ORIGINAL ARTICLE

Bioactivity Guided Fractionation and Elucidation of Anticancer Properties of *Madhuca longifolia* Leaf Extracts

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ABSTRACT

One of the leading causes of mortality in the globe is cancer. Cancer is a primary reason of death, with approximately 19.3 million novel cases expected to be diagnosed globally in 2022. The use of naturally generated anticancer chemicals must be developed as an alternative safe, affordable, and practical method for cancer treatment as traditional cancer medicines have failed to meet the requirements for successful cancer therapy. Phytochemicals and herbal extracts from various plants have been shown to have powerful chemopreventive properties. In this work, we studied the pharmacognostical and anticancer activity of the leaves of *Madhuca longifolia*. Aqueous, hydroalcoholic and ethanolic extracts of plants *M. longifolia* shows considerable movement beside breast cancer cell line MCF-7. The pharmacognostical study of leaves of *M. longifolia* shows existence of phenolic compounds, steroids, glycosides, saponins, alkaloids, flavonoids, amino acids, proteins, carbohydrates, and triterpenoids. The cytotoxicity of cancer cells has been linked to the induction of intracellular reactive oxygen species (ROS). The IC_{50} values of ethyl acetate fractions of ML is (14.37 µg/mL) and n-butanol fraction of ML (19.58 µg/mL) showed good activity which is compared with the standard drug tamoxifen (4.59 µg/mL). From the study, we concluded that the leaves of the plant *M. longifolia* show significant anticancer activity.

Keywords: Anticancer, Apoptosis, Flow cytometry, *Madhuca longifolia*, MTT assay, Reactive oxygen species.

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Conflict of interest: None

INTRODUCTION

Cancer is a disease in which abnormal cells expand out of control and can attack nearby tissues. Another way for cancer cells to spread is through the body's lymphatic and vascular systems. There are various primary cancer kinds.[1]

One of the leading causes of mortality in the globe is cancer. Cancer is a primary cause of death, with 19.3 million new cases expected to be diagnosed globally in 2022. According to information, worldwide estimated cancers in both sex with all ages person are breast lung (11.4%), cancer (11.7%), prostate (7.3%), liver (4.7%), cervix uteri (3.1%), stomach (5.6%), colorectum (10%), and other cancers (46%) Africa, Asia, Central and South America report for more than 60% of new cancer cases and 70% of cancer deaths worldwide. By 2025, it is estimated that 22 million new cancer cases would be diagnosed. One of the most important health issues of our day is breast cancer. Breast cancer accounts for around 24.2% of new cases in women overall. Millions of women throughout the world are affected by this form of cancer. Breast cancer is a specific form of cancer that starts in the cells of the breast tissue, either in the cells that line the milk-delivery ducts that go to the nipples (ductal cancer) or in the cells that line the lobules, which are milk-producing glands.[2,3]

Currently, various approaches are utilized to cure cancer; unfortunately, in most of these instances, the response to the therapy is relatively poor and results in significant unfavorable consequences as well as drug resistance with prolonged treatment. Finding innovative ways to manage cancer is crucial due to the rise in cancer-related deaths and the lack of advanced chemical treatment and radiation. Numerous studies on medicinal plants are now being conducted, and the development of medications using natural substances that are beneficial has opened up new opportunities for the association of doctors and chemists.[4] As a result, one-third of the currently used pharmaceuticals in the human population are manufactured from natural and herbal sources, and doctors think that patients should be encouraged to take herbal plants because of how well-tolerated and successful they are at stopping tumor growth. Therefore, naturally generated anticancer agents must be developed as an alternative

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safe, affordable, and practical method for cancer treatment as traditional cancer medicines have failed to satisfy the requirements for successful cancer therapy.[5]

Phytochemicals and herbal extracts from a variety of plants, fruits, vegetables, and spices have been shown to have powerful chemopreventive properties. Many plants, including *Anacardium occidentale, Asparagus racemosa, Abrus precatorius, Andrographis paniculata, Centella asiatica, Curcuma longa, and Cedrus deodara,* have been proven to have cancer-preventive properties in previous studies. For more than a half-century, natural compounds with amazing chemical diversity have been widely explored for their anticancer potential.[6] Despite the significant progress made by the community in bringing natural products to clinical use and uncovering novel therapeutic options, there are still obstacles ahead. With the dramatic changes in the landscape of cancer therapy and the increasing importance of cutting-edge technology, we may have reached a point where we need to reassess our tactics for understanding natural products and exploring their therapeutic potential. In this work, the anticancer activity of plant *Madhuca longifolia* was studied. The plant *M. longifolia* was shows good anticancer activity against cancerous cells. It will help the researcher to develop new anticancer formulations.

MATERIALS AND METHODS

Sigma Aldrich Co. in St. Louis, USA, provided 3-(4, 5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), 2′, 7′-dichlorofluorescin diacetate, ethanol, ethyl acetate, methanol, and dimethyl sulfoxide. All other substances were of an analytical grade.

Collection of Plant and Authentication

The leaf parts of the plant *M. longifolia* (ML) were collected from Aurangabad, India. Plant was authenticated by BSI, Pune (Specimen No.- NKML-1 (*M. longifolia* (J. Koenig ex. L.) J. E. Macbr. Var. *longifolia*) family sapotaceae). Collection of sample-washing with purified water 3–4 times-shade dried for 2 weeks 1-hour tray drying if needed-grinding using mixer-sieving using sieve no 36 (420 µm) and the plant material was then used for further investigation.

Pharmacognostical Studies

Extraction

The essential plant material was first separated for good and bad material. Only high-quality materials were used. After that, garbage and other extraneous particles were removed from the chosen materials. This was accomplished by removing sand and other foreign debris with sieves, followed by hand sizing. They were then dried in the shade at 35–40°C for around 10 minutes, reducing the moisture content to roughly 10%. The dry pieces were then crushed into smaller particles and processed as follows. It is done by using three solvent systems.[7,8]

- Aq-using hot maceration (soak powder sample in hot water for 24 hours with occasional stirring).
- Hydroalcoholic-using cold maceration (soak powder sample in equal proportion of water and ethanol for 24 hours with occasional stirring)
- Ethanolic-using soxhlet apparatus (treatment with ethanol for 8 hours)

Solid : solvent ratio= 1:10 (mL)

Muslin cloth and Whatman's filter paper were used to filter all extracts, and the filtrates were subsequently concentrated under decreased pressure using a rota evaporator.

Percentage Yield Estimation

Dried extracts were calculated for percentage yield estimation using the formula as follows;

% yield= Practical yield/Theoretical yield X 100

Preliminary Phytochemical Screening of the Extracts

All three extracts for *M. longifolia* plants were tested for the preliminary phytochemical analysis. The following phytochemical assays were used to screen all three extracts for presence of different phytoconstituents eg; phenolic compounds, alkaloids, tannins, glycosides, flavonoids, saponins, steroids, amino acids, proteins, carbohydrates, and triterpenoids.[9]

Anticancer Activity using MTT Assay

Colorimetric assay is based on mitochondrial succinate dehydrogenase enzymes' capability to convert spectrophotometrically measured insoluble, purple formazan product from yellow, water-soluble substrate 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into living cells. Since only metabolically active cells can reduce MTT, the amount of action provides gauge of viability of cells utilized in the study, which included MCF-7 (a human breast cancer cell line), DMEM with high glucose (Cat No. 11965-092), and FBS (Gibco, Invitrogen) Cat No. -10270106. For the investigation, an antibiotic called antimycotic 100X solution (Thermo Fisher Scientific)-Cat No. 15240062 was utilized.[10]

The DMEM medium added with 10% fetal bovine serum was utilized to sustain MCF-7 cell line. Cells were grown for 24 hours at 37°C after being plated at density of $1x10^4$ cells per well in a 96-well plate. Then, 5–80 µg/mL were exposed to the cells. Cell proliferation was assessed by adding 10 µL of MTT (thiazolyl blue tetrazolium bromide) dye (5 mg/mL in phosphate-buffered saline) each well to plates after 24 hours of incubation. The plates were incubated for further 4 hours at 37°C in a humid environment with 5% CO_2. DMSO of 200 µL was used to dissolve the formazan crystals that each well produced as a result of the live cells reducing the dye, and absorbance was measured at 490 nm.

Utilizing the following formula, the compound percentage of cytotoxicity was determined.[11]

$$\textbf{Percent cytotoxicity} = \frac{\text{reading of control - reading of treated cells}}{\text{reading of control}} \times 100$$

Fractionation

IC_{50} values of the ML-EA (14.37 µg/mL) showed good activity which is compared with standard drug tamoxifen (4.59 µg/mL).

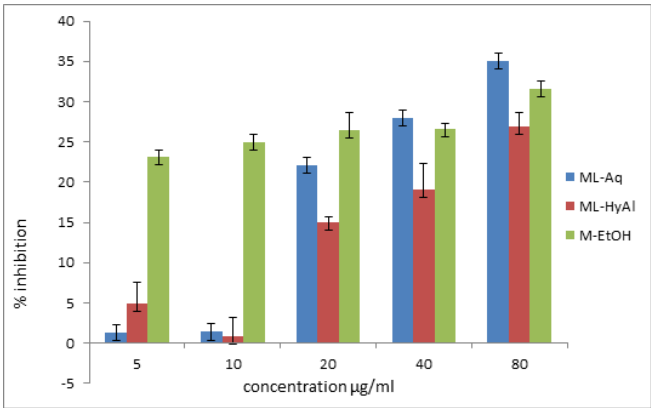


Figure 1: Graphical representation of % Inhibition of crude aqueous, Hydro-alcoholic and Ethanolic extract of ML (Each value represent the mean ± S.D., n=3)

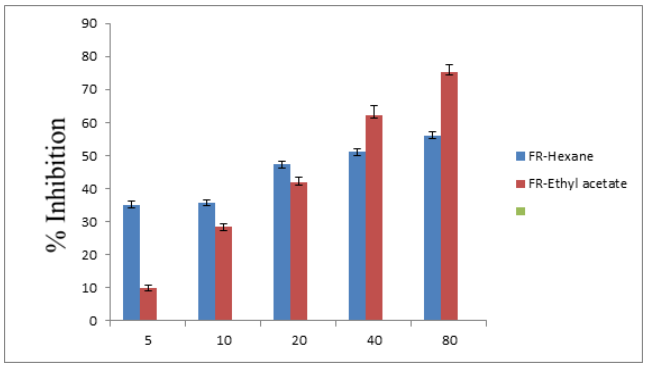


Figure 2: Graphical representation of % inhibition of ML fractions against MCF-7 using MTT assay

Hence, these ML-EA active fractions were taken for further phytochemical characterization and detailed anticancer screening.

Anticancer Screening

The active fractions from *M. longifolia* plant were further evaluated for detailed anticancer screening. The main purpose of evaluating active compounds/fractions using In-vitro anticancer screening assays is to examine the exact mechanism of cell death action. Here we have screened the active compounds/fractions using four *in-vitro* anticancer assays. Basically, these assays give idea about the percentage of cell death (% cytotoxicity); whether the cell death is occurring or not and in which phase it is occurring, such as necrosis, early apoptosis and late apoptosis; pathways/receptor mechanisms through which the compounds were responsible to cause the cell death; and various reasons of cell death such as rupturing the whole cell, rupturing the individual cell components like mitochondria, generation of reactive oxygen species (ROS), changing in the gene expression etc.[12]

Apoptosis

Use the chosen technique to cause apoptosis in the cells. Cells were incubated without an inciting substance to create the negative control. After incubation, cells were collected and rinsed in cold phosphate-buffered saline. After then, 1X

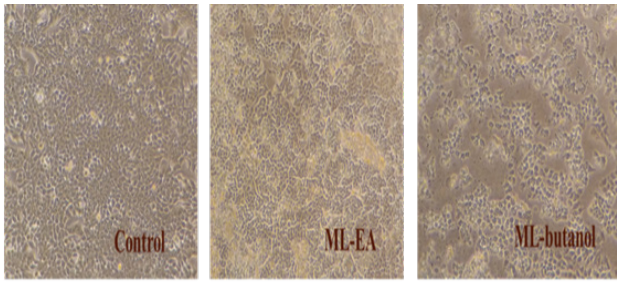


Figure 3: Microscopical images of ML fractions with control showing viable cell count using MTT assay

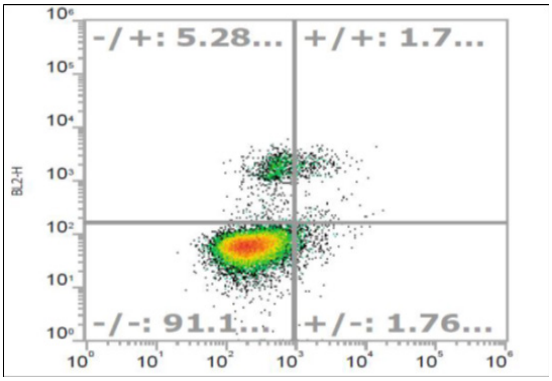


Figure 4: Apoptosis study using control strained

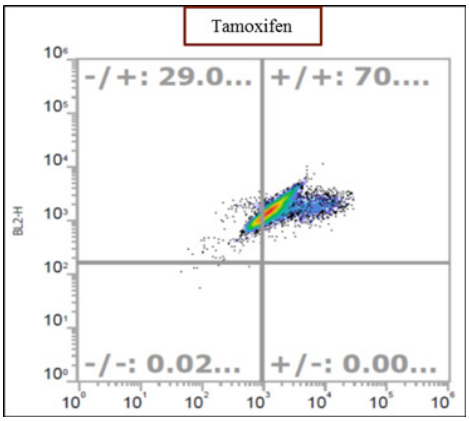


Figure 5: Apoptosis study using standard drug (Tamoxifen)

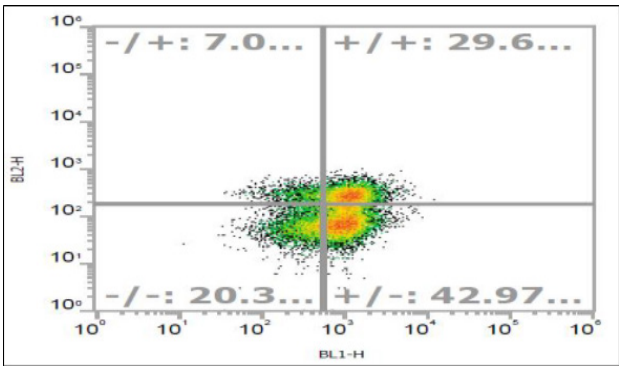


Figure 6: Apoptosis study using ML-EA fraction

annexin-binding buffer was made. By combining 5 µL of the 1 mg/mL PI stock solution (component B) with 45 µL of

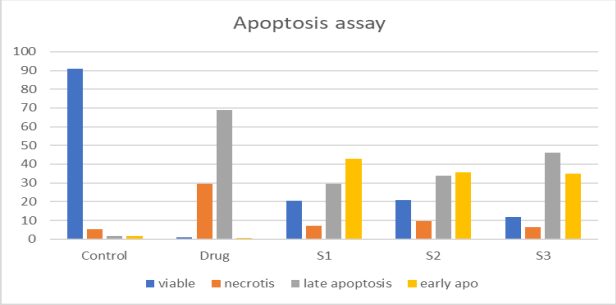


Figure 7: Graphical presentation of apoptosis (S1 represent ML-EA)

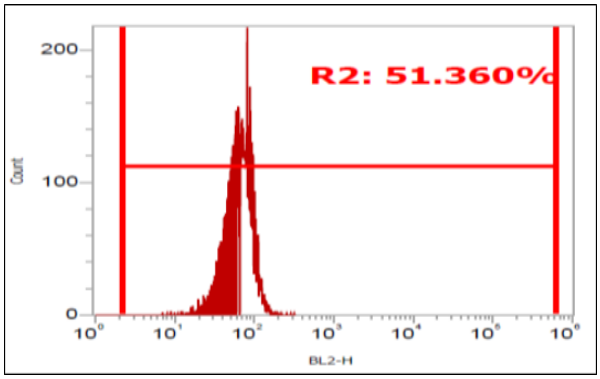


Figure 8: Effect of CCCP (negative control) on Mitochondrial Membrane potential

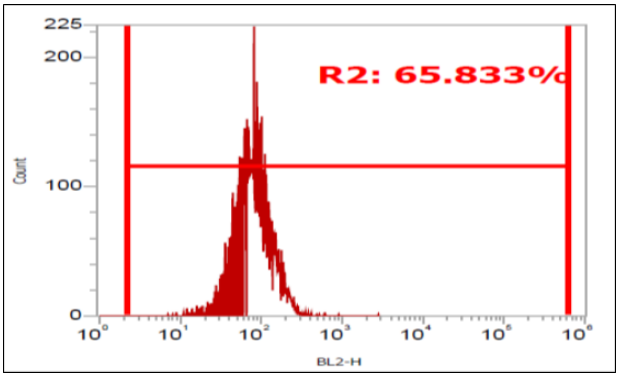


Figure 9: Effect of DiLC1 (positive control) on Mitochondrial Membrane potential

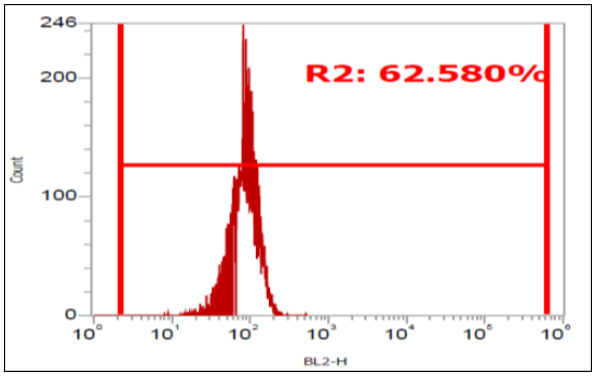


Figure 10: Effect of ML-EA fraction on Mitochondrial Membrane potential

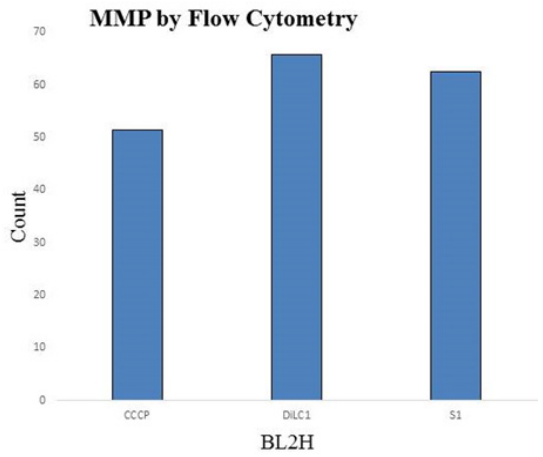


Figure 11: MMP by Flow Cytometry (S1 represent ML-EA)

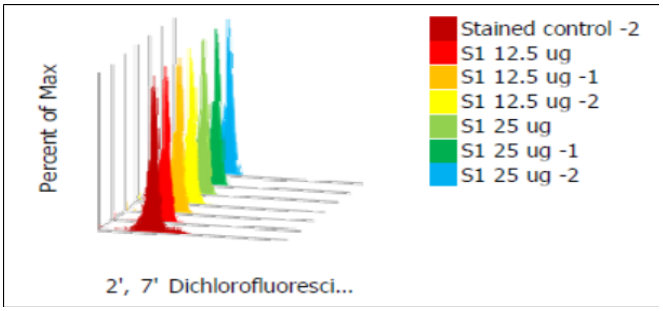
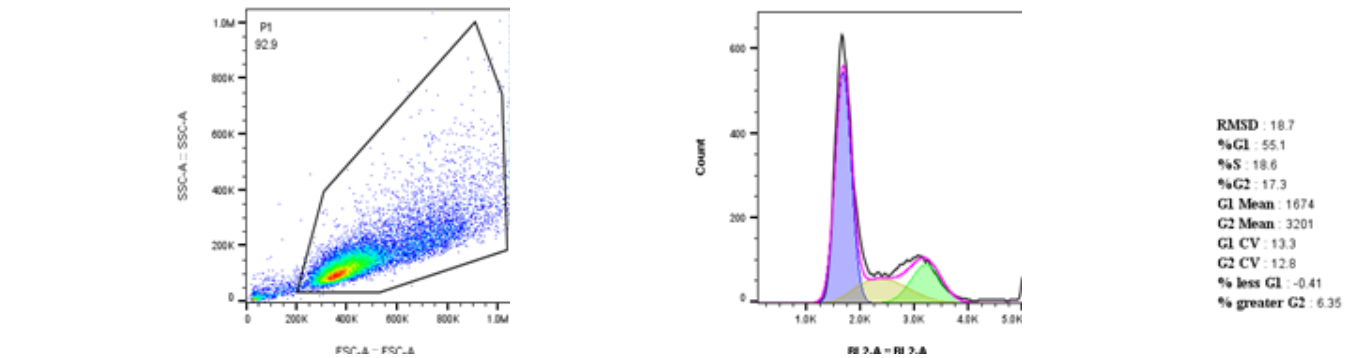


Figure 12:ROS Generation Assay of ML-EA fraction (S1 represent ML-EA)

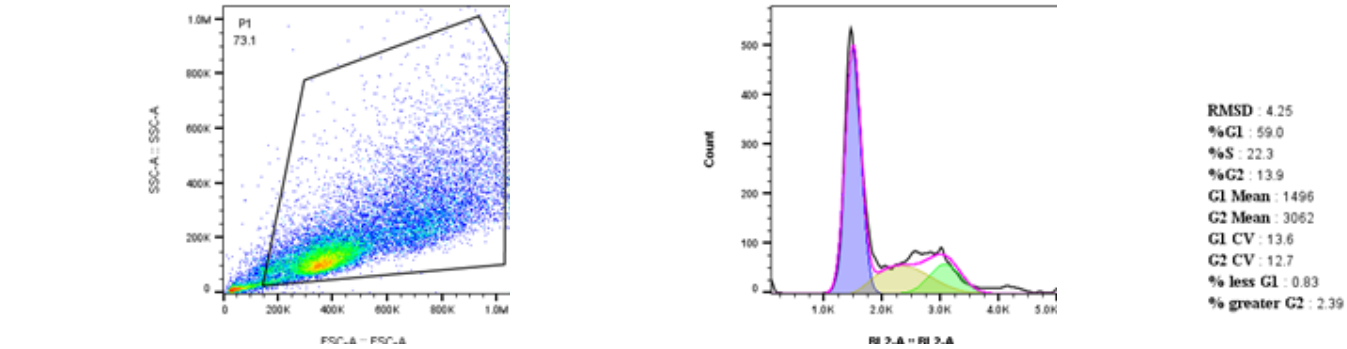
1X annexin-binding buffer, PI was diluted to an operational solution concentration of 100 µg/mL. After centrifuging washed cells and removing the supernatant, resuspending the cells in 1X annexin-binding buffer. Cell density was calculated and diluted to 1 X 106 cells/mL in 1X annexin-binding buffer. For every 100 µL of cell suspension, 5 mL of FITC annexin V (Component A) and 1-µL of 100 µg/mL PI functioning solution were supplemented. The cells were cultured for 15 minutes at room temp. Add 400 µL of 1X annexin-binding buffer after incubation, stir gently, and keep the samples chilled. Utilizing flow cytometry, examine the labeled cells and fluorescence emission at 530 nm (FL1) and above 575 nm (e.g., FL3).[13]

Cell Cycle Analysis

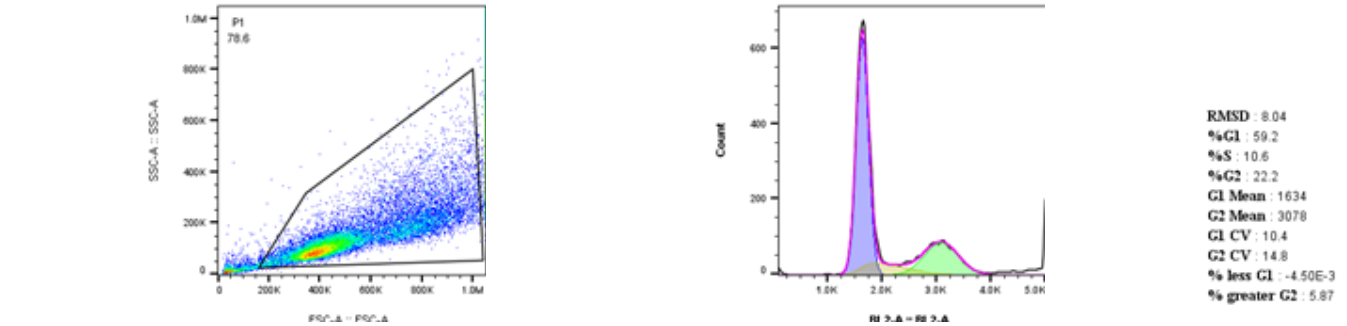
The right way to collect the cells was followed by a 1X PBS wash of the cells. A short diameter cell pellet made of supernatant was removed, and while it was being vortexed, cold 70% ethanol was added drop by drop. This should prevent clumping and guarantee that all cells are fixed. then set for 4°C overnight. Cells were rinsed in 2 X PBS following incubation. Then a centrifuge was used to spin the sample at 850 g while avoiding cell loss when removing the supernatant, particularly after spinning off the ethanol. The cells were given ribonuclease treatment. 100 µg/mL of RNase stock should be added in 50 µL. RNA was not stained; only DNA was, thanks to this. For a 200 µL suspension, 4 µL of PI (from a 1 mg/mL stock sol) was then added. The suspension was combined,



(a) Group_Control PI



(b) Group_ ML-EA - 6.25 ug



(c) Group_ ML-EA 3.12 ug

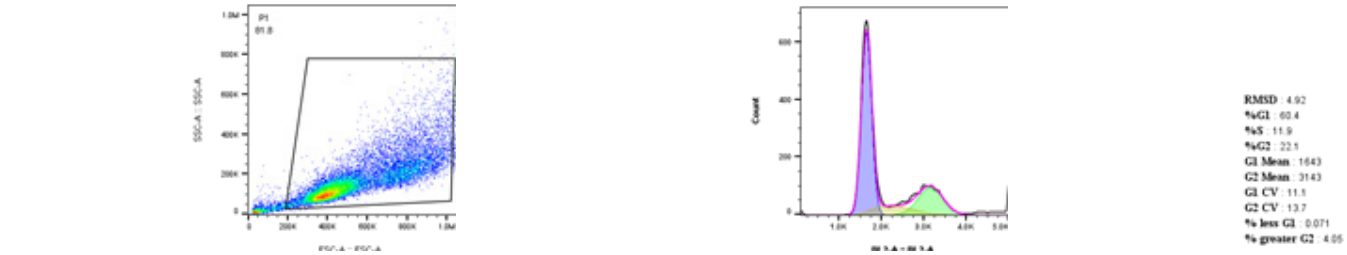


Figure 13: Cell Cycle Analysis of extract

transferred, and deposited at the sample lifter in a cuvette (Cat No. P10306 - Abdos). Cell doublets were removed from the data using pulse processing. PI may be measured with a proper bandpass filter because its greatest emission is at 605 nm.

Mitochondrial Membrane Potential

Approximately 1 x 106 cells/mL were suspended in 1-mL of warm, phosphate-buffered saline media. Before adding DilC1 dye to the control tube, 1-µL of 50 mM CCCP (included with kit, 50 µM final concentration) was added as negative control. DiIC1 dye was only added as a positive control, and cells were then incubated at 37°C for 5 minutes. Cells were then treated with 5 µL of 50 nM final concentration 10 µM DilC1 (provided with the kit) for 15 to 30 minutes at 37°C with 5% CO_2. Cells were cleaned in each tube of cells by adding 2 mL of warm

Table 1: Extractive values of *M. longifolia* plant in different solvent

S. no.	*Plants type of extraction*	*Plant 1 (ML)*
1.	Aqueous extraction	14.6 ± 2.5 %
2.	Hydroalcoholic extraction	19.4 ± 1.20 %
3.	Ethanolic extraction	10.4 ± 2.2 %

Table 2: Preliminary phytochemical screening of the extracts

S. No	*Test*	*ML*		
		Aq.	*HyAlc*	*Ethanol*
1.	Carbohydrates	+	+	+
2.	Alkaloids	+	+	+
3.	Steroids	+	+	+
4.	Glycosides	+	+	+
5.	Flavonoids	+	+	-
6.	Tannins and Polyphenols	+	+	+
7.	Proteins and Amino acid	+	+	+

+ Present, - Absent

Table 3: Anticancer activity using MTT assay of ML extract

S No	*Plant extract*	*Conc (µg/mL)*	*%Inhibition*
1.	ML-Aq	5	1.34 ± 3.73
		10	1.40 ± 0.36
		20	22.08 ± 3.25
		40	27.99 ± 2.69
		80	35.03 ± 0.92
2.	ML-HyAl	5	4.90 ± 0.71
		10	6.80 ± 0.44
		20	15.01 ± 0.69
		40	19.08 ± 3.22
		80	26.86 ± 1.8
3.	ML-EtOH	5	23.10 ± 0.91
		10	24.92 ± 0.99
		20	26.49 ± 2.15
		40	26.57 ± 0.75
		80	31.59 ± 0.98

Each value represent the mean ± S.D., n=3

phosphate-buffered saline (PBS). The centrifuged cells were then gently flicked back into suspension. Each tube was filled with 500 µL of PBS. After that, it was examined using a flow cytometer at 633 nm. The CCCP-treated sample was used to carry out the usual compensation.[14,15]

ROS Generation Assay

The cells were balanced in 1-mL of warm, phosphate-buffered saline media. Cells were then treated with 5 µL of 2′,7′-dichlorofluorescin diacetate (10 µm) for 15 to 30 minutes at 37°C with 5% CO_2. Add 1-mL of warm phosphate-buffered saline to wash the cells. Centrifuge the cells at 1400 rpm to pellet them and gently flip the tubes to resuspend them. Each tube should contain 500 µL of PBS (or another appropriate buffer). The samples were examined for green dye in emission filters on a flow cytometer at 504 nm.[16-17]

Table 4: IC_{50} value of Crude extract of *M. longifolia* against MCF-7 cell lines

S. No	*Drug/Plant*	*Type of crude extracts*	*IC_{50} value (µg/mL)*
1.	Tamoxifen	Standard drug	4.59 ± 1.762
2.	ML	Aqueous	20.58 ± 7.19
		Hydro-alcoholic (50:50)	31.37 ± 4.577
		Ethanolic	53.67 ± 2.626

Each value represent the mean ± S.D., n=3

Table 5: % inhibition of ML fractions against MCF-7 using MTT assay fractions against MCF-7

S. No	*Active fraction*	*Conc (µg/mL)*	*% Inhibition*
1.	ML-Butanol	5	12.85 ± 1.2
		10	18.87 ± 2.54
		20	24 ± 2.44
		40	31.64 ± 0.25
		80	87.35 ± 2.55
2.	ML-Ethyl acetate	5	14.99 ± 3.58
		10	23.35 ± 0.54
		20	31.79 ± 1.99
		40	42.79 ± 2.55
		80	91.79 ± 2.98

[Each value represent the mean ± S.D., n=3]

Statistical Analysis

The data were accounted as means of standard deviation, with each test run in triplicate. Using SPSS Statistics 17 for Windows (SPSS, Chicago, IL, USA) software, data were subjected to one-way analysis of variance and the consequence of the difference between means was assessed using Duncan's multiple-range test ($p < 0.05$).

RESULTS AND DISCUSSION

Pharmacognostical Studies

Extraction

Table 1 shows extractive values of *M. longifolia* plant in a different solvent. The hydroalcoholic extract of *M. longifolia* plants has a higher % of extractive value than that of aqueous and ethanolic extracts.

Preliminary Phytochemical Screening of the Extracts

All secondary metabolites, with the exception of flavonoids, are present in the ethanolic extract of ML, which is obtained from aqueous, hydroalcoholic, and ethanolic extracts of plants (Table 2).

Anticancer Activity using MTT Assay

The extracts were subjected to anticancer activity. As we see in our results (Table 3), all the *M. longifolia* extracts showed good % inhibition against cancerous cells. The % inhibition of ML-Aq extracts was in ranges of 1.34 ± 3.73% to 35.03 ± 0.92 %. In case of ML-HyAl extracts % inhibition was from 4.90 ± 2.71 to 26.86 ± 1.8. ML-EtOH shows higher % inhibition than ML-Aq and ML-HyAl, it shows 23.10 ± 0.91 to 31.59 ± 0.98 %

inhibition. In all the extracts, the % inhibition was increased as increased in ML concentration.

Table 4 indicates aqueous, ethanolic and hydroalcoholic extract of ML analysed for IC_{50} value. Tamoxifen (standard sample) shows 4.59 ± 1.762 IC_{50} value. In case of ML, IC_{50} value for aqueous extract was 20.58 ± 7.19%, hydroalcoholic was 31.37 ± 4.577% and ethanolic was 53.67 ± 2.626%. All the extract also shows good IC_{50} value when compared with tamoxifen as standard (Figure 1).

Anticancer Activity of Active Fractions Against MCF-7 using MTT Assay

Anticancer activities of vigorous fractions beside MCF-7 were studied by MTT assay (see Table 5). All the active fractions showed significant % inhibition. The ethyl acetate fractions of ML shows % inhibition from 14.99 ± 3.58 to 91.79 ± 2.98% and butanol fractions of ML shows 12.85 ± 1.2 to 87.35 ± 2.55% inhibitions as concentration increase from 5 to 80 µg/mL. the ML-ethyl acetate fractions show highest % inhibition than ML-Butanol fractions.

Figure 2 and 3 indicate the of MTT assay of ML fractions. As we compared with control groups, the fractions of ML shows good activity compared with the standard drug tamoxifen. Hence, these active fractions were taken for further phytochemical characterization and detailed anticancer screening.

Anticancer Screening

Apoptosis

Current cancer treatment methods, such as chemotherapy and radiotherapy, entail inducing cellular apoptosis in cancer cells because it plays a critical role in cancer growth. Control i.e., blank showed a maximum (91%) of viable cells (Figure 4). The drug-treated sample showed maximum cell death which occurs combinely in necrotic and late apoptotic phases (Figure 5). ML-EA fraction shows results of maximum cell death in the early and late apoptotic phase (Figure 6). Graphical representation of apoptosis also given in Figure 7.

In various cancer cell lines, it has been documented that extracts induction of intracellular ROS deposition results in cell membrane deterioration and death.

Mitochondrial Membrane Potential

Some apoptotic pathways' induction and activation have been connected to mitochondrial integrity (Figure 8-10). Figure 11 shows MMP by flow cytometry. The current study, CCCP was utilized as –ve control, DiLC1 was selected as the + control. From above results, ML-EA fraction showed certain loss of mitochondrial membrane potential which causes apoptosis.

ROS Generation Assay

The cytotoxicity of cancer cells has been linked to the induction of intracellular ROS (Figure 12). The current investigation showed that cancer cells accumulate intracellular ROS. ROS production increased in all individuals following extract consumption. Due to the interindividual heterogeneity in ROS production, all results were standardized to a baseline concentration of 100% (Figure 12).

Cell Cycle Analysis

The alcohol extract was applied to MCF-7 cells in the exponential development phase. Cell cycle investigations were carried out using flow cytometry to investigate the causes of the extract's antiproliferative effects. As shown in Figure 13, cells began to amass in the G1 phase, and their numbers steadily grew after being exposed to the test substance. The proportion of cells in S and G2/M phases also dropped.

CONCLUSION

Numerous plant species have been shown to contain phytochemicals and herbal extracts that have potent chemopreventive activities. This work investigated hydroalcoholic, aqueous, and ethanolic extracts of plant *M. longifolia*. The hydroalcoholic extracts of *M. longifolia* plants shows higher percentage of extractive value compare to aqueous and ethanolic extracts. The plants included a variety of phytoconstituents, including triterpenoids, alkaloids, glycosides, tannins, flavonoids, phenolic compounds, steroids, and saponins. In MTT assay, ML-aq extract have high % inhibition and good IC_{50} value. All the extracts also show good IC_{50} value when compared with Tamoxifen (4.59 µg/mL) as standard. Intracellular ROS production has been linked to the cytotoxicity of cancer cells. Study also shows a reduction in mitochondrial membrane potential. In cell cycle analysis, cells began to amass in the G1 phase, and their numbers steadily grew after being exposed to the test substance. The proportion of cells in S and G2/M phases also dropped. Our study established that intracellular ROS are accumulated by cancerous cells. From the study, we concluded that the presence of various phytoconstituents in plants *M. longifolia* shows significant anticancer activity.

FUNDING

No funding sources

CONFLICT OF INTEREST

None declared

ETHICAL APPROVAL

Not required

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