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Review article *Pharmacology*

Anticancer potential of isolated fractions of muntingia calabura l.using HT-29 cell line

Vinjavarapu. L. Anusha[1], D. Pravallika*[2], T. Bhargavi, B. Pavani[2], SK. Manisha[2], R. Bhavni[2]

[1]*Associate. Professor, Department of Pharmacology, SIMS college of Pharmacy, Mangaldas Nagar, Guntur, Andhra Pradesh, India.*
[2]*Department of Pharmacology, SIMS College of Pharmacy, Mangaldas Nagar, Guntur, Andhra Pradesh,India.*

Address of Correspondence: D. Pravallika
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ABSTRACT

Cancer is a large family of diseases that involve abnormal cell growth with the potential to invade or spread to the other parts. A neoplasm or tumor is a group of cells that have undergoneunregulated growth and will often form a mass or lump, but be distributed diffusely. To extract the leaves of *Muntingia calibra L.* using chloroform by cold maceration method. To isolate the phytoconstituents with column chromatography technique, and evaluate the anticancer activity of isolated fractions on HT-29 cell line using MTT assay method. Perturbing factor must first be investigated via a cell viability assay. The viability assay most commonly used throughout the world is the MTT assay, first described by Tim Mossman in 1983. This colorimetric assay uses reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity as a proxy for cell viability. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. Formazan crystals are then dissolved using a solubilising solution and absorbance is measured at 500- 600 nanometers using a plate-reader. The darker the solution, the greater the number of viable, metabolically active cells. Each condition should be done in triplicate or more. Phytochemical constituents of carbohydrates, proteins, glycosides, cardiacglycosides terpenoids, saponins, flavonoids and phenolic compound are present in the chloroform leaf extract. Three compounds re isolated from chloroform leaf extract of *Muntingia calubra L.* by using column chromatography. The isolated fractions F3M, F5MF8M have anticancer activity among these fractions F3M have more potent anti-cancer activity on HT-29 cell lines.

Keywords: Cancer, HT-29 cell line, MTT

INTRODUCTION

Cancer is a large family of diseases that involve abnormal cell growth with the potential to invade or spread to the other parts. A neoplasm or tumor is a group of cells that have undergoneunregulated growth and will often form a mass or lump, but be distributed diffusely.

Local symptoms

The common local symptoms of cancer include: cough or pneumonia, narrowing of esophagus, blockage in the bowel. Some cancers can cause a build up of fluid within the chest orabdomen.

Systemic symptoms

The common systemic symptoms of cancer include: unintentional weight loss, fever, excessive fatigue and changes to the skin. appearance of myasthenia gravis in thymoma and clubbing in lung cancer.

Metastasis: Cancer can spread from its original site by local spread to regional lymph nodes or by hematogenous spread via the blood to distant sites, known as metastasis.

CAUSES

Genetic mutations from environmental factors: 90-95%
Inherited genetics environmental: 5-10%

CLASSIFICATION OF CANCER

Cancers are classified by the type of cell that the tumor cell resembles and is therefore presumed to be origin of the tumor.theses types include,

>Carcinoma
>Sarcoma
>Lymphoma and leukemia
>Germ cell tumor
>Blastoma

How cancer is treated

Surgery: directly removing the tumor.
Chemotherapy: using chemicals to kill the cancer cells.

PLANT PROFILE

Muntingia is a genus of plant in the family *muntingiaceae,* comprising only one species, and *Muntingia calabura*. it is a native to the neotropics, from mexico south to Bolivia.

SCIENTIFIC CLASSIFICATION

Kingdom	:	Plantae
Angiosperms	:	Clade
Order	:	Malvales
Family	:	Muntingiaceae
Genus	:	Muntingia
Species	:	*Muntingia calabura*

Traditional medicinal uses

Lowering blood sugar
Preventing cancer
Lowering blood pressure and blocking pain.

Description

Muntingia calabura is a shrub or tree up to 12m tall with spreading branches. The leaves are alternate, distichous or lanceolate, 4-15cm long and 1-6cm wide, with toothed margins and covered in short hairs. The flowers are small up to 3 cm wide, solitary or in inflorescences of 2-3 flowers, with 5 lanceolate sepals, hairy, 5obovate white petals.

Distribution and habitat

M.calabura is native to southern mexico, the caribbean, central america, and western south america to Bolivia and Argentina. (58,59,61) it is present in tropical climate in disturbed low land areas from sea level to 1000 cm of elevation.

Ecology

This species colonized disturbed habitats in tropical lowland areas, becoming part of the secondary vegetation, as well as gallery forests. It thrives in poor soil, able to tolerate acidic and alkaline conditions and drought.

AIM AND OBJECTIVES

To extract the leaves of *Muntingia calibra L.*using chloroform by cold maceration method. To isolate the phytoconstituents with column chromatography technique, and evaluate the anticancer activity of isolated fractions on HT-29 cell line using MTT assay method.

MATERIALS AND METHOD

Plant authentication, collection and extraction

Muntingia calabura leaves are collected in the month of january gunu P. The plant was identified and authenticated by their botany, Dr. Sandhya M.phil. Ph.D. SIMS College of College Life Science, mangaldas nagar guntur. After that they are shade dried and powdered using mechanical grinder and passed through sieve mesh 40.powder (100g) was defatted using 500ml ethanal for 12h extractions were concentrated and controlled temperature (40-50c)three extracts were stored in tightly closed container in refrigerator and were screened for phytochemicals and pharmacological activity.

Phytochemical studies

Concentrated crude extracts were dissolved in the same solvent to determine the major phytochemicals by following the methods described earlier.
Test for glycosides
Test for flavonoids
Test for saponins
Test for tannins
Test for terpenoids

Column chromatography

Column chromatography is frequently used by organic chemists to purify liquids (and solids.) Animpure sample is loaded onto a column of adsorbent, such as silica gel or

alumina. An organic solvent or a mixture of solvents (the eluent) flows down through the column. Components of the sample separate from each other by partitioning between the stationary packing material (silica or alumina) and the mobile eluent. Molecules with different polarity partition to different extents, and therefore move through the column at different rates. The eluent is collected in fractions. Fractions are typically analyzed by thin layer chromatography to see if separation of the components was successful.

1. First we used a piece of wire to add a plug of cotton to the bottom of the column. Enough cotton is used so that the silica will not fall out of the column. However, too much cotton or cotton packed too tightly will prevent the eluent from dripping at an acceptable rate.
2. Clamp the column to a ring stand.
3. We placed a pinch clamp on the tubing, and then fill the column 1/4 to 1/3 full with the initial eluent. (The composition of eluent is often changed as the separation proceeds.) Then we prepared slurry of silica in the initial eluent by pouring dry silica into a beaker of eluent. (Add a volume of silica gel, such as 20 ml, to approximately double the volume of eluent, 40 ml.) CAUTION: keep the dry silica in your hood and be careful not to inhale the lightweight substance. Quickly but carefully pour the slurry into the column. Stir and pour immediately to maximize the amount of silica that goes into the column instead of remaining behind in the beaker. You may find a clean spatula or glass rod helpful in transferring the silica.
4. Then we removed the pinch clamp to allow solvent to drip into a clean flask and tap on the side of the column with a rubber stopper or tubing to help the silica settle uniformly.
5. Pasteur pipette is used to rinse any silica that is sticking to the sides of the column. Allow the silica to settle while eluent continues to drip into the flask.
6. Loading a sample onto the column:
7. Once the silica has settled, we carefully added dry silica gel to the top of the column. Sample is added to the column.
8. Drain eluent from the column until no sample remains above the surface of the silica.
9. Use-1 ml of cluent to rinse your container and pipette
10. Eluting the sample
11. We repeated step 12 two or three times to completely transfer your sample onto the silica gel.
12. Analysing the fractions: Analyse the fractions by thin-layer chromatography to determine if the fraction contains more than one component and b) if fractions can be Combined without affecting the purity of those fractions.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert backing. It may be performed on the analytical scale as a means of monitoring the progress of a reaction, or on the preparative scale to purify small amounts of a compound. TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity. and speed of separation. TLC functions on the same principle as all chromatography: a compound will have different affinities for the mobile and stationary phases, and this affects the speed at which it migrates. The goal of TLC is to obtain well defined, well separated spots.

RESULTS AND DISCUSSION

INVITRO PHARMACOLOGICAL SCREENING MTT ASSAY METHOD

Perturbing factor must first be investigated via a cell viability assay. The viability assay most commonly used throughout the world is the MTT assay, first described by Tim Mossman in 1983. This colorimetric assay uses reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity as a proxy for cell viability. Viable cells contain NAD(P) H-dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple color. Formazan crystals are then dissolved using a solubilising solution and absorbance is measured at 500-600 nanometers using a plate-reader. The darker the solution, the greater the number of viable, metabolically active cells. Procedure: Short 96 well assay: EACH condition should be done in triplicate or more.

1. DAY ONE: Trypsinized one T-25 flask and add 5 ml of complete media to trypsinized cells. Centrifuge in a sterile 15 ml falcon tube at 500 rpm in the swinging bucket rotor (~400 x g) for 5 min.
2. Remove media and resuspend cells to 1.0 ml with complete media.
3. Count and record cells per ml. Remember to remove the cells aseptically when counting.
4. DILUTE the cells (CV-CV) to 75,000 cells per ml. Use complete media to dilute cells.
5. Add 100 µl of cells (7500 total cells) into each well and incubate overnight.
6. DAY TWO: Treat cells on day two with agonist, inhibitor or drug. If removing media, do it very carefully. This is where most variation in data may occur. - Final volume should be 100 µl per well.
7. DAY THREE: Add 20 µl of 5 mg/ml MTT to each well, Include one set of wells with MTT but no cells (control). All should be done aseptically.
8. Incubate for 3.5 hours at 370C in culture hood.
9. Carefully remove media. Do not disturb cells and do not rinse with PBS.
10. Add 150 µl MTT solvent.
11. Cover with tinfoil and agitate cells on an orbital shaker for 15 min.
12. Read absorbance at590nm with a reference filter of 620nm. storage: the MTT reagent must be kept at 4°in the dark.

Phytochemical analysis showed the presence of different phytochemicals like flavonoids, cardiac glycosides, saponins, terpenoids, reducing sugars in the extracts.

Table 1: Phyto Constituents

Phytoconstituents	Chemical Test Method
Carbohydrates	+VE
Proteins	+VE
Alkaloids	-VE
Glycosides	+VE
Steroids	-VE
Cardiac glycosides	+VE
Flavonoids	+VE
Terpenoids	+VE
Tannin	-VE
Phenolic compounds	+VE
Saponins	+VE
Anthraquinone	-VE

Table 2: Chromatography results

Fractions	Solvent	Ratio	Colour
F3M	N-hexane:ethylacetate	5:1	Green
F5M	N-hexane :ethylacetate	1:5	Dark green
F8M	Ethyl acetate:methanol	10:1	Dark green

Table 3: Thin layer chromatography results

S.No.	Samplecode	Sample color	TLC samplecolor	TLC mobile phasesolvent ratio	Rf value
1.	F3M	Green	Red color	5:1/n-hex:et.ac	0.60
2.	F5M	Dark green	Red color	1:5/n-hex:et:ac	0.97
3.	F8M	Dark green	Red color	10:1/et.ac:meoh	1

MTT ASSAY

Principle:

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogenous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS). The MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles.

CELL CULTURE AND MTT ASSAY

The Colon cancer cell line (SW620) were plated separately using 96 well plates with the concentration of 1×10 cells/well in DMEM media with IX Antibiotic Antimycotic Solution and 10% fetal bovine serum (Himedia, India) in CO2 incubator at 37°C with 5% CO2. The cells were washed with 200 µl of IX PBS, and then the cells were treated with various test concentrations of compound in serum free media and incubated for 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5mg/ml MTT prepared in IX PBS was added and incubated at 37°C for 4 h using CO2 incubator. After the incubation period, the medium containing MTT was discarded from the cells and washed using 200 µl of PBS. The formed crystals was dissolved with 100 µl of DMSO and thoroughly mixed. The development of color intensity was evaluated at 570 nm. The formazan dye turns to purple blue color. The absorbance was measured at 570 nm using a microplate reader.

CONCLUSION

Phytochemical constituents of carbohydrates, proteins, glycosides, cardiacglycosides terpenoids, saponins, flavonoids and phenolic compound are present in the chloroform leaf extract. Three compounds re isolated from chloroform leaf extract of *Muntingia calubra L.* by using column chromatography. The isolated fractions F3M, F5MF8M have anticancer activity among these fractions F3M have more potent anti-cancer activity on HT-29 cell lines.

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