CHROMATOGRAPHIC ANALYSIS AND CYTOTOXIC EFFECT OF *ARGEMONE MEXICANA* LEAF METHANOL EXTRACT INCLUDING ITS ACUTE TOXICITY STUDY

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ABSTRACT

Argemone mexicana leaves (AML) contain a variety of bioactive components such as fatty acids, amino acids, phenolics and alkaloids, including Berberine, Isoquinoline, Scoulerine, Stylopine, Thalifone, Benzylisoquinolines, Protopine, and Tetrahydroberberine. With the objective to identify an additional source for anticancer treatment, this study investigated the Gas Chromatography and Mass Spectrometry (GC-MS) of AML extract, its cytotoxic effect against MCF-7 cell lines, and acute toxicity study on experimental rats. 9,12,15-Octadecatrienoic acid, Phytol, 1-methyl piperidine, n-Hexadecanoic acid (palmitic acid), and 9,12-Octadecadienoic acid were among the primary substances identified based on the peak area of GCMS. The percentage of cell viability(MTT) after AML extract treatment ranged from 28.50% to 64.28% for concentrations of 1000 to 125 µg/ml (IC50 = 258.87 µg/ml). Statistical analysis revealed a significant difference in cell viability ($p=0.05$). Cytoplasm condensation, nuclear fragmentation, membrane blebbing and cell shrinkage was observed in AO stain after exposure to AML extract. Acute toxicity study revealed that LD50 of AML extract is greater than 2000 mg/kg/b.w. Therefore, the present study offers a satisfactory preclinical proof of safety for AML extract, its cytotoxic effects appear to be caused by its bioactive components.

Keywords: AML extract, cytotoxicity, MTT assay, apoptotic assay, GC-MS analysis, Acute toxicity.

INTRODUCTION

Naturally occurring secondary metabolites of a plant, referred to as phytochemicals, and have the potential to inhibit diseases. Alkaloids, steroids, volatile oils, flavonoids, and other phenolic

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compounds are examples of phytochemicals [1]. Therefore, screening of active chemicals and assessing cytotoxic action from plants have helped identify and develop novel anticancer medications.Drugs made from herbal extracts are widely accessible, reasonably priced, safe, effective, and rarely cause side effects. GC/MS analysis is an advanced method which utilizes direct analysis to identify volatile essential oils, fatty acids, lipids, alkaloids, and nonpolar components present in medicinal plants and traditional remedies [2].

Figure 1: Argemone mexicana L

Argemone mexicana, commonly known as Mexican prickly poppy or yellow thistle, belongs to the Papaveraceae family. It was also reported to grow throughout the subtropical and tropical regions, in nearly every part of India. Lower leaves are alternate, sessile, auricled, and obovate in outline. The leaves are 5–22 cm long and 3–7 cm wide, with a wavy margin, sharp teeth, white variegation along the main veins, and scattered prickles along the margin and on the veins below [3]. In India, the yellow juice exuding from injured plants has been traditionally used as medicine for dropsy, jaundice, ophthalmia, scabies, and cutaneous affections. Moreover, the native population in India reportedly uses the root to treat inflammation and chronic skin diseases [4]. Leaves and seed juices are used to treat nonhealing wounds and skin diseases. They have also been reported to have applications in regulating blood circulation and cholesterol levels in the human body [5]. One of the most common cancers in women and one of the main causes of cancer-related fatalities globally is breast cancer. Cancer remains the world's most significant cause of mortality because it is a malfunction of the processes that control cell growth and division. Therefore, the present investigation aims to identify potential therapeutic compounds by performing GC-MS analysis of AML and studying its cytotoxic effects on the human breast cancer cell line (MCF-7) included acute toxicity study of AML extract.

MATERIALS AND METHODS

Collecting Plants: Authentication and Extraction Methods

Argemone mexicana leaves (AML) were collected from the Erode district, Tamil Nadu, between January2022 to June 2023. The procedure of plant authentication was carried out by the Botanical Survey of India, Coimbatore. The survey number was Tech/260/BSI/SRC/5/23/2021. After collecting fresh AML leaves and washing them, on paper towels, they were left to dry at room temperature for a week. During this period, the leaves became pliable, and the spines on the underside of the leaves softened. After drying, plant leaves were crushed into a powder using a pestle and mortar. The Soxhlet apparatus was used to extract 200 mg of dried leaves with 1200 ml of methanol. The contents were distilled using a boiling water bath. After being collected, the crude extract was used for further analyses and refrigerated at -4°C.

GC-MS Analysis

The methanolic extract of AML was subjected to GC-MS analysis using the protocol outlined by Hema et al. (2011) [6]. GC-MS, PerkinElmer mass spectrometer interfaced with a chromatograph and coupled with an Elite-1 fused silica capillary column (30 m, 0.25 mm, 1 µm df, mainly comprised of dimethyl polysiloxane). Helium gas (99.999%) was used as the carrier gas at a steady flow rate of 1 mL/min, and a split ratio of 10:1 was applied with an injection volume of 1.5 mL/min. The temperature of the ion source was 280°C, and the injector was 250°C. Starting at 110°C (isothermal for two minutes), the oven temperature was scheduled to increase by 10°C/min to 2000°C, then by 5°C/min to 280°C, culminating in a 9°C isothermal at 280°C. The detected compounds ranged from 50 to 550 amu, with a total GC running time of 36 minutes. By comparing the average peak area of each ingredient to the overall area, the relative amount of each ingredient was determined. Mass spectra from the Wiley-8 library and the NIST-0.8 L database, as well as chromatograms, were handled by Turbo Mass.

***IN-VITRO* CYTOTOXIC STUDIES**

Cell lines:

MCF-7 cell lines were purchased from National Centre for Cell Sciences, Pune, India. Cells was cultured in Dulbecco's Modified Eagle's Minimum (DMEM). Media supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 1 % antibiotic–antimycotic solution in a humidified incubator (5 % CO_2 in air at 37°C). Cells (2 x 10^5cells/ml) were cultured in T25 flasks and a stock cell suspension (1 x 10^5 cell/mL) was prepared. A 96-well flat bottom tissue culture plate was seeded with 50,000 cells in 0.1 mL of DMEM medium supplemented with 10 % FBS for 24 h in CO_2 incubator. Test compounds in 0.2 % DMSO were added to the cells and incubated for 24, 48 or 72 h. The study was performed in triplicate for each dose level. The cells in the control group received only the medium containing 0.2 %DMSO.

MTT Assay

For this examination, it was assumed that no reduction of tetrazolium occurs in dead cells or any of their byproducts. The assay was based on the highly successful principle of MTT being broken down by living cells into a blue formazan byproduct. The fundamental principle is that 3-(4,5-

dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is cleaved by the mitochondrial enzyme succinate dehydrogenase in a colorimetric test, yielding a blue-colored product (formazan). The amount of formazan was measured using a microplate reader, and the absorbance optical density (OD) was calculated at a wavelength of 540 nm. The results were presented as the average of three replicates, and the IC50 value was calculated [7]. Applying the following formula, the growth inhibition was calculated: Mean OD of Individual Test Group / Mean OD of Control Group x 100 = % of cell viability.

Acridine Orange (AO) Staining

Apoptosis analysis was conducted by following the procedure outlined by Mohan et al. (2016) [8]. 24 hours before to the start of the medication treatment, 50,000 cells were placed into each of the 24 well plates using culture media that included 10% fetal bovine serum. The cells were exposed to AML extract for 24 hours at doses of 250 µg/ml and 125 µg/ml. They were then incubated for the entire night at 37°C in a 5% CO2 atmosphere. After incubating for a full night, the cells were cleaned with PBS and the well media was disposed. One milliliter of 90% methanol was used to fix the cells for 20 minutes at -20°C. After the methanol was removed, it was air-dried. Ice-cold PBS was used to clean fixed cells two or three times. After that, the cells were maintained for 30 minutes at 37°C in phosphate-buffered saline (PBS) containing 1% BSA (bis(trimethylsilyl)acetamide) and 0.1% Triton X-100. 200 µl of acridine orange (0.01% in PBS, pH 7.4) was added to the plate and incubated for 20 minutes at 37°C after two or three PBS washes. After three PBS washes, the plate was examined under a fluorescence microscope to check for any nuclear alterations, and pictures were taken. Slides were observed within 30 minutes under a UV-fluorescence microscope before fluorescent color faded. The observations were as follows: (i) intact structures with green nuclei were observed in viable cells; (ii) condensation of chromatin in the nucleus was noted, exhibiting a bright green nucleus indicating early apoptotic cells; and (iii) dense orange areas were observed due to chromatin condensation, indicating late apoptosis [9].

Oral acute toxicity study:

Healthy female Sprague-Dawley (SD) rats (8-10 weeks old) weighing 120±5 g were used for this study. All the rats were maintained under the standard guidelines, in the JKKN Institutional Animal House, approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), Reg No. 887/PO/Re/S/2005/CPCSEA. The study was approved by the Institutional Animal Ethical Committee (IAEC) of JKK Nataraja Educational Institution, proposal number is JKKN/IAEC/Ph.D./04/2021. Rats were procured from Biogen Laboratory Animal Facility, Reg No. 971/PO/RcBt/S/2006/CPCSEA, Bangalore-562107.

Animals were divided into two groups (n = 3) and starved overnight. Group I (control) received only 2 ml of normal saline. Group II received *Argemone mexicana* crude methanol leaf extract administered orally (i.e., a single dose of 2000 mg/kg) on the first day of the experiment**.** Animals were observed for 14 days, and after being sacrificed, blood and tissue samples were collected for further analysis. Body weight was calculated for control and treated groups of animals on one day before the experiment (0 days), the 7th day, and the 14th day of the experiment. The

cage-side observation comprised behavior changes in animals, including fur, skin, gait, tremor, eye dullness, pupil dilatation, ptosis, and breathing difficulty. liver, kidney, and heart were removed from the control and experimental groups, weighed, and used for histological study. All the acute toxicity procedures and animal care were followed as per the guidelines of OECD-425. From the acute toxicity study, we can select the extract-safe doses of 1/10 and 1/5 for our experiment [10].

Statistical Analysis

The averages of the three analyses was used to express the data using one-way ANOVA by ***SPSS*** 28.0.1. followed by the test Turkey HSD for comparison between the groups. The cytotoxic activity (IC50) was determined by applying a linear or nonlinear regression equation to the percentage of cell growth and the extract's inhibitory action at different concentrations, with the F-statistic at $p \leq 0.05$. The results were expressed as mean ± SEM, and values with $P < 0.05$were considered statistically significant.

RESULTS

Gas Chromatography and Mass Spectrometry (GC-MS):

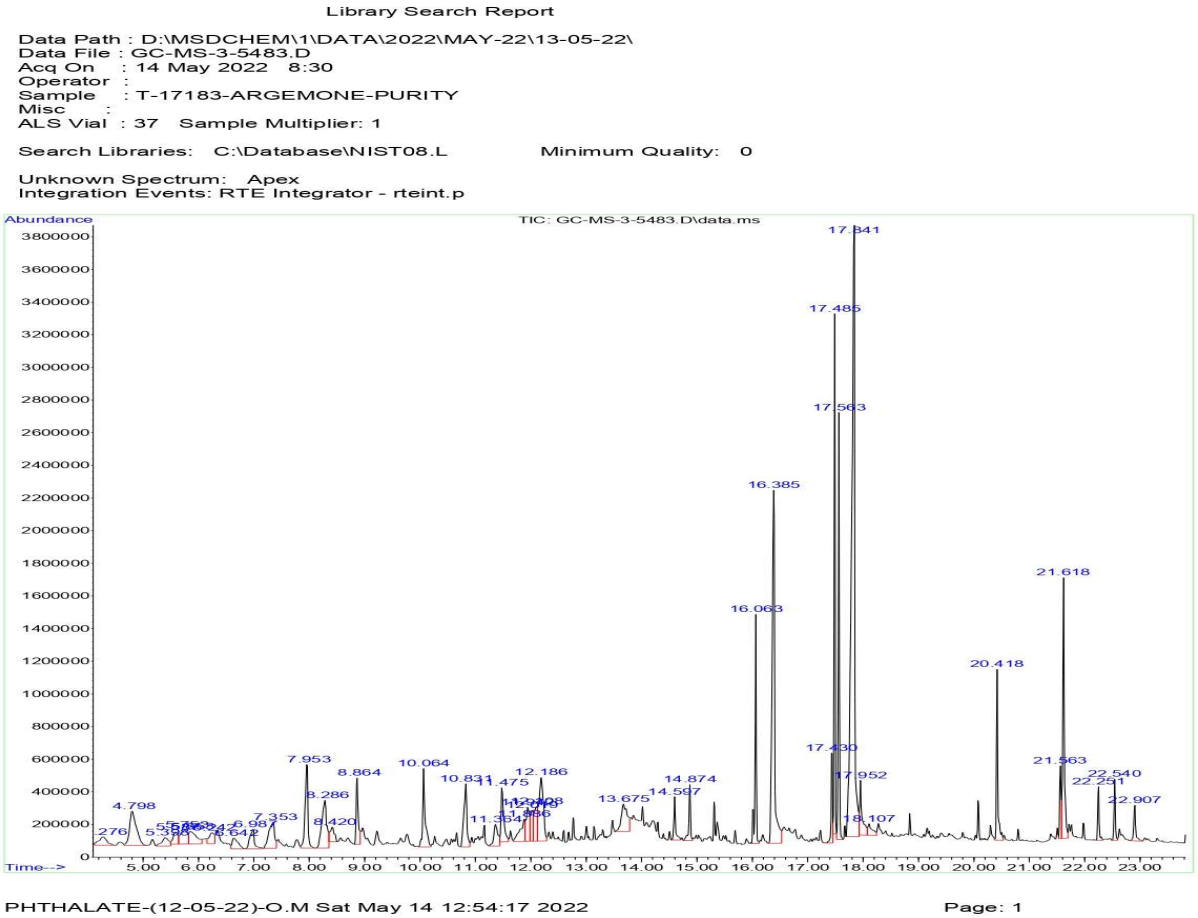

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Figure 2 shows GCMS chromatogram of AML extract (Chromopark Life Sciences).

Table 1: Vital Compounds Identified by GC-MS Analysis of AML Extract Along with Their Biological Activity

Name of the Compound (Name of Peaks)	Retention Time (Min)	Area of Peak (%)	Molecular Formula	Biological Activity
Benzoic acid	8.286	2.7	C_6H_5COOH	Antifungal activity [11].
2-Methoxy-4-vinylphenol	10.064	2.16	$C_9H_{10}O_2$	Inhibition of proliferative cell nuclear antigen [12].
N-Ethyl-2-carbome thoxyazetidine	10.831	2.2	$C_9H_{17}NO_2$	Potential antioxidant properties and cytotoxic activity have been reported [13].
Piperidine, 1-methyl-	12.186	3.19	$C_6H_{14}CIN$	Cytotoxic potential against tumor cells [14].
Hexadecanoic acid, methyl ester	16.063	3.33	$C_{18}H_{36}O_2$	Antioxidant, anti-inflammatory activity, and antimicrobial properties [15].
n-Hexadecanoic acid (palmitic acid)	16.385	10.55	$C_{16}H_{32}O_2$	Cytotoxic potential by inhibiting DNA topoisomerase-I [16]. Also found that the substance has antimicrobial, anticancer, and antioxidant properties [17].
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.430	1.43	$C_{19}H_{34}O_2$	Anti-inflammatory and anti-microbial activity [18].

Phytol	17.485	6.3	$C_{19}H_{32}O_2$	Cytotoxic activity against MCF-7 cell lines [19].
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	17.563	5.15	$C_{20}H_{40}O$	Anti-inflammatory and cancer preventive [20].
Octadecanoic acid (stearic acid)	17.841	22.28	$C_{18}H_{30}O_2$	Antitumor activity [21], antimicrobial activity, antioxidant [22].
9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	21.618	4.48	$C_{18}H_{32}O$	Anti-inflammatory and anxiolytic activity [23].

Bioactive compounds identified through GC-MS analysis

The GC–MS chromatogram (Figure 2) showed various peaks indicating the presence of bioactive compounds. Bioactive compounds were identified and characterized by comparing the mass spectra of fractions with the Wiley and NIST libraries. Total 41 compounds were detected in the crude extract of AML. Some of the major compounds identified include 9,12,15-Octadecatrienoic acid (Z,Z,Z) (22.28%); n-Hexadecanoic acid (palmitic acid, 10.55%); 9,12,15-Octadecatrienoic acid methyl ester (Z,Z,Z) (6.3%); Phytol (5.15%); 9,12,15-Octadecatrien-1-ol (Z,Z,Z) (4.48%); Piperidine, 1-methyl- (3.19%); Hexadecanoic acid methyl ester (3.33%); 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; and Benzoic acid (2.7%). Additionally, Hexadecanoic acid 2,3-dihydroxypropyl ester (2.68%); N-Ethyl-2-carbomethoxyazetidine (2.22%); 2-Methoxy-4-vinylpheno (2.16%); Isosorbide Dinitrate (1.94%); Butanedioic acid monomethyl ester (1.75%); 3H-Cyclopenta[c]pyridazin-3-one 2,5,6,7-tetrahydro (1.62%); Benzofuran 2,3-dihydro (1.64%); 9,12-Octadecadienoic acid (Z,Z)- methyl ester, 10,13-Octadecadienoic acid methyl ester (1.45%); Octadecanoic acid (stearic acid) (1.42%); and 9,12-Octadecadienoic acid (Z,Z)- 2-hydroxy-1-(hydroxymethyl)ethyl ester (1.34%) were presented with their retention time (RT) and peak area as a percentage. This analysis identified the active elements containing reported and non-reported phytochemical compounds. Furthermore, a few compounds were tabulated based on retention time and previously reported biological activities (see Table 1).

CYTOTOXIC ACTIVITY:

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Sample *Argemone mexicana* leaf extract for cell line study (Concentration used)	% Cell viability	IC50 value
1000 µg/ml	28.50	258.87 µg/mL
500 µg/ml	36.88	
200 µg/ml	48.89	
125 µg/ml	64.28	

Figure 3: Percentage of viable MCF-7 cells after treatment with AML extract.

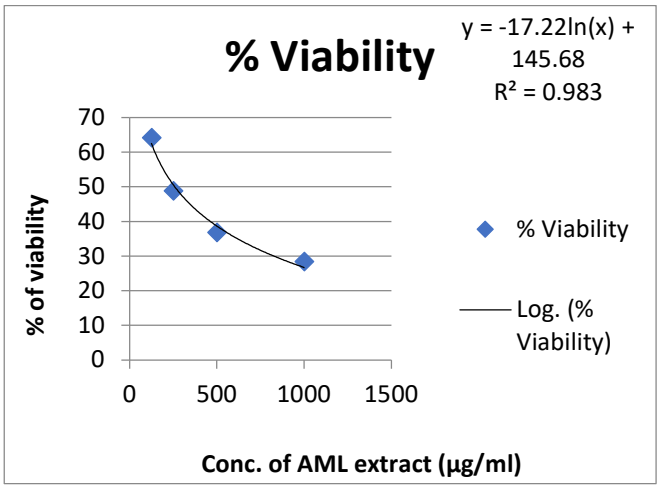

Figure 4 shows the effect of the extract after 48 hours (data not shown). However, after 72 hours, a dose-dependent antiproliferative activity was observed with a correlation coefficient of r2 = 0.983 for MCF-7. Statistical analysis indicated a significant difference in cell viability for the MCF-7 cell line between 1000 µg/mL and 125 µg/mL ($p < 0.05$).

Table 2: Concentration of extract and percentage of cell viability.

Table 2 shows that the percentage of cell viability after treatment with sample AML extract ranged from 28.50% to 64.28% for concentrations of 1000 to 125 µg/ml. The inhibitory concentration of 50% (IC50 value) for the extract was 258.87 µg/mL. Any concentration below the IC50 value can be used for further molecular-based studies.

Inhibition of growth and proliferation of MCF-7 cell lines by AML extract

Human breast cancer cells (MCF-7) were treated with various concentrations (ranging from 1000 to 125 µg/mL) of AML extract for different incubation periods. AML extract decreased cell growth and survival by inducing cell death. The effect of AML extract on MCF-7 cells depended on the dosage and duration of exposure. Cells treated with a higher concentration exhibited a significant reduction in the number of viable cells. After 72 hours of incubation, a maximal inhibition of cell growth was recorded at 1000 µg/mL, and the extract's IC50 value was 258.87 µg/mL. Figure 4

and Table 2 demonstrate that AML extract caused a statistically significant ($p < 0.05$) reduction in the cell viability of MCF-7 cells in a concentration-dependent manner. The sample of Argemone mexicana leaf extract has shown potential cytotoxic effects against human breast cancer cells.

Figure 4: Acridine Orange staining of MCF7 cells

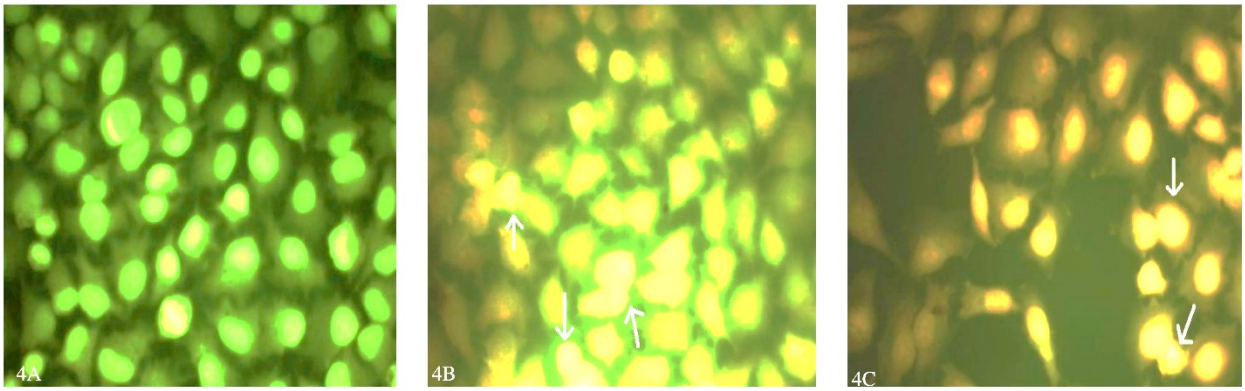

Figure 4 shows, 4A: Control MCF7 cells without drug treatment; 4B: MCF7 cells treated with AML extract at a concentration of 125 µg/ml for 48 hours; 4C: MCF7 cells treated with AML extract at a concentration of 250 µg/ml for 72 hours. Magnification: 40X; The arrow indicates cells exhibiting membrane blebbing, cytoplasmic condensation, and nuclear fragmentation.

Induction of apoptosis in MCF-7 cells by AML extract

Apoptosis occurred in MCF-7 cells after being treated with AML extract, as identified by the acridine orange (AO) staining method. All cells are susceptible to acridine orange (AO) staining, wherein the nucleus turns green, while untreated (control) cells remain round-shaped, brightly intact, green in color, and do not exhibit any signs of apoptosis (Figure 4A). Compared to the control group, the AML extract-treated group exhibited increased apoptosis, which was observed to rise with time and concentration of the extract. The round-shaped cells started to shrink, and apoptosis became visible at 48 hours (Figure 4B). Early apoptosis is characterized by a light green-orange nucleus with fragmented chromatin and condensed features. Apoptosis was evident following the 72-hour treatment, as characterized by a bright orange nucleus, membrane blebbing, and chromatin condensation (Figure 4C). The single cell was surrounded by blebs, which indicated the onset of apoptosis. Additionally, due to acridine orange's binding to the denatured DNA in the latter stages of apoptosis, alterations such as a reddish-orange hue were observed. In this study, it was found that the drug induces the self-destruction of cancer cells, which is considered an advantage of the AML extract's anticancer application (Figures 4A, 4B, 4C).

ACUTE TOXICITY STUDY:

Table 3: Cage side observation of control and treated groups:

Parameter	Cage side observation

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	Control	2000mg/kg	4000mg/kg
Mortality	Nil	Nil	Nil
Food intake	Normal	Normal	Normal
Water intake	Normal	Normal	Normal
Nasal bleeding	Nil	Nil	Nil
Hair loss	Nil	Nil	Nil
Behavior	Normal	Normal	Normal
Feces color	Normal	Normal	Normal
Diarrhea	Nil	Nil	Nil
Urination	Normal	Normal	Normal
Breathing difficulties	Nil	Nil	Nil
Locomotion	Normal	Normal	Normal
Eye dullness/ Ptosis	Nil	Nil	Nil

Table 4: Mean body weight of control and experimental groups (grams)

Group	0 day	7th day	14th day
Group I-Control	150.0 ± 1.15	160.16±2.74 [b]	162.3±1.60 [a]
Group II 2000 mg/kg	151.66 ± 2.02	157.0±3.60	163.0±2.10 [b]
Group III 4000 mg/kg	151.5 ±1.60	157.66±1.44[b]	168.33±1.62 [a]

Values are expressed as mean ± SEM (n=3)

[a]P<0.001; [b]P<0.05 Vs 0'day body weight of respective group. Data were analysed by One Way ANOVA followed by Dunnett's test post hoc analysis.

Table 5: Mean organ weights in control and experimental groups

Group	Liver (g)	Kidney(g)	Heart (g)
Group I-Control	3.80 ± 0.06	1.5 ± 0.08	2.19±0.04
Group II 2000 mg/kg	3.80 ± 0.13	1.55 ± 0.07	2.25 ±0.08
Group III 4000 mg/kg	4.08 ± 0.15	1.57 ±0.12	2.59 ±0.07

Values are expressed as mean ± SEM (n=3)

All the values are not significant when compared to control group. Data were analysed by One Way ANOVA followed by Dunnett's test analysis.

Table 6: Hematological parameters of control and experimental groups

Group	Hemoglobin (g/dl)	RBCs (10^6/mm^3)	WBCs (10^3/mm^3)	Platelets (10^6/mm^3)	Mean corpuscular volume-MCV (fL)	Mean corpuscular—(g/dl)??
Group I-Control	12.32 ± 0.42	6.80 ± 0.21	12.31 ± 0.23	735.90 ± 5.72	56.23± 1.14	33.40 ±0.26
Group II 2000 mg/kg	13.70 ± 0.95	6.96 ±0.18	13.70 ±0.13	729.56 ± 6.45	58.30 ± 1.26	34.49 ±0.32
Group III 4000 mg/kg	13.50 ± 0.87	7.20 ±0.52	13.9 ±0.46	762.90 ± 4.98	58.40 ± 1.05	33.20 ±0.38

Values are expressed as mean ± SEM (n=3). All the values are not significant when compared to control group. Data were analysed by One Way ANOVA followed by Dunnett's test analysis.

Figure 5: Histological features of liver, kidney and heart of control and experimental rats

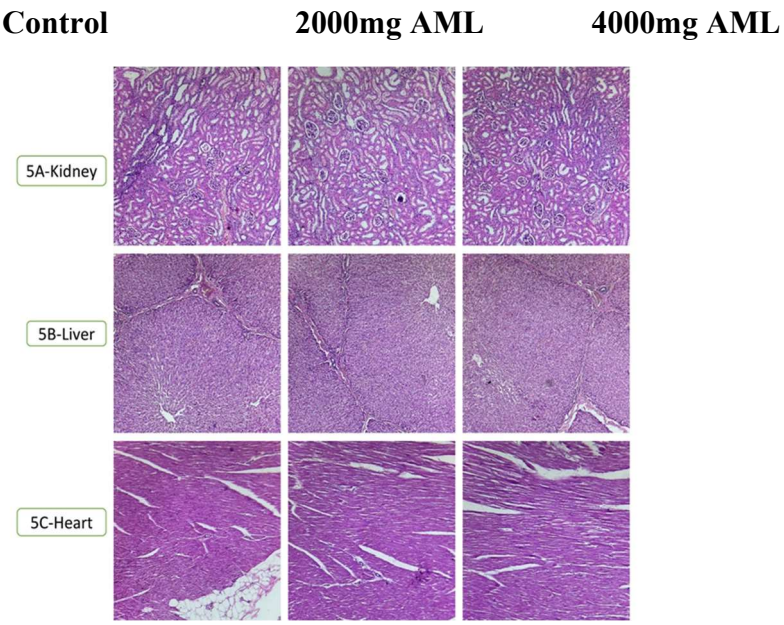

Figure 5: 5A, 5B, 5C shows kidney, liver and heart of control, 2000mg and 4000mg of AML extract treated groups respectively. Histologically no difference between control and treated groups (H&E stain, 10X magnification). 5A: Intact glomeruli and tubules normal

architecture as in similar to the control group. 5B: Hepatocytes in lobules, has central vein. 5C: Heart muscles also showed normal branching pattern and striated, no necrotic changes.

Acute toxicity study

The acute toxic study results of AML extract showed no noticeable signs of acute toxicity and lack of death at all doses up to 4000 mg/kg body weight. Behavior changes of treated rats carried out by general inspection of each animal on a day- to- day basis from the 0 days of study to the 14th day of the study at all dose levels showed no significant changes between control and treated groups (Table 3). The body weight of all treated group rats was slightly diminished in 7^{th} day, but on 14^{th} day treated animals weight has increased, when compared with control, but the increase was not statistically significant (Table 4). Organ weights between control and treated rats showed no statistical significant difference, the P value is >0.05 (Table 5). The blood parameter values showed slightly increased, but not a statistically significant change of HB, WBC, RBC, HCT, MCV, MCH, MCHC & PLT in the treated groups compared to the control group the P value is >0.05 (Table 6). Histological section of liver, kidney and heart were evaluated for pathological changes. In kidney demonstrated intact glomeruli and tubules normal architecture as in similar to the control group (Fig: 5A). Normal liver with hepatic lobules has central vein, clearly visible portal triad seen (Fig 5B). Heart muscles also showed normal branching pattern and striated, no necrotic changes (Fig:5C).

DISCUSSION

In the present study, 41 chemicals were identified from the crude extract of AML with GC-MS analysis. Compounds such as 2-Methoxy-4-vinylphenol have demonstrated anticancer effects against pancreatic cancer cell lines by inhibiting the expression of the cell nuclear antigen (PCNA) protein and reducing the phosphorylation of Focal Adhesion Kinase (FAK) and AKT [11]. Rejinthala et al. (2024) reported that piperidine is a pharmacophore. Its derivatives inhibit triple-negative breast cancer cells by influencing cell viability and arresting the cell cycle at the M2/G phase in K562 cells [24]. Hexadecanoic acid (palmitic acid) induces cell apoptosis in tumor cells through the mitochondrial pathway, facilitated by the promotion of intracellular reactive oxygen species (ROS) generation [25]. Hexadecanoic acid and phytol extracted from microalgae inhibit the growth of MCF7 breast cancer cells in vitro [26]. The primary component in the AML extract with the highest concentration is stearic acid, or 9,12-octadecadienoic acid, constituting 22.28% of the sample. Reza et al. (2021) studied the chloroform fraction of Achyranthes ferruginea *via* GC-MS and discovered various forms of octadecanoic acid methyl esters. The compound has demonstrated potent cytotoxic activity against HeLa cells. Additionally, an *in-silico* study further supports that octadecanoic compounds are significantly associated with the target cancer cells [27]. Stearic acid, also known as octadecadienoic acid, has been shown to inhibit the growth of breast tumor cells and induce apoptosis [28]. N-hexadecanoic acid is one of the bioactive substances found. The literature review indicates that octadecadienoic acid actively inhibits DNA topoisomerase I activity, which triggers apoptosis and hinders cell growth [29]. Plant-based

anticancer and cytotoxic compounds have enormous therapeutic potential as they can achieve the desired results with fewer side effects. Further exploration of plant-derived therapeutics is needed today.

In the cytotoxicity study, the cell viability percentage decreased from 64.28% to 28.50% as the concentration of AML extract increased from 125 µg/mL to 1000 µg/mL. The results denoted that MCF-7 cell growth was significantly inhibited, and cell viability markedly decreased after exposure to AML extract in a dose-dependent manner. The potent cytotoxic activity of AML extract could be attributed to its secondary metabolites, which have a wide range of actions, including antioxidant, antibacterial, antimutagenic, and antiproliferative properties. Datkhile et al. (2020) also reported higher cytotoxicity of Argemone mexicana leaves and stems, which exhibited maximum inhibitory activity against MCF-7 cell lines compared to previous findings [30]. In vitro data suggests that the AML extract sample exhibits potent cytotoxic activity against MCF-7 breast cancer cells. Cells treated with the AML extract were stained with acridine orange (AO) to examine whether apoptosis was a significant factor in inducing the cell death of MCF-7.

In this study, acridine orange (AO) staining morphological analysis distinguished the stages of apoptosis and differentiated viable, apoptosis, and necrosis. Fluorescence microscope investigations demonstrated that the extract caused morphological changes and apoptosis in the AO-stained cells. Numerous studies involving plants and anticancer agents on cancer cells have reported apoptotic-morphological features, such as cytoplasm condensation with a marked decrease in cell volume, chromatin condensation and fragmentation, plasma membrane blebbing, and degeneration of the nucleus into the membrane [31]. It refers to that the main components of the apoptotic process are influenced by the polyphenolic and flavonoid compounds found in food plants, which exhibit cytotoxicity. However, research on how AML extract affects the expression of apoptotic marker enzymes is required to confirm this finding (p53, caspases, etc.).

During the observation period of 14 days in acute toxicity study, noticed that the doses of AML extract supplied to the rats (even the maximum dose of 4000 mg/kg body weight) did not produce any mortality, morbidity or signs of toxicity or behavioral changes. Rats body weight and organ weight of all treated group rats was slightly increased when compared with control, but the increase was not statistically significant. There is no statistically significant change of HB, WBC, RBC, MCV, MCHC & PLT in the treated group compared to the control group. (P value is > 0.05). Histological section of liver, kidney and heart were appeared to be normal, there is no sign of pathological changes. Above the results were supported by many *invivo* analysis on experimental animals. Crude leaf powder suspension of *Argemone mexicana* have not shown any mortality, even at the maximum dose (2500 mg/kg, p.o.) reported by SOURABIE et al (2009) [32], at the dose of 5000 mg/kg, p.o. reported by Prasad and Venugopal (2016) [33]. The acute toxicity study revealed that LD50 of methanol extract of AML is greater than 4000 mg/kg/b.w. Therefore, the present study offers a satisfactory preclinical proof of safety for *Argemone mexicana* leaves extract.

CONCLUSION

Argemone mexicana leaves, stems, and roots have been frequently used in Indian traditional medicine for various conditions. Metabolites detected from GCMS of AML extract, mainly fatty acids, that have anticancer potential are octadecanoic acid (stearic acid); n-Hexadecanoic acid (palmitic acid); phytol, 9,12,15-octadecatrienoic acid; hexadecanoic acid; and methyl ester. According to the study, apoptosis has a role in inhibiting MCF-7 breast cancer cells' proliferation by AML extract and extract has not shown any mortality at the highest dose in *in-vivo*. According to the results, AML extract shows potential as a chemotherapeutic agent for the treatment of cancer.

Future scope:

The separation of many flavonoid fractions using high-performance liquid chromatography and the cytotoxic analysis of each fraction will assist in identifying the active component in AML. Additionally, research on the apoptotic transduction pathway in cancer cells treated with this plant extract could provide insights into the mechanism underlying the activation of cell death.

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