PHYTOCHEMICAL AND CYTOTOXIC EVALUATION OF *Calopogonium mucunoides* EXTRACT AGAINT HUMAN BREAST CANCER CELLS

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ABSTRACT

Background: Breast cancer is the leading cause of cancer-related deaths globally, and it is caused by the uncontrolled proliferation of abnormal cells in breast tissue, primarily in the milk ducts or lobes. While traditional treatments like chemotherapy and radiation therapy are effective, they are frequently associated with severe side effects, emphasizing the need for alternative therapeutic techniques. Herbal drugs, such as *Calopogonium mucunoides* extract, offer a promising route due to their bioactive components, which have cytotoxic, anti-inflammatory, and antioxidant characteristics with potentially less side effects than synthetic treatments.

Aim: This study aims to prepare ethanolic extract of *Calopogonium mucunoides*, perform phytochemical assessments, and evaluate its in-vitro anticancer activity against breast cancer cell lines.

Methodology: Soxhlet extraction was utilized to produce petroleum ether, hexane, ethanol, and aqueous extracts of *Calopogonium mucunoides*, which were then subjected to routine phytochemical evaluations. MCF-7 breast cancer cells were used to test the in-vitro anticancer activity, and the IC_{50} value was determined.

Results: The ethanol extract was selected for the studies due to its high yield (11.62%). The extract contains phenols, flavonoids, tannins, and other compounds required for anti-cancer activities. The plant extract produced a concentration-dependent decrease in cell viability. The IC50 was found to be 59.52 µg/mL.

KEYWARDS: *Calopogonium mucunoides,* proliferation, flavonoids, chemotherapy

INTRODUCTION

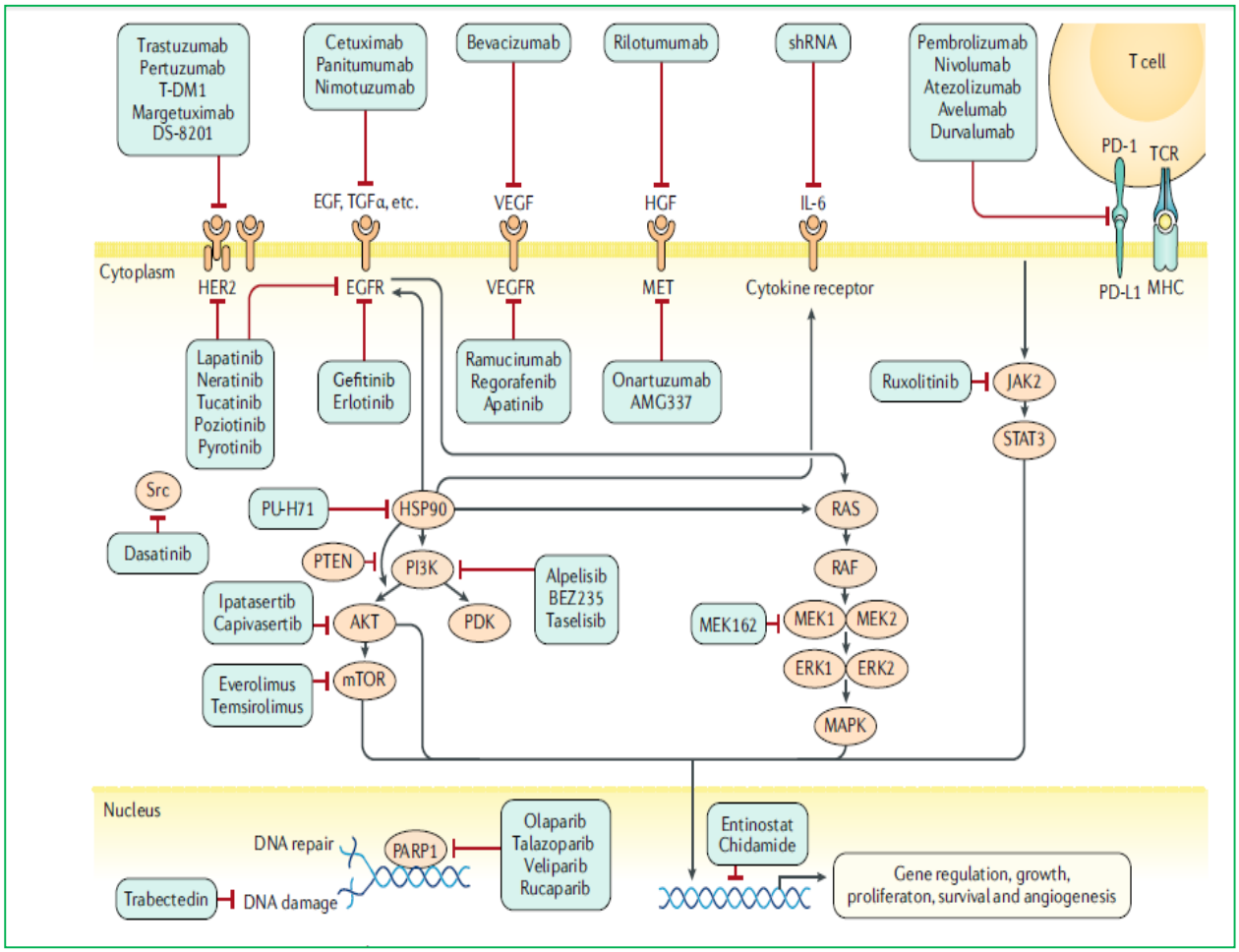
CANCER

Cancer is a disease of cells characterized by uncontrolled proliferation of cells that have transformed from the normal cells of the body. Cancer cells growth anywhere in a body. Cancer is a group of diseases caused by loss of cell cycle control. Cancer is associated with abnormal uncontrolled cell growth. Cancer is caused by both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism). Cancer is a significant worldwide health problem generally due to the lack of widespread and comprehensive early detection methods, the associated poor prognosis of patients diagnosed in later stages of the disease and its increasing incidence on a global scale. Cancer related deaths with an estimated number of 2.3 million new cases worldwide according to the GLOBOCAN 2020 data[12].

BREAST CANCER:

Breast cancer is a kind of cancer that develops from breast cells. Breast cancer usually starts off in the inner lining of milk ducts or the lobules. Although men can also breast cancer, cases of male breast cancer account for less than 0.05% of all diagnosed. Breast cancer is caused when abnormal tissue in the breast begins to multiply uncontrollably. These cancerous cells can travel to other locations in the body and cause further damage. Breast cancer is represents 16% of all cancers in women. Reactive oxygen species (ROS) are involved in a variety of important pathophysiological conditions including mutagenesis and carcinogenesis. Oxidative stress has the potential to cause cellular DNA damage, lipid peroxidation, and membrane disruption. Human body is equipped with various antioxidants such as superoxide dismutase (SOD), glutathione peroxidise (GPx), catalase (CAT), glutathione (GSH), ascorbic acid (vitamin C), atocopherol (vitamin E), and so on, which can counteract the deleterious action of ROS and protect from cellular and molecular damage. Bioactive compounds from plant origin have the potential to subside the biochemical imbalances induced by various toxins associated with free radicals. Therapeutic interventions include Radiation therapy (radiotherapy), Surgery, Biological therapy (targeted drug therapy), Hormone therapy, Chemotherapy. Though such treatment is associated with substantial cost and numerous side effects, it has led a way to search plant based treatments for breast cancer. Herbal plants have been used since centuries to prevent and/or reduce the oxidative stress and DNA damage[11].

Targetable pathways in breast cancer



HERBS AS MEDICINE

The pharmacological behaviour of illness began long ago with the use of therapeutic plants Methods of folk curative during the world commonly used herbs as part of their custom. Ayurveda is a medical system primarily skillful in India that has been recognized for almost 5000 years. It includes food and herbal remedies, while emphasizes the organization and brain in sickness prevention and management. Flora and their secondary metabolite components have an extended history of use in contemporary medicine and in certain systems of conventional medicine and are the sources of significant drugs. The World Health Organization (WHO) estimates that four billion people, 80% of the world's population presently use herbs for some aspect of primary care. They have stood the test of time for their protection, effectiveness, literary suitability and minor side effects. The chemical components present in them are a part of the physiological functions of living plant life and hence they are whispered to have better compatibility with the human body.

INDIAN HERBS USED TO TREAT BREAST CANCER

List of some Indian herbal plants that are used to treat breast cancer[12]

S.NO	PLANT NAME	FAMILY	PLANT PART
1.	*Berberis vulgaris L.*	Berberidaceae	Root bark
2.	*Citrullus colocynthis L.*	Cucurbitaceae	Leaves
3.	*Curculigo orchioides Gaertn.*	Amaryllidaceae	Root
4.	*Ocimum gratissimum L.*	Lamiaceae	Stem and leaves
5.	*Semecarpus anacardium L.*	Anacardiaceae	Dried fruits
6.	*Tiliacora racemosa Coleb.*	Menispermaceae	Root
7.	*Alstonia scholaris L.*	Apocynaceae	Stem
8.	*Bauhinia variegate L.*	Caesalpiniaceae	Stem

CALOPOGONIUM MUCUNOIDES

Synonyms[11,12,13]

Calopogonium brachycarpum (benth), Calopogonium orthocarpum Urb, stenolobium brachycarpum

Habitual names

English – Calopo, Wild ground nut, Caloponiums
Spanish – Cama, Jequirana, Calopogonio indico
Portuguese – Mielillo
Indonesia – Kacang asu
Colombia – picapica , gusanillo

Taxanomic Hierarchy

Kingdom – Plantae

Phylum –Spermatophyta
Clade- Angiosperms
Class - Dicotyledonae
Order – Fabales
Family – Fabaceae
Sub-family – Faboideae
Genus – Calopogonium
Species – C.mucunoides
Botanical name- *Calopogonium mucunoides*

Topographical description

About six to eight species of the genus Calopogonium are endemic to tropical continental America and have spread far throughout tropical and subtropical regions of the world. Updated checklists, however, regard Cuba and Puerto Rico as naturalized habitats for this species. From Mexico to Argentina, as well as several West Indies islands, tropical America is home to C.mucunoides. In India, it is widely distributed in the states of Kerala, Karnataka and tamilnadu[13].

Morphological overview

Calopogonium mucunoides Desv. is an annual trailing legume of 3-5 m long, slightly woody, twining vine with many branches. Alternate trifoliate with obtuse apex measuring about 2-10 x 1.5-6.5 cm assymetrical leaflets were seen. Both the surfaces are dull pubescent with prominent venation at lower side. Axillary pseudo-racemous inflorescence of about 10cm long with bracts is present. Leguminous fruits which are flattened that have reddish-brown quadrangular seeds are found. Succulent stems with long, brown hairs covering them with twning at the upper part. A dense shallow roots with nodules were seen[13,14].

MATERIALS AND METHODS:

Collection and Authentication of Plant: Plant materials were collected from Thiruvananthapuram district during the month of October to November and authenticated Dr. N Mabel Starlin, Botony department, Nesamony Memmorial Christion College , Marthandam. After washing with fresh water, plant materials were dried under shade. Dried samples were coarsely powdered and stored in suitable plastic air tight containers.

Extraction: Dried powdered samples were used for extraction. Extraction is carrying out in an increasing order of polarity (Pet ether, Hexane, Ethanol, and Aqueous). Pet ether extraction of *Calopogonium mucunoides* is done.

Method of Extraction (Soxhlet Extraction Method)

Procedure

Dried sample was powdered to obtain a coarse powder and placed inside a thimble, which is loaded into the main chamber of the Soxhlet extractor. The extraction of weighted leaf powder with respective volume of the solvent was carried out with its boiling point. Selected solvents were carried for the extraction depending on their increasing polarity. The Soxhlet extractor is positioned into a flask containing the extracting solvent. The Soxhlet is then fitted with a condenser. The solvent is heated to reflux. The solvent vapor travels up a distillation tube and condensed into the extractor housing the thimble holding the solid. The condenser ensures that solvent vapor condenses and drips back down into the chamber housing the plant material. The chamber containing the material slowly fills with warm solvent. Some of the desired compound then dissolves in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the extracting flask. This cycle may be allowed to repeat many times, over hours or days, until the solvent gets colorless in extracting chamber[7]. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. After extraction the solvent is removed typically by means of a rotary evaporator yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded after extraction running with all the selected solvents.

Physical properties of sample

The physical properties of sample, like consistency, colour and percentage yield of extract were recorded.

Qualitative Phytochemical analysis[8,9]

1. **Phenols**
   Two ml of plant extract was taken in a test tube and add 1% lead acetate solution. Formation of white precipitate indicates the presence of phenolic compounds.
2. **Flavonoids**
   Two ml of plant extract was treated with 2ml of 10% Lead acetate solution. Appearance of yellowish green colour indicated the presence of flavonoid.
3. **Alkaloids**
   (Wagner’s reagent): Take 2 ml of plant extract and add 2ml Wagner’s reagent. Test tubes were observed for the appearance of reddish brown precipitate
4. **Tannins**
   About 0.5 g of plant extract was boiled in 20 ml of distilled water in a test tube and then filtered. 1ml of the leaf extract added with 5 % FeCl3 (1 ml) was added to the filtrate. Appearance of brownish green coloration showed the presence of tannins.
5. **Glycosides**
   In 5ml plant extract, 2ml glacial acetic acid, one drop of 5% FeCl3 and conc. H2SO4 were added. Brown ring appears, indicating the presence of glycosides.

6. **Quinones**
   Two ml of each extract of plant was mixed with 3 or 4 drops of concentrated HCl. A yellow colour precipitate indicates the presence of quinones.
7. **Saponins**
   To about 1ml of each extract was added to 2ml of distilled water in a test tube and shaken vigorously with few drops of olive oil. Foam which persisted was taken as an evidence for the presence of saponins.
8. **Steroids**
   One ml of extract was dissolved in 10ml of chloroform and equal volume of concentrated sulphuric acid was added by the sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicates the presence of steroids.
9. **Terpenoids**
   2ml of each extract of plant samples was mixed with 2ml of chloroform .Then allow evaporating and adding 2ml of concentrated sulfuric acid, then heat for 2 minutes. Reddish brown colour indicates the presence of terpenoids.
10. **Fatty Acids**
    Plant extract (0.5 ml) was added to 5ml of ether and allowed it to evaporate on filter paper. Then the filter paper was dried and the appearance of transparency on filter paper is the indication of presence of fatty acids.

Specifications of Instruments used in this study:

Soxhlet : Heating Mantle Rotek 23148, B & C Industries, 300W, 230V**.**
Water bath: 1990 RotekPlus Cat No: PSW-07.
Hot air oven: Rotek 2333 B & C Industries Cat No: RHOM-120

IN VITRO ANTICANCER EFFECT DETERMINATION BY MTT ASSAY[18,19]

MCF-7 (Human Breast Cancer) cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1mg of sample was weighed and dissolved in 1ml 0.1% DMSO using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Anticancer Evaluation:

After 24 hours the growth medium was removed, freshly prepared each compounds were added at concentrations of 100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml of DMEM. Each concentration were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Untreated control cells were also maintained.

Anticancer Assay by Direct Microscopic observation:

Entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Anticancer Assay by MTT Method[3,4]:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization..

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).

RESULT AND DISCUSSION:

Extraction Yield

Sample code	Solvent used (500ml)	Weight of sample (g)	Weight of extract (g)
CML	Petroleum Ether	50	0.17
	Hexane	50	2.43
	Ethanol	50	5.81
	Aqueous	50	4.04

Physical properties of sample

The physical properties of sample, like consistency, colour and percentage yield of extract were recorded.

Sample code	Solvent used	Consistency	Extract colour	Yield (%)
CML	Petroleum Ether	Sticky	Green	0.34
	Hexane	Sticky	Black	4.86
	Ethanol	Sticky	Dark Brown	11.62
	Aqueous	Sticky	Brown	8.08

Phytochemical Analysis

Name of secondary metabolites	Name of test	Observation
Phenol	Lead acetate Test	Formation of whiteprecipitate
Tannin	Ferric chloride Test	Formation of brown precipitate
Flavonoid	Ferric chloride Test	Formation of yellowish green color
Saponin	Foam Test	Persistant foam
Terpenoid	Liebermann-Burchard Test	Formation of reddish brown color
Alkaloid	Wagner’s Reagent	Appearance of reddish brown precipitate
Glycoside	Kellar-Killani Test	Formation of brown ring
Quinones	HCl method	Appearance of yellow precipitate
Steroid	Liebermann-Burchard Test	Upper reddish layer and greenish yellow acid layer
Fatty acid	Filter paper Test	Appearance of transparency in filter paper

Sl. No:	Name of test	Sample Code- CML			
		Hexane	PE	EtOH	H_2O
1	Phenol	-	-	+	+
2	Tannin	+	-	-	+
3	Flavonoid	+	-	+	+
4	Saponin	-	-	+	+
5	Terpenoids	+	-	+	+
6	Alkaloid	-	+	-	+
7	Glycoside	-	-	-	+
8	Quinones	+	-	-	+
9	Fatty acid	+	+	+	-
10	Steroid	++	+	+	+

"+" = Positive (Presence of Phytochemical detected qualitatively)
" –" = Negative (Presence of Phytochemical not detected qualitatively)

In vitro study

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

Sample Concentration (µg/ml)	OD I	OD II	OD III	Average Absorbance @ 540nm	Percentage Viability
control	0.6128	0.6036	0.6174	0.6113	100.00
SAMPLE CODE- CMLE					
6.25	0.5954	0.5824	0.5847	0.5875	96.11
12.5	0.5355	0.5218	0.5237	0.5270	86.21
25	0.4614	0.4523	0.4519	0.4552	74.46
50	0.3351	0.3236	0.3246	0.3278	53.62
100	0.2064	0.2152	0.2134	0.2117	34.63

LC50 Value of Sample (CMLE): 59.5248 µg/mL (Calculated using ED50 PLUS V1.0 Software)

STATISTICAL ANALYSIS

Treatment	% Viability
Control	100
6.25	94.70 ±1.27
12.5	86.22 ± 1.30
25	74.48 ± 1.22
50	53.62 ± 1.05
100	34.64 ± 0.99

Cell line - MCF-7

SAMPLE CODE- CMLE

	OD1	OD2	OD3	Percentage viability 1	Percentage viability 2	Percentage viability 3	Average	Stdev	Std error
Control	0.6128	0.6036	0.6174	100	100	100	100	0	0
6.25	0.5954	0.5824	0.5847	97.1606	96.4877	94.7036	96.1173	1.26969	0.73305
12.5	0.5355	0.5218	0.5237	87.3858	86.448	84.8235	86.2191	1.29641	0.74848
25	0.4614	0.4523	0.4519	75.2937	74.9337	73.194	74.4738	1.12286	0.64828
50	0.3351	0.3236	0.3246	54.6834	53.6117	52.5753	53.6235	1.0541	0.60859
100	0.2064	0.2152	0.2134	33.6815	35.6528	34.5643	34.6328	0.98743	0.57009

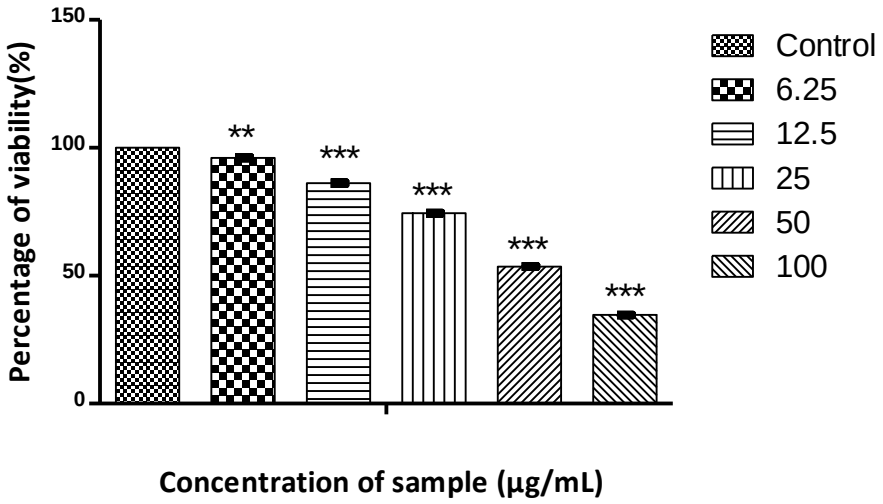


Figure: Graphical representation depicting the anticancer effect of CMLE by MTT assay- Along Y axis Percentage viability, Along X axis varied concentration of CMLE. All experiments were done in triplicates and results represented as Mean+/- SE. One-way ANOVA and Dunnets test were performed to analyse data. ***p< 0.001 compared to control group.

DISCUSSION

The study aimed to evaluate the solvent extraction yields, phytochemical content, and anticancer activity of a sample (CML) using various solvents, followed by an in vitro evaluation of its potential as an anticancer agent against the MCF-7 breast cancer cell line.

Extraction and Yield Analysis

The weight of the extract varied significantly depending on the solvent used. Ethanol provided the highest yield (5.81g) compared to the other solvents, followed by aqueous (4.04g), hexane (2.43g), and petroleum ether (0.17g). These results align with the nature of ethanol, which is a polar solvent capable of extracting a wider range of bioactive compounds, including alkaloids, flavonoids, and phenols, compared to non-polar solvents like petroleum ether.

The physical properties of the extracts also varied. All extracts exhibited a sticky consistency, with ethanol yielding a dark brown extract, while aqueous and hexane extracts appeared brown and black, respectively. The variation in color can be attributed to the different classes of compounds extracted by each solvent, as some phytochemicals may have specific color profiles. Notably, the percentage yields of extracts ranged from 0.34% for petroleum ether to 11.62% for ethanol, which reflects the solvent's efficiency in extracting phytochemicals.

Phytochemical Analysis

The qualitative phytochemical tests revealed the presence of various secondary metabolites, with notable variations across different solvents. For instance, phenols were detected in the ethanol and aqueous extracts, while tannins were present in hexane and aqueous extracts. Alkaloids, saponins, and terpenoids were found in ethanol and aqueous extracts, while flavonoids were present in hexane and ethanol, indicating their potential therapeutic properties.

Interestingly, glycosides were only present in the aqueous extract, suggesting that this solvent might be particularly effective for extracting water-soluble compounds. The presence of fatty acids in all extracts except the aqueous one is consistent with the non-polar nature of these compounds, which are better solubilized in organic solvents like hexane and petroleum ether.

Anticancer Activity

The anticancer activity of the CML extract was evaluated using the MTT assay on MCF-7 human breast cancer cells. The results showed a concentration-dependent decrease in cell viability across all concentrations of the CML extract, with an LC50 value of 59.52 µg/mL. The cytotoxic effects became more prominent at higher concentrations (50 µg/mL and 100 µg/mL), with a 53.62% and 34.64% reduction in cell viability, respectively. These results suggest that the CML extract possesses significant anticancer potential, which may be attributed to the bioactive compounds present in the extract, such as flavonoids, alkaloids, and terpenoids, which have previously been linked to anticancer activity.

The microscopic observations revealed morphological changes such as cell rounding, shrinking, and granulation, further supporting the extract's cytotoxic effects. The presence of various phytochemicals, particularly those with known anticancer properties (e.g., flavonoids and terpenoids), could explain these observed effects.

Statistical Analysis

The percentage viability of the cells was calculated, and the results were analyzed using one-way ANOVA and Dunnets test, with statistical significance observed at higher concentrations of the extract ($p<0.001$). This supports the effectiveness of the CML extract in inhibiting the growth of MCF-7 cells, highlighting its potential for further exploration in cancer therapy.

CONCLUSION

This study demonstrates that the CML extract, particularly when extracted with ethanol, exhibits significant anticancer potential against the MCF-7 breast cancer cell line. The varying yield and phytochemical composition across different solvents suggest that ethanol is the most effective solvent for extracting bioactive compounds. The results also highlight the importance of further exploring the individual compounds present in the extract to better understand their contributions to the observed anticancer effects. Further in vivo studies and mechanistic investigations are warranted to confirm the therapeutic potential of CML in cancer treatment.

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