NY), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, Burlington, MA), 4′,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, Burlington, MA), methanol, hexane, ethyl acetate, DMSO (Sigma Aldrich, Burlington, MA), annexin-V/propidium iodide (PI) apoptosis detection kit (BD- Pharmingen, Bergen, NJ), CM-H_2DCFDA (Invitrogen, Carlsbad, CA), JC-1 kit (Invitrogen, Carlsbad, CA), propidium iodide (PI, Himedia, Maharashtra, India), caspase detection kit (BioVision, Waltham, MA), and primary and secondary antibodies against poly (ADP- ribose) polymerase (PARP) and b-actin (Abcam, Cambridge, UK) were purchased from the respective manufacturers.

2.3. Preparation of mushroom extracts

The samples were dried under a shed and then ground using a mixer grinder. Each mushroom powder sample was extracted by selectively applying a solvent system based on solvent polarity, and 50 g of starting material was primarily percolated in hexane for 3 d to remove the fatty substances. The residual material was then sequentially extracted with ethyl acetate followed by methanol solvent. Each extract was evaporated in a rotary evaporator, lyophilized at reduced pressure, and finally stored at 4°C for further experiments.

2.4. Cell culture and maintenance

The MOLT-4 (T-cell acute lymphoblastic leukemic), Reh (B-cell acute lymphoblastic leukemic), and NALM6 (B-cell acute lympho- blastic leukemic) cell lines were kindly gifted by Dr. Santu Ban- dyopadhyaya and Dr. Chitra Mandal (IICB, Kolkata, India). Hep G2 (human hepatocellular carcinoma) cells were gifted by Prof. Sanjit Dey from the Department of Physiology, University of Calcutta (Kolkata, India). The A549 (human lung carcinoma), MCF-7 (human breast cancer), and BEAS-2B (human nontumorigenic lung epithelial) cell lines were procured from the National Centre for Cell Sciences (NCCS, Pune, India). All cell lines were maintained in either DMEM or RPMI 1640 supplemented with 2 mM L-glutamine, 10% (v/v) FBS (heat-inactivated), and 10 U/mL penicillin/streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO_2 incubator (HF90, Heal Force, Hong Kong, China).

2.5. Cell viability assay

The antiproliferative effect of the crude extracts of the 5 mushroom species was assessed against 6 different cancer cell lines, that is, three leukemic cell lines (MOLT-4, Reh, and NALM-6), one hepatocellular carcinomic cell line (Hep G2), one lung carci- nomic cell line (A549), and one breast cancer cell line (MCF-7) as well as against one normal cell line (BEAS-2B) and PBMCs from a healthy donor by the MTT assay.[15,16] In brief, 5×10^4 cells were incubated with various concentrations (10e100 mg/mL) of mush- room extract in a 96-well plate for 24 h at 37°C. Subsequently, 3 h before completion, 25 mL MTT solution (5 mg/mL in PBS) was added to each well, and finally, formazone complex was dissolved in DMSO, and the optical density (OD) was recorded at 560 nm. The absorbance of fully lysed (100%) cells was measured by treating them with 5% sodium dodecyl-sulfate (SDS) lysis buffer before measurement at 560 nm. The percentage of cell viability was calculated using the following equation:

$$\text{Cell viability } (\%) = \frac{OD_{\text{sample}} - OD_{100\%\ \text{lysis}}}{OD_{0\%\ \text{lysis}} - OD_{100\%\ \text{lysis}}} \times 100$$

Regarding the isolation of PBMCs, the percoll density gradient centrifugation (d 1.082 g/L) method was applied to heparinized venous blood from a healthy donor and desired PBMCs were recovered from the interface layers. The recovered cells were washed in PBS and resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, gentamicin, and 10% heat-inactivated FBS, for further study.[17]

2.6. Effect of methanol extract on cellular morphology-DAPIstaining

To investigate the effect of the methanol extract of *A. hygrometricus* on the nuclei of MOLT-4 cells, DAPI staining was performed.[18,19] The cells were incubated with increasing concentrations (0e25 mg/mL) of mushroom extract for 24 h at 37°C. Cells were fixed with 4% paraformaldehyde, followed by washing with PBS. The cells were then stained with 2 mg/mL DAPI for 15 min in the dark and viewed under a fluorescent microscope at 400 1 magnification (Dewinter Optical, Delhi, India). Based on the status of the nucleus, cells were categorized as apoptotic (condensed or fragmented chromatin) or normal (smooth nuclear structure).

2.7. Cell cycle profiling assay by propidium iodide staining

For this experiment, MOLT-4 cells and PBMCs were treated with increasing concentrations of mushroom extract (0e25 mg/mL) for 24 h. Subsequently, cells were harvested into a single-cell suspension and incubated overnight at —20°C with 70% ethanol. Cells

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were resuspended in PBS and incubated for 2 h with RNaseA (20 mm) at
37°C. Eventually, PI was added, and after incubation for 20 min at 25°C, cells were subjected to flow cytometry analysis.[4,19]

2.8. Measurement of cellular apoptosis by annexin V-PI staining

Induction of apoptosis by the mushroom extract was measured by flow cytometry after annexin V-FITC/PI staining.[16,20] Briefly,
1.5 × 10^5 MOLT-4 cells and PBMCs were incubated with an increasing concentration (0e25 mg/mL) of methanol extract for
24 h. After incubation, cells were washed with PBS and resuspended in 100 mL of binding buffer for 30 min. Finally, 5 mL annexin V-FITC and 5 mL PI were added to cells and incubated for 15 min,and fluorescent signals were recorded. The cell population negative for both annexin V and PI was recorded as viable cells. Based on the annexin V/PI ratio, stained cells were categorized as early apoptotic (positive for annexin V/negative for PI) or late apoptotic (positivefor annexin V/positive for PI).

2.9. Measurement of mushroom-induced cellular ROS using DCFDA

The levels of intracellular ROS were measured using the DCFDA method according to the manufacturer's instructions. In brief,
1.5 × 10^5 MOLT-4 cells were incubated with different concentra- tions of mushroom extract (0, 5, 10, 25, and 50 mg/mL) for 6 h. After
incubation, cells were washed with PBS and reincubated in serum-free media containing 5 mM CM-H_2DCF-DA for another 30 min at 37°C. Finally, fluorescent signals of the intracellular production of
ROS were recorded using a flow cytometer. (Attune NxT, Boston, MA).[17]

2.10. Measurement of mushroom-induced intracellular RNS byDAF-2

The levels of intracellular RNS induced by the mushroom extract were measured using the DAF-2 method according to the manu- facturer's instructions. For the measurement of the concentration- dependent formation of RNS, 1.5 10^5 MOLT-4 cells were incubatedwith different concentrations of mushroom extract (0, 5, 10, 25, and50 mg/mL) for 6 h. To evaluate the time-dependent formation of
RNS, 1.5 10^5 MOLT-4 cells were induced with 5 mg/mL of mush- room extract every 60 min for up to 300 min. After incubation, the cells were washed with PBS and reincubated in serum-free media containing diaminofluorescein-FM (DAF-FM) for another 30 min. The cells were then subjected to flow cytometry and fluorescent signals of intracellular production of RNS were recorded.

2.11. Measurement of mitochondrial permeability

To explore the involvement of mitochondria in the apoptosis process, we measured the mitochondrial membrane potential (DJm), using JC-1. In this procedure, 1.5 10^5 MOLT-4 cells were incubated with increasing concentrations of mushroom extract (0e25 mg/mL) for 6 h. The cells were then stained with JC-1 (10 mM in PBS), incubated for 15 min, and subjected to flow cytometry. The recorded data was further analyzed by applying a quadrant plot to distinguish monomers from J-aggregates and by using the FlowJo software (FlowJo, Ashland, OR). Results were presented as the ratio of J aggregates/monomers, effectively indicating the cellular mito- chondrial transmembrane potential.[17]

2.12. Determination of caspase activity

The activity of caspase-3, -8, and -9 was measured in cell lysates (100 mg protein in 50 mL lysis buffer) using a colorimetric assay according to the manufacturer's instructions. In brief, 5 × 10^5 MOLT-4 cells were incubated with 5 mg/mL of methanol extract for 0e180 min at 37°C. Cell lysates were then prepared, according to
the manufacturer's instructions, and the protein concentration wasmeasured, spectrophotometrically. Lysates were supplemented with 50 mL reaction buffer (10 mM DTT) and DEVD-para- nitroanilide (pNA, 4 mM, 5 mL; caspase-3 substrate) or LEHD- pNA (4 mM, 5 mL; caspase-9 substrate) or IETD-pNA (4 mM,
5 mL; caspase-8 substrate) and incubated at 37°C for 0e3 h. The
activity of each caspase was evaluated by quantitatively measuring the levels of the resulting chromophore pNA at 405 nm every 30 min for 3 h. For validation of the mushroom extract-induced activation of each caspase, MOLT-4 cells were further coincubated with methanol extract and a pancaspase inhibitor, Z-VAD-FMK, for 24 h, and cell viability was measured by the MTT assay.[18,21]

2.13. Immunoblotting

Cell lysates were prepared from MOLT-4 cells treated with 5 mg/ mL of mushroom extract for 12 and 24 h using the RIPA buffer method.[4] For immunoblotting, 20e35 mg proteins were resolved on 8%e15% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Sigma Aldrich, Burlington, MA). Mem- branes were blocked with 5% BSA in 1 TBST and probed with respective primary monoclonal antibodies, followed by incubation with the corresponding secondary antibodies. Finally, the blots were visualized using the ECL method on the Chemidoc MP system (Bio-Rad, Hercules, CA).

2.14. Data analysis

All experiments were performed independently in

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duplicates or triplicates. All numerical data are expressed as mean (standard deviation). One-way ANOVA followed by Dunnett's multiple com- parison test or Turkey's test (wherever applicable) was used to compare differences among the experimental groups. In a specific case, the Student's *t*-test was also performed. Statistical significance was based on the following *P* value thresholds. All analyses were performed using GraphPad Prism 7.00 (GraphPad Software, San Diego, CA).

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3. Results

3.1. Differential antiproliferative activity induced by ethyl acetate and methanol extract of 5 different mushrooms against a panel of cancer cell lines

We aimed to study the antiproliferative efficacy of methanol and ethyl acetate extracts of 5 different mushrooms against 6 cancer cell lines and normal cells by performing MTT assays. We cultured the 6 cancer cell lines, that is, NALM6, Reh, MOLT-4, A549, Hep G2, and MCF-7, one normal cell line BEAS-2B, and PBMCs isolated from a healthy donor with different concentra- tions (0, 10, 25, 50, and 100 mg/mL) of mushroom extracts for 24 h. The results showed that the majority of extracts exhibited a dif- ferential pattern of dose-dependent inhibition of cancer cell pro- liferation (Fig. 1A). All tested extracts exhibited minimal cytotoxicity against normal cells, that is, PBMCs, and BEAS-2B cells. The antiproliferative efficacies of 2 representative mush- room extracts, in terms of "half-maximal inhibitory concentration" (IC_{50}), are presented in Fig. 1B and C. We categorized the resulting IC_{50} values as promisingly good (IC_{50} less than 25 mg/mL), moderately good (IC_{50} between 25 mg/mL and 250 mg/mL), or nonconsiderable (IC_{50} more than 250 mg/mL). The Selectivity Index (SI), which implies the effectivity and safeness of any drug, was calculated by dividing the "half-maximal inhibitory cytotoxic concentration" (CC_{50}) value of this drug in normal cells with the IC_{50} value of this drug in each cancer cell line. The respective SI values are presented in Supplemental Tables 1 and 2.

Among all the 5 tested ethyl acetate mushroom extracts (Fig. 1B), *A. hygrometricus* showed the highest effectivity against the MOLT-4 cell line with an IC_{50} of 16.10 (1.22) mg/mL. The ethyl acetate extract of *A. hygrometricus* also exhibited a high selectivity index against the MOLT-4 cell line, in contrast to other cell lines, with SI values of 43.63 (1.30) against BEAS-2B, and 34.52 (1.77) against PBMCs. The ethyl acetate extracts of all 5 mushrooms were more effective against leukemic than other cancerous cell lines. The same trend was observed in the methanol extracts (Fig. 1C). The meth- anol extract of *A. hygrometricus* was the most effective against the MOLT-4 cell line with an IC_{50} value of 7.25 (0.69) mg/mL, exhibiting SI values of 64.20 (0.59) and 127.67 (1.57) against BEAS-2B and PBMCs, respectively.

Both ethyl acetate and methanol extracts of *A. hygrometricus* produced promising to moderately good efficacy against all cancerous cell lines, except for the A549 cell line, which appeared to be the most resistant when treated with ethyl acetate extracts. Compared with other mushrooms, both extracts of *Lentinus* sp. showed the least efficacy against all cell lines. The methanol ex- tracts of *Phallus* sp. and *Tricholoma* sp. were the most effective

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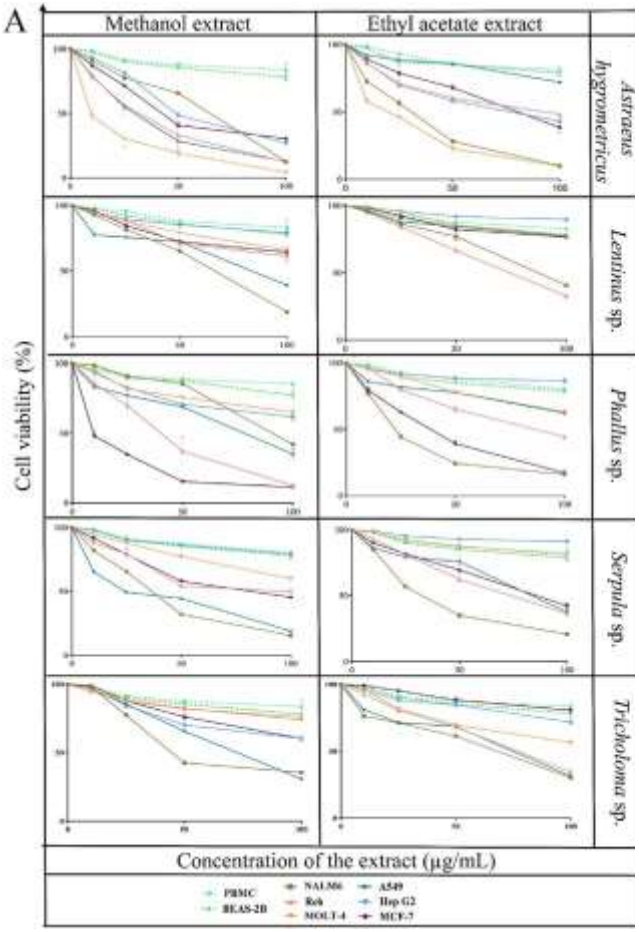

Fig. 1. *In vitro* antiproliferative efficacy of both ethyl acetate and methanol extracts of 5 mushrooms were measured by MTT assay in the presence of an increasing concentration of the extract (0e100 mg/mL) in various cell lines, including NALM6, Reh, MOLT-4, A549, Hep G2, and MCF-7; one normal cell line BEAS-2B and PBMC isolated from a healthy donor (A); the IC_{50} value of different cancer cell lines, treated with increasing ethyl acetate extract of 5 different mushrooms (B); the IC_{50} value of different cancer cell lines treated with increasing methanol extract of five different mushrooms (C).
Note: PBMC: peripheral blood mononuclear cell.

against the breast cancer cell line MCF-7 with IC_{50} values less than
10 mg/mL. Methanol extract of all the 5 mushrooms produced moderately good efficacy against Reh and NALM6 cell line. Both extracts of all 5 mushrooms showed mediocre efficacy against the NALM6 cell line. We concluded that the methanol extract of
A. hygrometricus was the most effective and safe anticancer extract,and MOLT-4 was the most sensitive cell line; hence, we performed further experiments on MOLT-4 cells using the methanol extract of
A. hygrometricus.

3.2. Mushroom extract induced nuclear condensation on MOLT-4 cells in a concentration-dependent manner

Following DAPI staining, MOLT-4 cells treated with an increasing concentration of the methanol extract (0e25 mg/mL) for 24 hexhibited characteristic features of increased apoptosis in a concentration-dependent manner (Fig. 2AeC) with respect to control. Treated cells displayed bright, fragmented, condensed, and unorganized nuclei, whereas the nuclei of control cells appeared dimly blue and organized (Fig. 2A and B). The apoptotic effect of the extract was initiated at the concentration of 5 mg/mL, resulting in 26.33% morphologically altered MOLT-4 cells and increased concentration-dependently. The percentage of apoptotic MOLT-
4 cells at the highest treatment concentration reached 74.66% (Fig. 2C).

3.3. Methanol extract interfered with cell cycle progression bytargeting cells at sub-G0/G1 phase

To verify the effect of the methanol extract of *A. hygrometricus* on the cell cycle distribution profile, we treated both MOLT-4 cells and PBMCs with increasing concentrations of the extract (0e25 mg/ mL) for 24 h, and acquired the cell cycle histogram by flow cytometry. The results showed that the methanol extract specif- ically targeted cells residing at the sub-G0/G1 phase, whereas no significant effect was noticed in normal cells (Fig. 3AeC). A signif- icant decrease occurred in the numbers of the cell population at the G0/G1 phase in cells treated with 5 mg/mL extract, which further increased analogously in a concentration-dependent manner (Fig. 3A). There was a drastic shift in the number of cells from the G0/G1 phase to the sub-G0/G1 zone, which indicated that the extract specifically targeted the G0/G1 population, as evidenced bycell cycle analysis. The sharp shift observed in the number of cells in the sub-G0/G1 phase indicated nuclear fragmentation resulting from apoptosis-like cell death. This finding suggested that these wild edible mushrooms may promote apoptosis-like cell death, specifically in MOLT-4 cells but not in normal cells. Overall, our findings suggested that the methanol extract of *A. hygrometricus* demonstrated antileukemic activity against MOLT-4 cells by tar- geting the sub-G0/G1 population.

3.4. Methanol extract of A. hygrometricus augmented robust apoptosis, specifically in MOLT-4 cells, had negligible impact onnormal cells

Using annexin V/PI dual staining, we further corroborated the *A. hygrometricus*-induced apoptosis in cancer cells. We treated both MOLT-4 cells and PBMCs with or without increasing concentrations of the methanol extract (0e25 mg/mL) for 24 h, and subjected them to flow cytometry after staining with annexin V/PI. Our analysis revealed a dose-dependent increase in the number of apoptotic MOLT-4 cells; whereas, the methanol extract exhibited minimal activity against PBMCs isolated from healthy donors (Fig. 3D and E).At

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24 h after treatment, the percentage of apoptotic cells was increased from 52.22% at the lowest treatment concentration of 5 mg/mL to 94.93% at the highest treatment concentration of 25 mg/ mL. The apoptotic-related IC_{50} value of the *A. hygrometricus* extractwas 6.91 (0.91) mg/mL, which was very close to the IC_{50} concen- tration obtained by the MTT assay. At the same range of treatment concentrations, PBMCs showed very modest apoptosis. With these results, we inferred that the methanol extract of *A. hygrometricus* specifically induced robust apoptosis in MOLT-4 cells but not in normal PBMCs.

3.5. Methanol extract of A. hygrometricus induced the production ofhigh levels of ROS in leukemic cells

ROS may trigger the intrinsic apoptotic cascade via interactions with proteins of the mitochondrial permeability transition complex. Therefore, we aimed to test the involvement of intracellular ROS in the *A. hygrometricus* methanol extract- induced apoptosis in MOLT-4 cells. We treated MOLT-4 cells with increasing concentrations (0e50 mg/mL) of methanol extract for 6 h, followed by labeling with CM-H_2DCFDA (5 mM) and measurement of the number of DCF^b cells by flow cytom-
etry. The methanol extract triggered the generation of a high

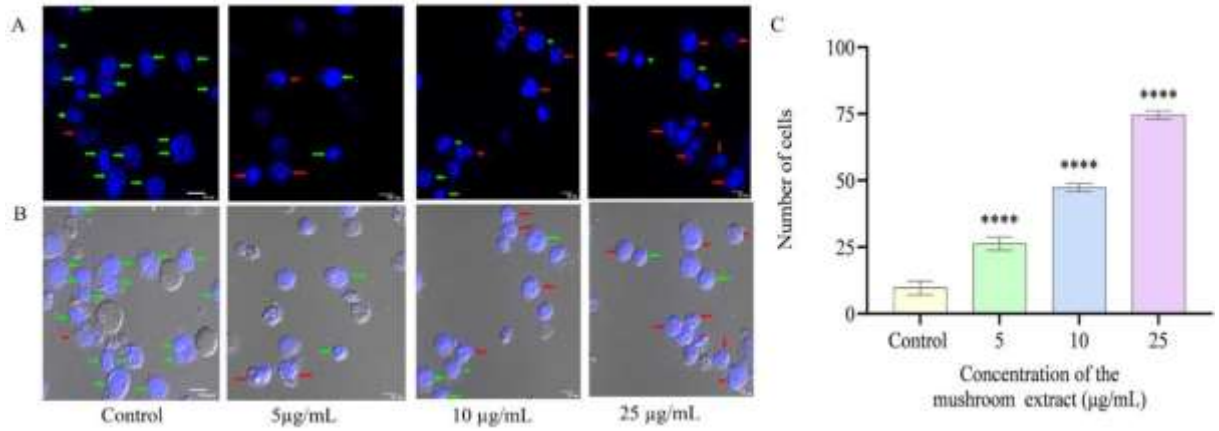

Fig. 2. Impact of methanol extract of *A. hygrometricus* on cellular and nuclear morphology in MOLT-4 cells: dose-dependent change in the condensed nuclear structure of MOLT- 4 cells treated with 5,10, and 25 mg/mL extract for 24 h and studied by DAPI (A); changes in the cellular shape and membrane blebbing were clearly observed in MOLT-4 cells in adose-dependent manner (B); the bar graph indicates a concentration-dependent increase in mushroom-induced altered morphology containing MOLT-4 cells (C). Notes: Data are expressed as mean (standard deviation). Magnification: 400 × 1. Scale bar ¼ 10 mm. ****$P < .0001$ *vs.* control group.

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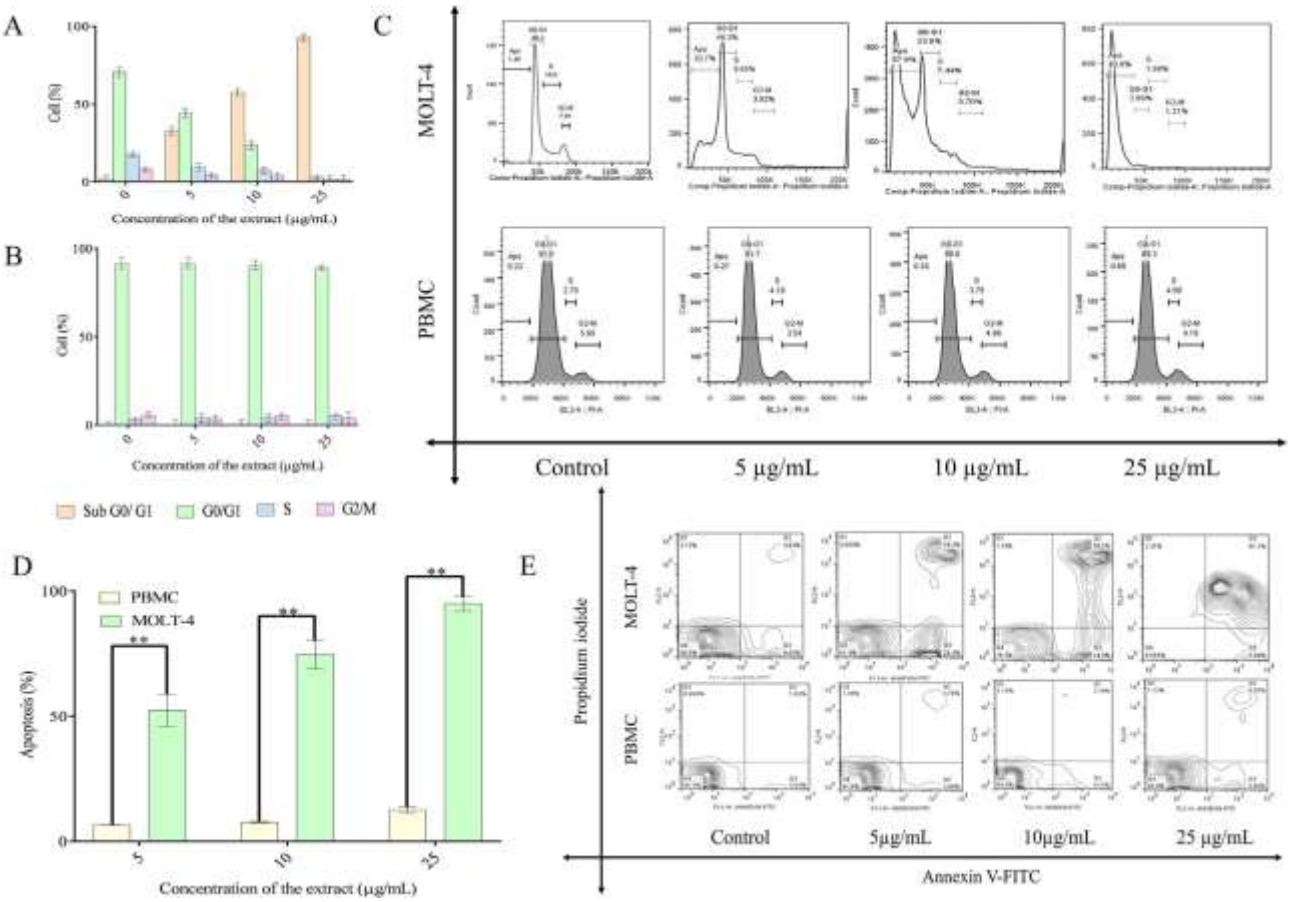

Fig. 3. MOLT-4 and PBMC, isolated from healthy donor cells, were treated with an increasing concentration of mushroom extract (0, 5, 10, and 25 mg/mL) for 24 h: The bar graph represent a dose-dependent augmentation of the cells at the G0/G1 phase in MOLT-4 cells (A); in contrast, no noticeable change was observed in PBMC (B); posttreatment, cells were harvested and fixed with ethanol and stained with PI. the representive histogram showing the distribution profile of the cell cycle analyzed by flow cytometry (C); A concentration-dependent increase in the annexin V-FITC/PI-positive population of MOLT-4 cells was observed in response to increasing concentration of methanol extract treated for 24 h, whereas a very light effect was observed in PBMC cells (D); The contour plot depicts that with increasing concentration of the extract, the percentage of apoptosis significantly increased in MOLT-4 cells in contrast to PBMC (E).
Notes: PBMC: peripheral blood mononuclear cell. *t*-test was performed to compare multiple groups means (MOLT-4) *vs.* PBMC control. $^{**}P < .01$ *vs.* control group.

yield of ROS in a concentration-dependent manner as the fluorescence intensity of DCF was increased with the increase in the concentration of the extract (Fig. 4A and B). To confirm that the induction of apoptosis was a downstream event of the increased production of intracellular ROS, we preincubated a set of MOLT-
4 cells with the antioxidant, L-acetyl cysteine (L-NAC). The percentage of apoptotic cells decreased from 90.56% to 22.83% after L-NAC treatment, indicating that L-NAC rescued cells from apoptosis by scavenging ROS. In another set of MOLT-4 cells, media was supplemented with GSH, hence inhibiting the depletion of intracellular GSH. We found that the percentage of apoptotic cells decreased from 90.56% to 28.56% following GSH supplementation in the medium (Fig. 4C). This further confirmed the involvement of intracellular ROS in inducing apoptosis following treatment of MOLT-4 cells with the methanol extract*of A. hygrometricus*.

3.6. Methanol extract of A. hygrometricus induced the production ofhigh levels of RNS in leukemic cells

As reactive nitrogen species (RNS) are essential signaling and effector molecules that play a role in apoptosis along with ROS, we examined the effect of the methanol extract of *A. hygrometricus* on the production of RNS using DAF-2DA. We observed that MOLT-4 cells treated with an IC_{50} concentration, that is 7.25 (0.69) mg/mL of methanol extract of *A. hygrometricus* showed a time-dependent increase in the generation of RNS from the basal level, which peaked at 180 min, and subsequently plateaued (Fig. 5C). The production of RNS reached a maximum after treatment with 25 mg/ mL methanol extract, and gradually decreased with increasing concentrations

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(Fig. 5A and B). There was a gradual increase in the fluorescence of DAF^{b} cells along with concentration gradient treatment, suggesting that the methanol extract of *A. hygrometricus* also induced the generation of RNS along with ROS in MOLT4 cells.

3.7. Depolarization of mitochondrial membrane potentials in MOLT-4 cells treated with methanol extract of A. hygrometricus

Loss of mitochondrial membrane potential (DJm) is one of the significant consequences of ROS-induced apoptosis. The mitochondrial disruption includes an imbalance in the mitochondrial membrane potential and associated changes in the oxidation- reduction state inside the mitochondria. Depolarization of the mitochondrial membrane potential prevents the accumulation of the JC-1 dye in the mitochondria and results in the dispersion ofthe dye throughout the cell leading to a shift from red (J-

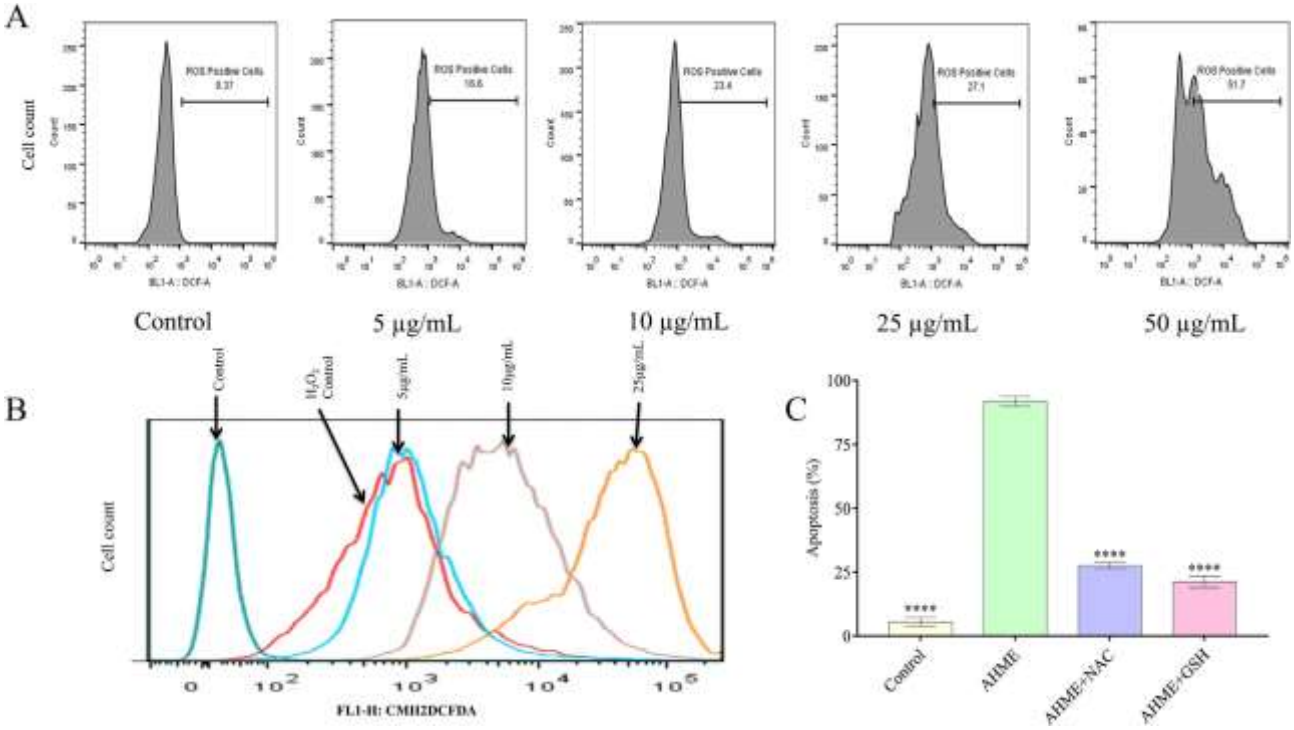

Fig. 4. Flow cytometric data indicated a concentration-dependent increase in intracellular ROS in MOLT-4 cells in response to the increasing concentration of the mushroom extract: the fluorescent signals from the cells were acquired by flow cytometer (A, B); The bar graph depicts a significant decrease in apoptosis, after treating with ROS scavenger NAC andGSH (C).

Notes: Posttreatment, DCFDA (5 mM final) in serum-free media was added and incubated for 30 min; cells were washed in 1 × PBS. Finally, the fluorescent signals from the cells
were acquired by flow cytometer. One-way ANOVA, followed by Dunnett's multiple comparisons tests, were performed to compare multiple groups means (treated sets) *vs.* control.
****$P < .0001$, *vs.* control group.

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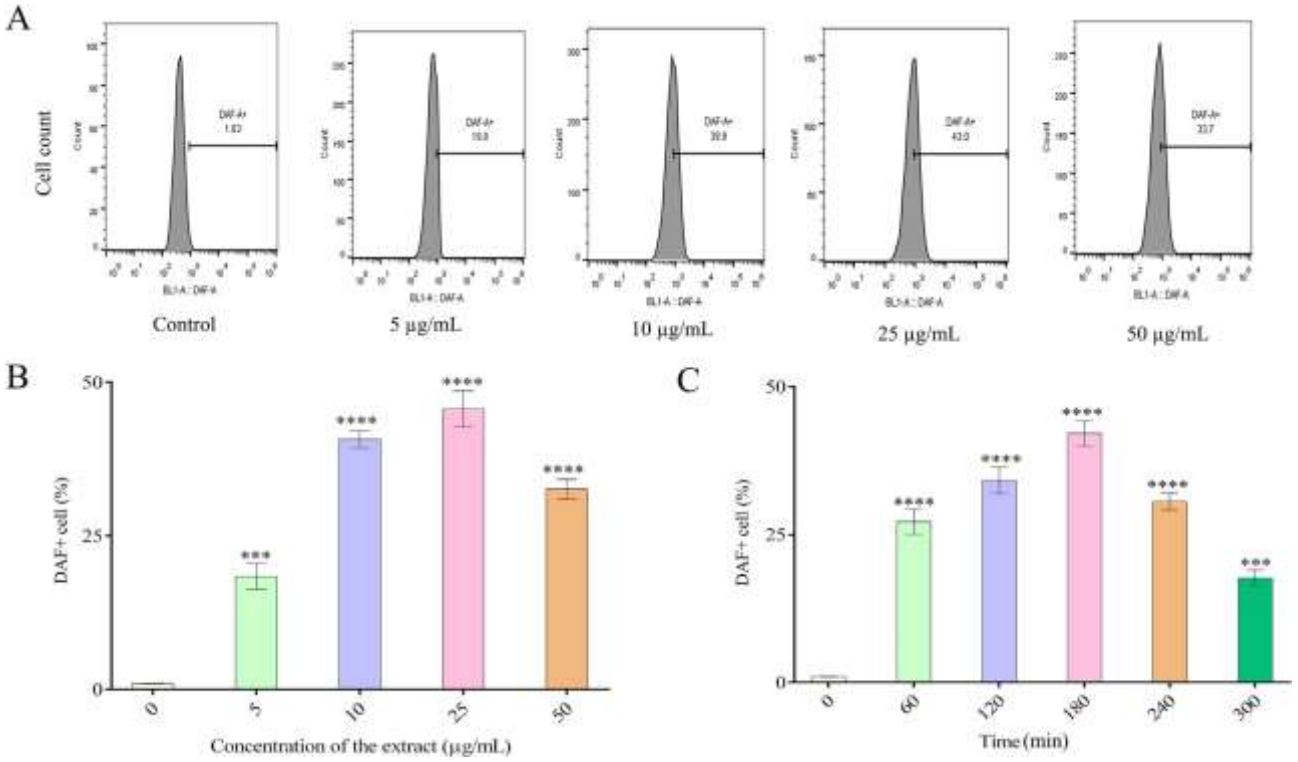

Fig. 5. Flow cytometric data indicated both concentration-dependent (A, B) and time-dependent (C) increases in intracellular RNS production in MOLT-4 cells.
Notes: One-way ANOVA, followed by Dunnett's multiple comparisons tests, was performed to compare multiple groups' means (treated sets) *vs.* control. ****$P <$ .0001, *vs.* control group.

A. hygrometricus methanol extract-treated cells. The results revealed a shift in the fluorescence emission from red to green, indicating a massive loss of DJm in *A. hygrometricus* methanol extract-treated MOLT-4 cells (Fig. 6A and B). Consequently, this decrease in the red/green fluorescence intensity ratio indicated mitochondrial depolarization (Fig. 6C). We detected that the per- centage of green monomers increased from 18.7% to 89.0% in a concentration-dependent manner. We thus concluded that the methanol extract induced the depolarization of mitochondrial membranes, leading to apoptosis.

3.8. Methanol extract of A. hygrometricus induced cleavage of PARP

The DNA repair enzyme, poly (ADP) ribose polymerase (PARP) plays a vital role in DNA repair. However, when cells receive an apoptotic stimulus and activate their caspase cascades, PARP servesas a substrate for active caspase-3, thus preventing the DNA repairprocess. Hence, the cleavage of PARP is considered a prerequisite for apoptosis.[17] Therefore, we evaluated the effect of the methanolextract on PARP cleavage. The amount of cleaved-PARP was significantly increased at 12 and 24 h in MOLT-4 cells treated with 5 mg/mL of methanol extract compared with that in the untreated control MOLT-4 cells (Fig. 7B).

3.9. Methanol extract of A. hygrometricus induced caspase-dependent apoptosis

Caspases, a group of proteases, are effector molecules of the apoptotic pathway and play a significant role in programmed cell death. Therefore, we measured the activities of caspase-3, -8, and
-9 after treatment with the methanol extract of *A. hygrometricus* (5 mg/mL) every 30 min for 3 h. There was an exponential increase in the activities of two caspases (caspase-3 and -9) up to 90 min of treatment. In contrast, there was no significant change in the level of activity of caspase-8 (Fig. 7A). To validate the methanol extract- induced activation of caspases, we coincubated MOLT-4 cells with a nontoxic concentration of Z-VAD-FMK (20 mM), a pancaspase in- hibitor, and measured cell viability using the MTT assay. We found that Z-VAD-FMK attenuated the extract-induced cytotoxicity in MOLT-4 cells by increasing the IC_{50} of the methanol extract *of A. hygrometricus*. Hence, we concluded that the methanol extract of
A. hygrometricus induced apoptosis in a caspase-dependent manner.

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3.10. Methanol extract of A. hygrometricus triggered mitochondria-dependent apoptosis in MOLT-4 cells

The increased production of intracellular reactive oxygen and nitrogen species (RONS), followed by the disruption of mitochon- drial membrane potentiality and elevated levels of caspase-3 and
-9, is correlated with the induction of the mitochondria-involved intrinsic apoptotic pathway. To confirm our hypothesis, we checked 3 other proteins related to mitochondria-dependent apoptosis. One of the related consequences of intrinsic apoptosisis the downregulation of the antiapoptotic proteins and upregula- tion of proapoptotic proteins.[17,22] We found that the levels of the anti-apoptotic Bcl-2 protein were downregulated in a time- dependent manner in methanol extract-treated MOLT-4 cells; in contrast, the levels of the proapoptotic protein (Bax) were upre-gulated (Fig. 7B). Cytochrome c is a significant mediator molecule of

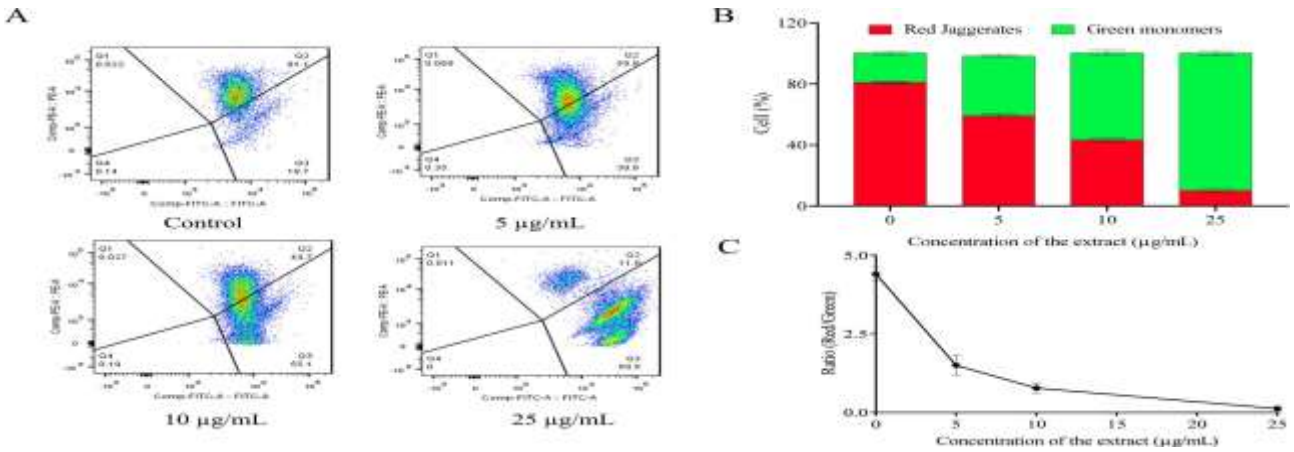

Fig. 6. Flow cytometric data indicated a concentration-dependent increase in green monomer and a decrease in Red J-aggerates in MOLT-4 cells (A, B), a sharp decrease in Red togreen ratio was observed in a concentration-dependent manner (C).

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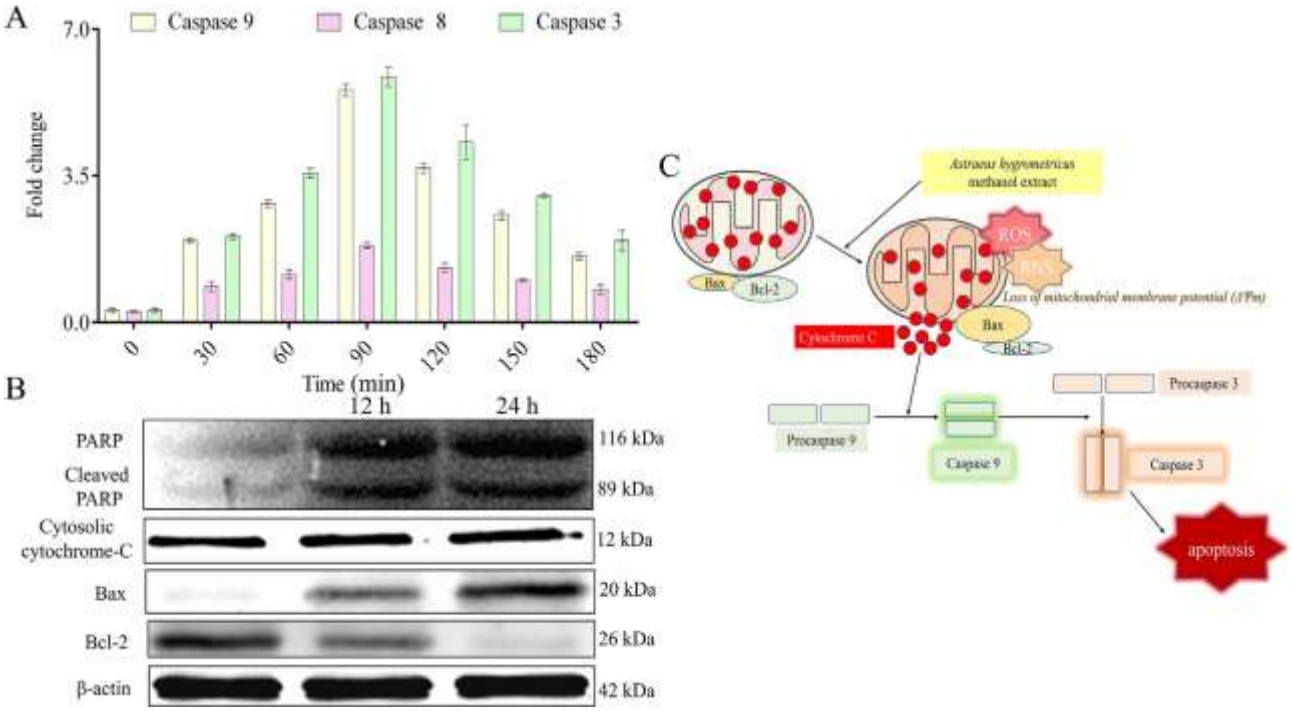

Fig. 7. Lysates of MOLT-4 treated with mushroom extract in a time-dependent manner were subjected to study the caspase profile (caspase 8, caspase 9, and caspase 3): both Caspase 9 and 3 upregulated in a time dependant manner (A); western blot images showing the expression profile of pro-apoptotic (Bax), and anti-apoptotic Bcl-2 protein involved in time-dependent extract induced apoptosis in MOLT-4 cells, the expression profile of cytochrome-c and PARP were also measured (B); the mushroom extract selectively induced the RONS in MOLT-4 cells and positively induced pro-apoptotic Bax protein, followed by the release of cytochrome-c, which ultimately exhibited cellular apoptosis through the intrinsic pathway (C).
Note: Data are expressed as mean (standard deviation).

the intrinsic apoptotic pathway. In mammalian cells, various apoptotic stimuli cause the release of cytochrome c from mitochondria, inducing a series of biochemical reactions, and finally cellular death through the induced activation of caspases.[23] Hence, an increased amount of cytosolic cytochrome c confirms the occurrence of intrinsic apoptosis. The extract-treated MOLT-4 cells showed an elevated amount of cytosolic cytochrome c in a time- dependent manner (Fig. 7B).

4. Discussion

Fatalities caused by cancer, particularly during the recovery phase, are mostly attributable to the ineffectiveness and adverse effects of currently available cancer treatments.24, 25 Poor individuals, particularly in less developed nations, may be unable to afford cancer treatments due to their high cost.26 Consequently, there is a need for new anticancer medications that are more selective for cancer cells and have fewer adverse effects.

The anticancer properties of many biomolecules originating from mushrooms have been shown. These include b-glucans, b-proteoglycans, ergosterol, lectins, triterpenes, ergothioneine, and selenium.27 Few studies have examined mushrooms' medicinal potential, especially in the context of drug development. We set out to assess the anticancer potential of mushrooms—an upcoming arsenal for possible anticancer medications—in order to circumvent one of the major roadblocks to cancer chemoprevention: the unwanted toxicity of pharmaceuticals to normal cells, which causes unpleasant side effects.

We screened ethyl acetate and methanol extracts of five wild mushrooms for antiproliferative potential: A. hygrometricus, Phallus sp., Lentinus sp., Tricho-loma sp., and Serpula sp. This was motivated by the extensive ethnobotanical history

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of mushroom usage as alternative medicine. Out of the five species we examined, our research found that the new edible fungus A. hygrometricus had the least amount of carcinogenic chemicals. No studies have shown the impact of astrakurkurone and astrakurkurol on leukemic cells, while prior research has investigated their effects on hepatocarcinoma (Hep G2, Thle 2, and Hep 3B) and renal cell carcinoma (RCC) cell lines.28e30 Prior to this, we documented that an A. hygrometricus methanol extract was effective in killing a moderately leukemic cell line.

In 2022, leukaemia accounted for 7% of all cancer cases and 7% of all cancer deaths worldwide, according to cancer statistics.3 The incidence of acute lymphoblastic leukaemia (ALL) is 1.6 per 100,000 in the US, making it a very common malignancy. There is an estimated 80% incidence rate of ALL, making it the most prevalent childhood cancer.3,31 The antileukemic effects of wild edible mushrooms have been the subject of few investigations so far. The antileukemic potential of A. hygrometricus, a wild edible fungus, was investigated in the current research. More specifically, we detail the molecular mechanism by which A. hygrometricus induces cell death in drug-resistant robust T-cell acute lymphoblastic leukaemia cells (MOLT-4). Our research showed that A. hygrometricus methanol extract had strong apoptogenic potential, and that after 24 hours of treatment with different quantities of mushroom extract, MOLT-4 cells showed a dramatic increase in cell population in the sub G0-G1 stage. Nonetheless, normal cells did not show any discernible alterations. Nandi et al. found that the A. hygrometricus compound astrakurkurol showed the same

Halting in the G0/G1 phase in Hep G2 cells.29 This discovery provides further evidence that A. hygrometricus may interrupt the cell cycle and cause cells to die in cancer cells, but it has no effect on normal cells.

There are two well-established routes for signalling cell death: intrinsic and extrinsic.32 An important component of the extrinsic apoptotic pathway is the recognition and propagation of extracellular stimuli by specific membrane receptors. On the other hand, RONS successfully triggers mitochondria-centered apoptosis via the intrinsic route, which is primarily a caspase-dependent process involving a regulatory system centred on mitochondria. After caspase-8 mediates the extrinsic route and caspase-9 initiates the intrinsic pathway, caspase-3 cleaves the downstream executor protein to activate apoptosis.33 Mushroom extract increased caspase-9 and -3 levels but had no discernible effect on caspase-8 levels. Based on our findings, it seems that the intrinsic apoptotic pathway may have been responsible for the strong apoptosis in MOLT-4 cells that was triggered by mushrooms. An integral part of the intrinsic apoptotic route includes the release of cytochrome c, collapse of mitochondrial membrane polarity, and mitochondrial malfunction mediated by reactive oxygen species (ROS).34 Nevertheless, proteins belonging to the Bcl-2 family regulate the last effector molecules of apoptosis, caspase-3 and caspase-9. Notably, the ratio of proapoptotic (Bax) to antiapoptotic (Bcl-2) proteins determines the extent of apoptosis.16 Our results show that the mushroom extract confirmed a time-dependent cleavage of the apoptosis controller Bcl-2 proteins, leading to cell death. The Bcl-2 protein and caspase-9, the last effectors of apoptosis, are connected via cytochrome c. Inferring from the increased ROS production by the A. hygrometricus methanol extract, we found that it exerted its antileukemic effects against MOLT-4 cells via the mitochondria-dependent apoptotic pathway (Fig. 7C). Additionally, it was shown

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that the levels of RNS were elevated after extract administration.

5. Conclusion

A. hygrometricus methanol extract significantly inhibited the proliferation of MOLT-4 cells relative to BEAS-2B and PBMCs derived from a healthy donor, out of five wild mushrooms evaluated against six human cancer cell lines. The aggressive, resilient, and resistant T-cell acute lymphoblastic leukaemia known as MOLT4 is characterised by PI3K-AKT deregulation. By controlling Bcl-2 and Bax, our results revealed that the intrinsic apoptotic route, which is mediated by cytochrome c and caspases, is the principal mechanism by which A. hygrometricus induces apoptotic-like cell death in MOLT-4 cells. In MOLT-4 cells, the mushroom extract increased ROS and RNS production and produced strong apoptosis via the mitochondria-dependent route. Since A. hygrometricus is a native mushroom, we infer that it contains active myco-compounds that may be useful in the fight against leukaemia.

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