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Evaluation of Phytochemical Analysis, Antioxidant, Anti Inflammatory and Antimicrobial Activities of Acalypha Indica L (Leaf) and Its Cytotoxic Effect on MCF-7 (Breast Cancer) Cell Lines

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Abstract- Breast cancer has emerged as the most common female cancer in developing countries. Each year 10.9 million people suffer from breast cancer worldwide that result in 6.7 million deaths from the disease. A recent study of breast cancer risk in India revealed that 1 in 28 women develop breast cancer during her lifetime. Breast cancer is a disease in which certain cells in the breast become abnormal and multiply without control or order to form a tumor. In some cases, cancerous tumors can invade surrounding tissue and spread to other parts of the body often in the bones, liver, lungs, or brain.

Acalyphaindica (Euphorbiaceae) is used as a medicinal plant and occurs throughout tropical Africa South Africa, India and Sri Lanka. The active medicinal compounds like Acalyphine and Triacetoneamine are extracted from this plant along with cyanogenic glucoside and alkaloids. The pharmacological properties of Leaf extracts of *Acalyphaindica* are anti-cancerous, anti-inflammatory, anti-helmintic, anti-bacterial, anti-fungal, anti-tubercular, ant-ioxidant, anti-molluscicidal, neuro-protective, neuro-theurapeautic, anti-venom and antiulcer activity. The leaf extract compounds appear to be non-polar in nature as they were extracted into non-polar solvents like hexane, ethyl acetate, ethanol and methanol. Further studies, however, are needed to identify the specific molecules involved in the inhibition of these enzymes and other receptors in breast cancer. Hence the present study is taken to systematically evaluate the anti-cancer properties of the pure extracts and also to isolate and characterize the active principles of *Acalyphaindica*.

The crude extracts of whole plant using different organic solvents will be screened for their anti-inflammatory and cytotoxic activity. This kind of assessment is further helpful for identification of active molecule and effective drug designing. In the present study, an attempt is being made to enrich the knowledge of anti-cancer activity of plant extracts from Acalyphaindica L. on human breast cancer cell lines MCF-7.

I. INTRODUCTION

Acalyphaindica (English: Indian acalypha, Indian nettle, three-seeded mercury) is an erect, simple or branched, slightly hairy annual herb. *Acalyphaindica* is a species of plant having catkin type of inflorescence. It occurs throughout tropical Africa and South Africa, in India and Sri Lanka, as well as in Yemen and Pakistan. It has possibly been introduced elsewhere as a weed. In West and East Africa the plant is used as a medicinal plant. It is a common herb growing up to 40-75 cm tall with ovate leaves. Flowers are green, unisexual found in catkin inflorescence. In West Africa the leaves are cooked and eaten as a vegetable. It is also browsed by cattle. This plant is held in high esteem in traditional Siddha medicine as it is believed to rejuvenate the body.

Figure 1: *Acalyphaindica* plant

Medicinal value of *Acalyphaindica*

Medicinal plants represent rich sources of antimicrobial agents. In recent years; multiple drug resistance has developed in many microbes, which has resulted in search for new antibiotic sources [1]. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. A wide range of medicinal plant parts is used for extracts as raw drugs and they possess varied medicinal properties. The plant part used include root, stem, flower, fruit twigs exudates and modified plant organs. While some of these raw drugs are collected in smaller quantities by communities and folk healers for local used, many other raw drugs are collected in larger quantities and traded in the market as the raw material for many herbal industries. Medicinal plants have been of age long remedies for human diseases because they contain components of therapeutic value. Some of them are also used for prophylactic purposes. Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products and used in the traditional systems of medicine. Approximately 20% of the

plants found in the world have been submitted to pharmaceutical or biological test and a sustainable number of new antibiotics introduced on the market are obtained from natural or semi synthetic resources it has been reported that between the year 1983 and 1994. The systematic screening of antibacterial plants extracts represents a continuous effort to find new compounds with the potential to act against multi resistant bacteria.

Methanol extract of the whole plant has potential analgesic and anti-inflammatory actions in rats and mice [3] [10] [13]. The Root extracts of *AcalyphaIndica* Linn have potential Nitric Oxide Scavenging Activity [4]. The ethanol, chloroform and hexane extracts of *Acalyphaindica* were known for inhibition of the α-amylase activity [5].

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. Medicinal plants are of great importance to the health of individuals and communities in general. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. Many of the indigenous medicinal plants are used as spices.

It has been reported to be useful in treating pneumonia, asthma, rheumatism and several other ailments. The dried leaves of *Acalyphaindica* was made into a poultice to treat bedsores and wounds and the juice of *Acalyphaindica* is added to oil or lime and used to treat a variety of skin disorders. The leaves of *Acalyphagrandis* have also been reported to possess contraceptive activity. Several chemical and biological investigations have been carried out on this plant. The *Acalyphaindica* root is prescribed as a tonic, astringent, febrifuge and strong purgative. The leaves extract reduced mutagenecity in *E. coli*. Extract of the root bark with alcohol can be used externally as emollient; a poultice is used for chilblains, in insect bites, swelling rheumatism and facial paralysis. Leaves possess anti-periodic and laxative properties; the leaves are used in jaundice, piles, rheumatism ulcers and also externally skin eruptions, ring worms, eczema. The leaves extract are applied to pustules, insect bites. The roots are used in chest pain, joint pain, and migraine and blood dysentery and the extract of the root lowered the blood sugar level up to 30%. A decoction of the leaves is giving in earache.

The juice extracted from the leaves, mixed with lime and applied on skin to cure diseases caused by Ringworm. Fresh juice of leaves mixed with oil and salt is used for Rheumatoid arthritis and to cure Scabies. Powdered leaves are used to cure bedsores and infected wounds. The active medicinal compounds like Acalyphine and Triacetoneamine are extracted from this plant. They contain cyanogenic glucoside and alkaloids. The paste of the leaves can be applied to burns. Results of leaf extract of *A. indica* showed promising larvicidal and ovicidal activity against malaria vector *A. stephensi*. Results of water extract study showed *A. indica* hasneuroprotective and neurotherapeutic effects exvivo on m. gastrocnemius frog. The ethanolic extracts of *Acalyphaindica* were evaluated for their wound healing activity in rat. The acetone and ethanol extracts were found to be effective in causing significant antimicrobial activity. In medicine, diuretics are used to treat heart failure, liver cirrhosis, hypertension, water poisoning, and certain kidney diseases. Some diuretics, such as acetazolamide, help to make the urine more alkaline and are helpful in increasing excretion of substances such as aspirin in cases of overdose or poisoning. *Acalyphaindica* is a natural diuretic agent [15]. Whole plant extract of *Acalyphaindica* has capacity of Post-coital antifertility activity (can be used as a contraceptive). The petroleum ether and ethanol extracts were found to be most effective in causing significant anti-implantation activity [14].

The plant contains kaempferol, a cyanogenetic glycoside, a base triacetonamine and an alkaloid, acalyphine. It is also contains the amide, acalyphamide and some other amides, 2-methyl athraquinone, tri-O-methyl elegiac acid and γ-sistosterol, β-sistosterol, glucoside, stigmaterol, n-octacosanol, quinine, tannin, resin and essential oil.

Uses in traditional medicine

It is use as a purgative for which purpose the plant is boiled and the extract drunk. It is a very good remedy in the treatment of piles. Root is used to remove worms in children, and given in the morning empty stomach work as mild laxative and also remove worms. Leaves of this with turmeric can give relief from acne and pimples.

***Acalyphaindica* in Homeopathy**

It is indicated in incipient phthisis, with hard, racking cough, bloody expectoration, arterial hemorrhage, but no febrile disturbance. Leaf extracts of this plant can relieve from weakness and headache.

***Acalyphaindica* in Ayurveda**

Preparations - Infusion of root, powder, decoction, cataplasm, succus (juice expressed), tincture and liquid extract. Uses Leaves possess laxative properties; “are used as a substitute for senega”; are used in the form of powder or decoction; mixed with garlic they are used as Anthelmintic in worms. Mixed with garlic they are applied to scabies; and their juice mixed with oil forms an application in rheumatic arthritis. Expressed juice of the leaves is a safe, certain and speedy emetic for children in one teaspoonful (I drachm) doses, in cases of croup; in smaller doses it is expectorant, and is useful in chronic bronchitis, asthma and constipation.

Anti-bacterial and anti-fungal activity

The ethanol extract of *Acalyphaindica*has maximum inhibition against *Bacillus cereus, Bacillus subtilis, Escherichia coli, Salmonella typhi, Vibrio cholera* and *Pseudomonas aeruginosa* but proved to be resistant against *Pseudomonas aeruginosa, Shigellaflexneri, Staphylococcus aureus, Klebsiella pneumoniae, Vibrio cholerae* and *Bacillus cereus*. The ethyl acetate extract of *Acalyphaindica* has maximum inhibition against *Staphylococcus aureus, Klebsiella pneumoniae* and *Shigellaflexneri* ethyl acetate was resistant to *Vibirocholerae* and *Bacillus cereus*. *Pseudomonas aeruginosa* was resistant to ethyl acetate extract of *Acalyphaindica*. Studies proved that ethanol and water extract of leaves, stems, seeds and roots from *Acalyphaindica* were effective against two bacterial *Escherichia coli* (Gram-negative bacteria), *Staphylococcus aureus* (Gram-positive bacteria) and for anti-

fungal activity against three fungi, *Aspergillus fumigatus*, *Microsporumcanis* (molds) and *Candida albican* (yeast). *Microsporumcanis* showed dose-dependent sensitivity towards aqueous leaves and roots extract, but resistant to both ethanol and water stems, roots and seeds extracts. *Aspergillus fumigatus* and *Candida albican* were resistant to both ethanol and water extract of all *Acalyphaindica*. Vasoconstriction is the narrowing of the blood vessels resulting from contraction of the muscular wall of the vessels, in particular the large arteries and small arterioles, which is particularly important in staunching hemorrhage and acute blood loss. When blood vessels constrict, the flow of blood is restricted or decreased, thus retaining body heat or increasing vascular resistance. Leaf extracts of *Acalyphaindica* has natural vasoconstrictor activity [12]. A nosocomial infection is an infection whose development is favored by a hospital environment, such as one acquired by a patient during a hospital visit or one developing among hospital staff. Acaricides are used both in medicine and agriculture, although the desired selective toxicity differs between the two fields.

Acalyphaindica has a caricidal activity [7]. This herb has anti-cancerous activity against various types of cancers [9] [10].Other properties of the herb include anti-inflammatory, anti-helmintic [8], anti-bacterial [6], anti-fungal [16], anti-oxidant [9], neuro-protective, anti-venom and antiulcer activity

Novelty Importance in the context of current status

The present proposed study may contribute for understanding the effect of the plant based drugs as anti-apoptotic and cytotoxic agents on the breast cancer. In view of rising incidence of breast cancer among and drug resistance by the women this would be a new path to the discovery of more effective therapeutic drugs. The outcome of the study may help in clinical management and in the development of target based therapy of breast cancer.

Breast cancer statistics

Breast cancer is the most common cancer in women worldwide, with nearly 1.7 millon new cases diagnosed in 2012 (second most common cancer overall). This represents about 12% of all new cancer cases and 25% of all cancers in women.

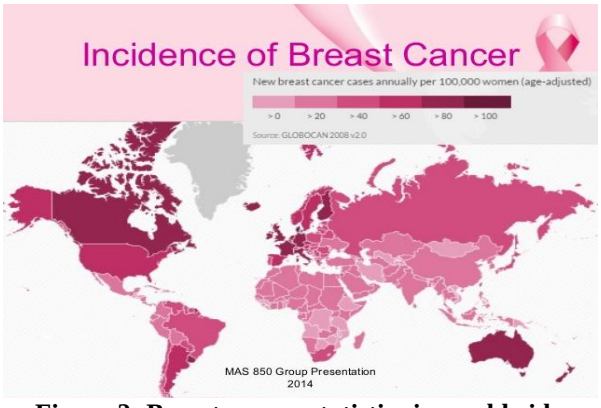


Figure 2: Breast cancer statistics in worldwide

Belgium had the highest rate of breast cancer, followed by Denmark and France. Slightly more cases of breast cancer were diagnosed in less developed countries (53%). The highest incidence of breast cancer was in Northern America and Oceana; and the lowest incidence in Asia and Africa. Coming to Indian statistics for breast cancer, about 1.3 lakh fresh cases of breast cancer are reported every year whereas 54,000 were counted a decade ago.

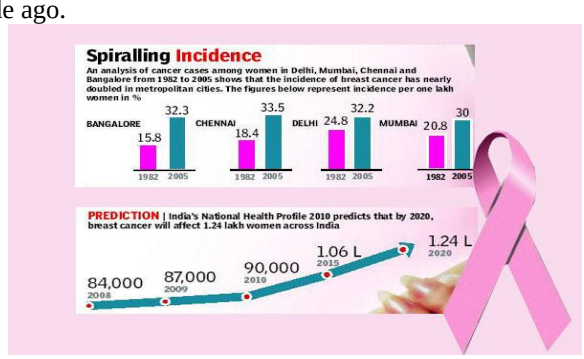


Figure 3: Breast cancer statistics in India

Aim & Objectives

1. To obtain pure plant leaf powder and obtaining the leaf extracts using soxhlet apparatus with different organic solvents.
2. To perform phytochemical analysis for different solvent extracts and list out the types of phytochemicals have actively dissolved in different organic solvents.
3. To perform anti-oxidant activity of different solvent extracts
4. To perform the anti-inflammatory activity of different solvent extracts.
5. To perform rotor vapor for different solvent extracts and to obtain crude extract.
6. To perform anti-microbial activity of different crude extracts.
7. To perform Cell Culture and Cytotoxic Assay (MTT Assay) by crude extracts.

II. MATERIALS AND METHODS

- ***Materials Required***

Materials required for Soxhlet Extraction

Plant powder, Soxhlet apparatus (condenser, extraction chamber and round bottom flask), Solvents (Methanol, Ethyl Acetate, Petroleum Ether and Hexane), Cotton, Pipes, Heater and Glass bottles

Materials required for phytochemical screening

Equipments Required: Water Heater

1. Detection of alkaloids

Test Tubes, Test Tube stand, Plant Extract, Micropipette, Micro Tips, Dilute Hydrochloric Acid, Mayer's Reagent (Potassium Mercuric Iodide), Wagner's Reagent (Iodine in Potassium Iodide).

2. Detection of carbohydrates

Test Tubes, Test Tube Stand, Plant Extract, Micropipette, Micro Tips, Distilled Water, Dilute Hydrochloric Acid, Benedict's reagent, Alkali, Fehling's A & B solutions.

3. Detection of Phytosterols

Test Tubes, Test Tube stand, Plant Extract, Micropipette, Micro Tips, Chloroform, Conc. Sulphuric acid

4. Detection of phenols
Test Tubes, Test Tube Stand, Plant Extract, Micropipette, Micro Tips, Ferric Chloride Solution
5. Detection of flavonoids
Test Tubes, Test Tube Stand, Plant Extract, Micropipette, Micro Tips, Sodium Hydroxide Solution, Dilute Acid,Lead Acetate Solution
6. Detection of proteins and aminoacids
Test Tubes, Test Tube Stand, Plant Extract, Micropipette, Micro Tips, 25% W/V Ninhydrin Reagent
Materials required for Anti-Oxidant Activity
Test Tubes, Test Tube Stand, Plant Extract, Micropipette, Micro Tips, 0.2M Phosphate Buffer (Ph 6.6), 1% Potassium Ferricynide, Eppendorf Tubes, Centrifuge Tubes, Solvents (Methanol, Ethyl Acetate, Petroleum Ether and Hexane), TCA (Trichloroaceticacid), Distilled Water, $Fecl_3$
Equipments Required: Water Heater, Centrifuge and Spectrophotometer.
Materials required for Rotary Evaporator
Round bottom flasks, Plant solvent extacts, distilled water.
Equipments Required: Roatary Evaporator
Materials for anti-inflammatory activity
Test Tubes, Test Tube Stand, Plant Extract, Micropipette, Micro Tips, BSA (Bovine Serum Albumin) (0.2%), Phosphate Buffer, Eppendorf Tubes, Centrifuge Tubes Solvents (Methanol, Ethyl Acetate, Petroleum Ether and Hexane), Distilled Water.
Equipments Required: Water Heater, Centrifuge and Spectrophotometer
Materials required for Anti-Microbial Activity
a) Preparation of LB Agar petriplates
LB Media Mix, Agar, Conical Flask, Cotton Plug, Petriplates, Spirit, Cotton, and Paraffin
Equipments Required: Weighing Machine, Autoclave and Laminar Airflow Chamber
b) Inoculation of microbial cultures and crude plant extract
Micropipette, Micro Tips, Crude Compound, Microbial Cultures, Petriplates, Cotton, Gel Puncher, Spreader and Paraffin
Equipments Required: Laminar Airflow Chamber and Biological incubator
Materials required for cell culture and trypsinization
MCF-7 Cell Lines, T-25 Flasks, DMEM Media (Dulbecco's Modified Eagle's Medium), PBS (Phosphate buffered saline), Trypsin, FBS (Fetal Bovine Serum), Antibiotics solution, Centrifuge tubes, Micropipette (100µl and 1ml), Micro Tips(100µl and 1ml).
Equipments Required: Inverted Microscope, Centrifuge and CO_2 Incubator
Materials required for MTT Assay
MTT Reagent, DMSO, PBS, Centrifuge tubes, Micropipette (100µl and 1ml), Micro Tips (100µl and 1ml)
Equipments Required: Microplate Reader, Centrifuge and CO_2 Incubator

- ***Methods***

Collection of Plant Sample
Different plant materials of *Acalyphaindica* were collected and leaves were separated.
The separated leaves were washed in a tray and shade dried for 3-5 days. This shade dried leaves after 3 days were milled to obtain a fine powder. Always shade drying is preferred as shade drying prevents denaturation of important phytochemicals when compared to sun drying. The weight of this fine powder was calculated.

Figure 4: Collection of *Acalyphaindica* plants, shade dried leaves, fine powder (from left to right)

Extraction from the plant powder
This fine powder was subjected to extraction using soxhlet apparatus. Soxhlet apparatus containing condenser, extraction chamber and round bottom flask. About 250ml of solvent was loaded in the round bottom flask. About 10 grams of dried fine plant powder was placed in between cotton in extraction chamber. This whole setup was subjected to continuous extraction for 48 hours. Four different kinds of solvents were used individually for extraction namely methanol, ethyl acetate, petroleum ether and hexane. Liquid plant extract was obtained in the bottom flask. This plant extract obtained from four different solvents were collected in four different glass bottles.

Figure 5: starts extraction using soxhlet apparatus, phytochemicals dissolves in solvent (left to right)

Table 1: different solvents have different boiling points

Solvent	Boiling point (°C)
Methanol	64.7
Ethyl acetate	77
Petroleum ether	70
Hexane	64-67

Figure 6: after 24hrs and 48hrs extraction completed (left to right)

Phytochemical screening:

Phytochemical examinations were carried out for all the extracts as per the standard methods [11].

1. Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered [2]. The following tests were carried out.

a) Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids [2].

b) Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids [2].

2. Detection of carbohydrates: Extracts were dissolved individually in 5ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates [2].

a) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars [2].

b) Fehling's Test: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars [2].

3. Detection of phytosterols

a) Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes [2].

4. Detection of phenols

a) Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols [2].

5. Detection of flavonoids

a) Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids [2].

b) Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids [2].

6. Detection of proteins and aminoacids

a) Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid [2].

Anti-Oxidant Activity:

1. 10 test tubes were taken and to each 2.5ml of 0.2M phosphate buffer (pH 6.6) was added.
2. 2.5ml of 1% potassium ferricynide was added to all test tubes.
3. 10 eppendorf tubes were taken and volumes of 10µl, 20µl to 100µl of plant extract was added to tubes individually and volume was made up to 1ml by adding respective solvent. This makes a total 1ml of plant extract.
4. Then this each 1ml plant extract in 10 different eppendorf tubes were added to respective test tubes and reaction mixture was incubated for 20mins at 50°C.
5. Then 2.5ml of TCA was added to all tubes and centrifuged at 3000rpm for 10 minutes.
6. After centrifuging, 2.5ml of supernatant liquid was collected and 2.5ml of distilled water and 0.5 ml of FeCl3 was added to all test tubes.
7. UV absorbance was recorded at 770nm.

Anti-Microbial Activity:

Collection of crude plant extract

Liquid plant extract was obtained in the bottom flask. This plant extract obtained from four different solvents were collected in four different glass bottles.

The content in each bottle was transferred to individual round bottom flasks and subjected to rotor vapor individually to obtain respective solvent crude extract [ex: methanolic crude extract] etc. Each plant crude extract from different flasks were collected in different tubes by scraping the flask and DMS was added and respective solvent labeled for further analysis of the plant crude extract.

Figure 7: Rotary evaporator used for removal of the solvents in crude extract

Anti-Microbial Activity:

LB Agar medium was prepared by taking a conical flask and dissolving 4 grams of agar and 4 grams of luria-beurtni medium mix in 250ml of distilled water and was subjected to autoclaving at 121^0C temperature and 15lb pressure for 15 minutes. After autoclaving, in each petriplate around 10ml of LB Agar media was poured the petriplates were closed and paraffin was wrapped to each petriplate and media in petriplates were subjected to solidification for about 24 hours.

In the present work four microbial cultures were used for study namely *Bacillus sps E.coli Psuedomonassps* and *Streptococcus sps.* After solidification of media in petriplates, 100µl of each culture was taken and spread plate technique was performed.

Using gelpuncher, in each petriplate 4 wells were punched and into each well respective labeled amount of plant crude (like 10µl, 25µl and 50µl) extract was added. Then the petriplates were closed and sealed using parafilm. These petriplates were subjected to incubation in a biological incubator at 25 degrees centigrade for 48 hours after which results were examined.

Anti Inflammatory Activity:

1. 10 test tubes were taken and 5ml of 0.2 % BSA was added to each.
2. To all the test tubes, 2.5ml of 0.2M phosphate buffer (pH 6.6) was added
3. 10 eppendorf tubes were taken and volumes of 10µl, 20µl to 100µl of plant extract was added to tubes individually and volume was made upto 1ml by adding respective solvent. This makes a total 1ml of plant extract.
4. This 1ml of plant extract was added to all the test tubes.
5. Then the tubes ere heated in water bath for 10-15 minutes at $100^{O}C$
6. O.D. values were taken at 660nm wavelength.

Cell culture and trypsinization

Preparation of complete media for cells

500ml of DMEM media was taken and to this 5ml of antibiotic solution was added. This makes the whole volume to 505ml and from this 45 ml of volume is taken and to this 45ml, 5ml of FBS was added making a total of 50ml of complete media. Plain media is direct usage of DMEM only.

Trypsinization

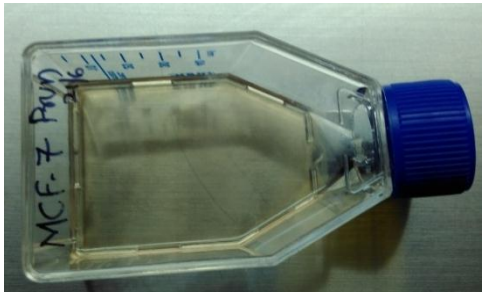


Figure 8: Trypsinization of MCF-7 cells in T25 plask

MCF-7 Cells from mother cultures, which are initially stored at -$80^{o}C$, are sub cultured into T-25 flask with complete media. After 6 hours, the media used by the cells (spent media) is discarded and cells are washed with 3ml of PBS. Then PBS is discarded and 2ml of trypsin is added and the cells in the flask are incubated at $37^{o}C$ in CO_2Incubator for 3 minutes. The cells in the flasks are detached and digestion of extracellular matrix (which holds cells together) is done by trypsinization. Then the cells are visualized under inverted microscope. 2ml of fresh complete media is added and contents in the T-25 flask are transferred into centrifuge tubes and centrifuged at 2000 rpm for 2 minutes. The supernatant (which contains media and trypsin) is discarded. The pellet which contains is washed with 3ml PBS and PBS is discarded. To the Pellet with cells, 3ml of complete media is added.

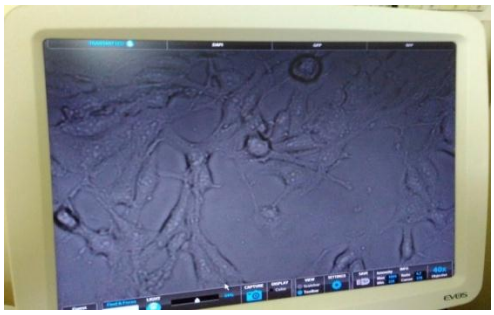


Figure 9: MCF -7 cells in under fluorescence microscope

MTT Assay

To the 96 wellplate, 100µl of media with pellet cells is added to 18 wells (6 wells are working wells, with three for compound addition and three for control and the work is done for triplet hence a total of 18 wells). This was subjected to 6 hours of incubation in CO_2Incubator at $37^{o}C$. Three concentrations of plant extracts 10mg/ml, 25mg/ml and 50mg/ml were prepared by dissolving the crude leaf extract, obtained after rotary evaporator, in PBS.

Figure 10: 1196 – well plate for MTT assay

To the 1^{st} triplet (1^{st} three vertical wells), 100µl of 10mg/ml plant extract was added. Similarly to the 2^{nd} and 3^{rd} triplets 100µl of 25mg/ml and 100µl of 50mg/ml of leaf extract was added. Only PBS was added to the control wells without leaf extracts. Now this 96 wellplate is subjected to 21 hours of incubation in CO_2Incubator at $37^{0}C$. After 21 hours of incubation in CO_2Incubator at $37^{0}C$, 20µlof MTT reagent was added to all the wells was incubated in CO_2Incubator at $37^{0}C$ for 3 hours. The concentration of MTT used is 0.5mg/ml. After 3 hours of incubation, 100µlof DMSO was added to all the wells. O.D values were noted using ELISA reader at 570nm.

Figure 11: Co2 Incubator, ELISA Reader (left →Right)

III. RESULTS AND DISCUSSION

PHYTOCHEMICAL ANALYSIS

Phytochemical analysis was performed using standard protocols. The results are in table 2.

Table 2: Phytochemical analysis

Test	Phyto chemicals	Methanolic extract	Ethyl acetate	Petroleum ether	Hexane extract
Mayer's test	Alkaloids	++	++	+	+++
Wager's test	Alkaloids	++	++	++	++
Benedict's test	Carbohydrates	-	-	-	-
Fehling's test	Carbohydrates	-	-	-	-
Salkowski's test	Phytosterols	-	-	-	-
Ferric chloride test	Phenols	++	-	-	-
Foam test	Saponins	+	++	-	-
Alkaline Reagent Test	Flavanoids	++	-	-	+++
Lead acetate test	Flavanoids	-	+	+	-
Ninhydrin test	Amino acids	+	+++	-	+
- =Absence; + =Presence; ++=Moderate Presence; +++ =More Presence					

Hexane extract

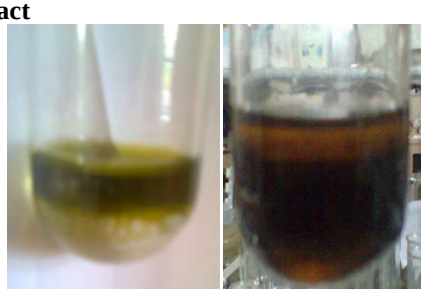


Figure 12: Mayer's test: Cream colored precipitate, Wagner's test: Dark brown precipitate

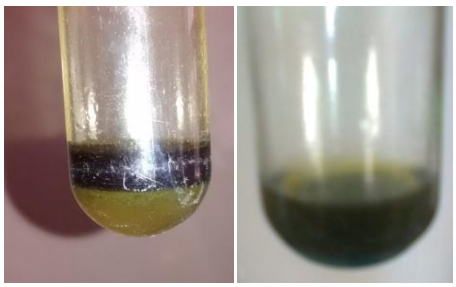


Figure 13: Alkaline test: Yellow Color, Ninhydrin test: Purple Color

Ethyl acetate extract

Figure 14: Mayer's test: Cream precipitate, Wagner's test: Dark brown precipitate

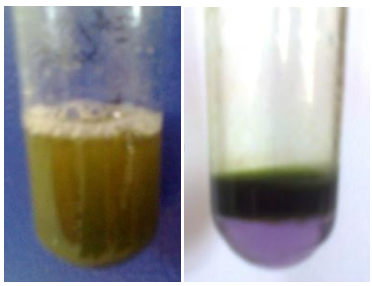


Figure 15: Foam test: Foam Presence, Ninhydrin test: Purple Color

Methanolic extract

Figure 16: Mayer's test: Cream precipitate, Wagner's test: Dark brown precipitate

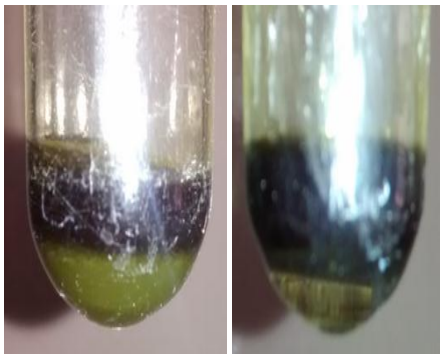


Figure 17: Alkaline test: Yellow Color, Ninhydrin test: Purple Color

Petroleum ether extract

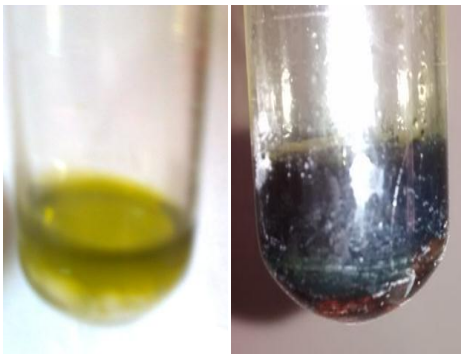


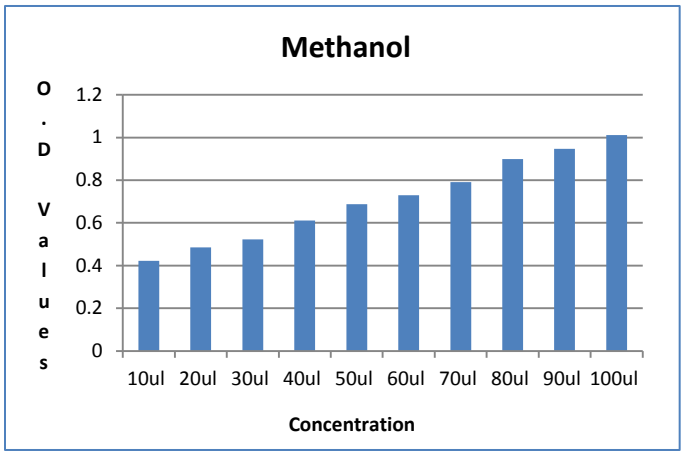
Figure 18: Mayer's test: Cream precipitate, Wagner's test: Dark brown precipitate

Figure 19: Alkaline test: Yellow Color

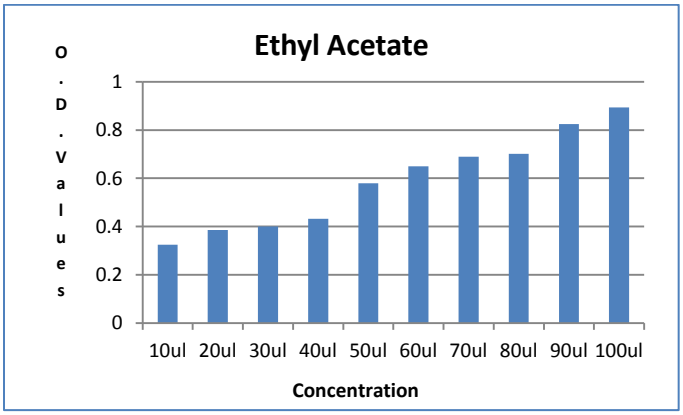
Anti-Oxidant Activity

Table 3: shows the results obtained by performing antioxidant activity with plant extract obtained from respective solvent

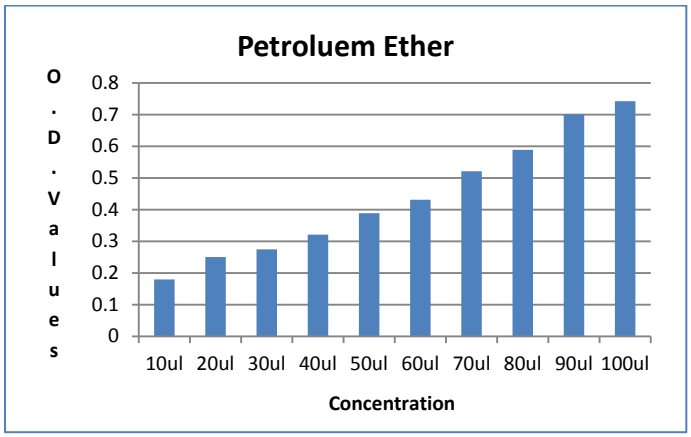
Concentration	O.D Values of Methanolic extract	O.D Values of Ethyl acetate extract	O.D Values of Petroleum ether extract	O.D Values of Hexane extract
10µl	0.422	0.325	0.180	0.321
20µl	0.485	0.386	0.250	0.479
30µl	0.523	0.401	0.275	0.582
40µl	0.611	0.432	0.321	0.611
50µl	0.687	0.58	0.389	0.684
60µl	0.729	0.649	0.431	0.729
70µl	0.791	0.689	0.521	0.791
80µl	0.899	0.702	0.589	0.836
90µl	0.947	0.825	0.701	0.893
100µl	1.012	0.893	0.743	0.979



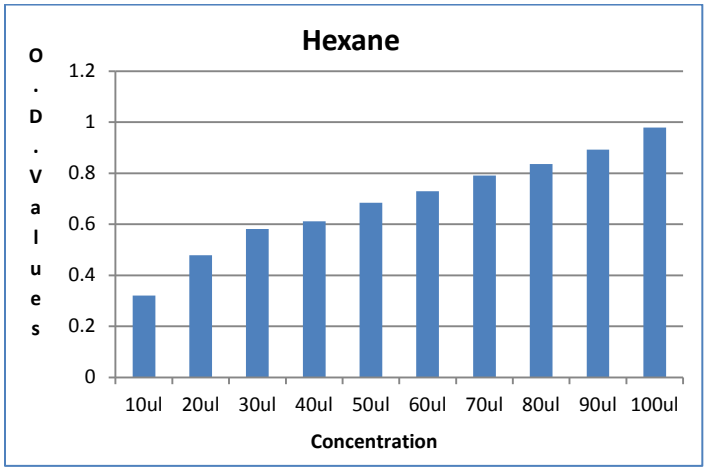
Graph 1: showing the plots of concentration versus O.D Values of methanol extract



Graph 2: showing the plots of concentration versus O.D Values of ethyl extract



Graph 3: showing the plots of concentration versus O.D Values of petroleum ether extract



Graph 4: showing the plots of concentration versus O.D Values of hexane extract

Antimicrobial Activity

Methanolic extract

Table 4: Anti-microbial activity of Methanolic crude compound with different concentrations and different microbial cultures

Microbial Culture	Concentration Of Plant Extract	Zone Of Inhibition (In Mm)
Bacillus sps	50µl	6±1
	25µl	4±1
	10µl	2±1
E.coli	50µl	7±1
	25µl	4±1
	10µl	3±1
Psuedomonas sps	50µl	4±1
	25µl	2±1
	10µl	1±1
Streptococcus sps	50µl	4±1
	25µl	4±1
	10µl	1±1

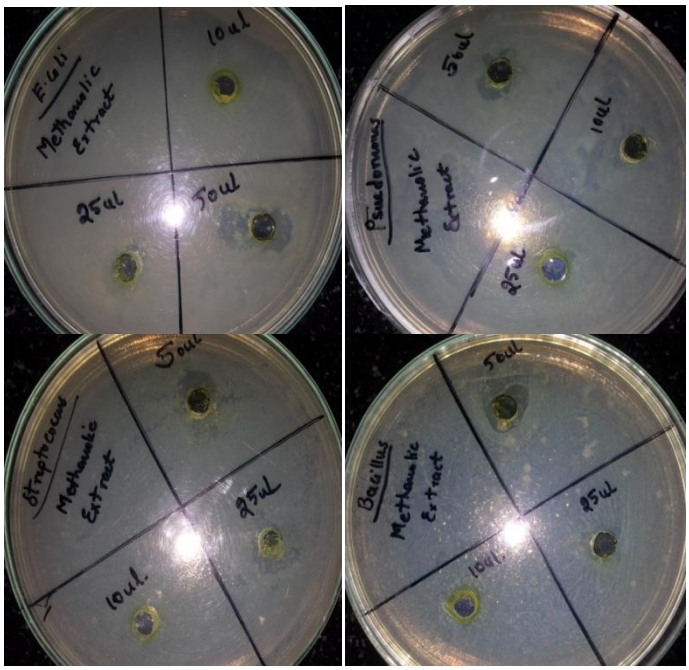


Figure 20: Antimicrobial activity of different microbial cultures in methonolic crud extract

Hexane extract

Table 5: Anti-microbial activity of Hexane crude compound with different concentrations and different microbial cultures

Microbial Culture	Concentration Of Plant Extract	Zone Of Inhibition (in mm)
Bacillus sps	50µl	5±1
	25µl	5±1
	10µl	2±1
E.coli	50µl	5±1
	25µl	4±1
	10µl	1±1
Psuedomonassps	50µl	4±1
	25µl	3±1
	10µl	1±1
Streptococcus sps	50µl	5±1
	25µl	3±1
	10µl	1±1

Petroleum Ether extract

Table 6: Anti-microbial activity of Petroleum Ether crude compound with different concentrations and different microbial cultures

Microbial Culture	Concentration of Methanolic Plant Extract	Zone Of Inhibition (in mm)
Bacillus sps	50µl	2±1
	25µl	1±1
	10µl	1±1
E.coli	50µl	2±1
	25µl	2±1
	10µl	1±1
Psuedomonassps	50µl	3±1
	25µl	1±1
	10µl	1±1
Streptococcus sps	50µl	2±1
	25µl	1±1
	10µl	1±1

Ethyl Acetate extract

Table 7: Anti-microbial activity of Ethyl acetate crude compound with different concentrations and different microbial cultures

Microbial Culture	Concentration of Methanolic Plant Extract	Zone Of Inhibition (in mm)
Bacillus sps	50µl	5±1
	25µl	3±1
	10µl	1±1
E.coli	50µl	4±1
	25µl	3±1
	10µl	1±1
Psuedomonassps	50µl	4±1
	25µl	2±1
	10µl	1±1
Streptococcus sps	50µl	4±1
	25µl	1±1
	10µl	1±1

Anti-Inflammatory Activity

Table 8: shows the results obtained by performing anti-inflammatory activity with plant extract obtained from respective solvent

Concentration	Mean Values of Methanolic extract	Mean Values of Ethyl acetate extract	Mean Values of Petroleum ether extract	Mean Values of Hexane extract
10µl	0.045	0.035	0.033	0.011
20µl	0.076	0.052	0.038	0.042
30µl	0.098	0.072	0.042	0.045
40µl	0.155	0.122	0.043	0.055
50µl	0.225	0.185	0.055	0.062
60µl	0.280	0.230	0.078	0.099
70µl	0.355	0.285	0.089	0.135
80µl	0.380	0.322	0.0122	0.138
90µl	0.425	0.385	0 0145	0.158

Trypsinization

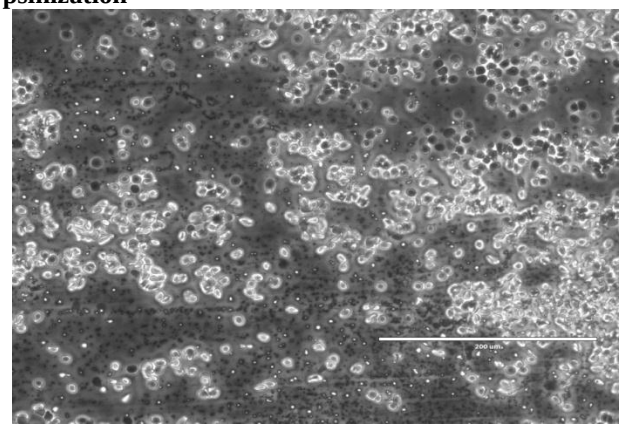


Figure 21: Trypsinization

MTT Assay

The following are the O.D values obtained for hexane solvent extract at 570nm:

Table 9: different O.D values of hexane solvent extract at 570nm

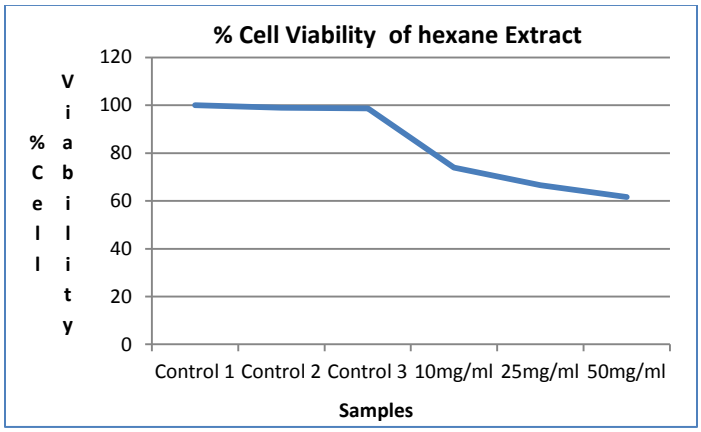
Sample	10 mg/ml	25 mg/ml	50 mg/ml	Control 1	Control 2	Control 3
O.D Value	0.358	0.304	0.293	0.457	0.468	0.472
O.D Value	0.347	0.319	0.282	0.462	0.459	0.452
O.D Value	0.338	0.299	0.279	0.447	0.443	0.461

Table 10: Average O.D values hexane solvent extract at 570nm

Sample	Control 1	Control 2	Control 3	10 mg/ml	25 mg/ml	50 mg/ml
Average values	0.461	0.456	0.455	0.341	0.307	0.284

Table 11: % Cell Viability

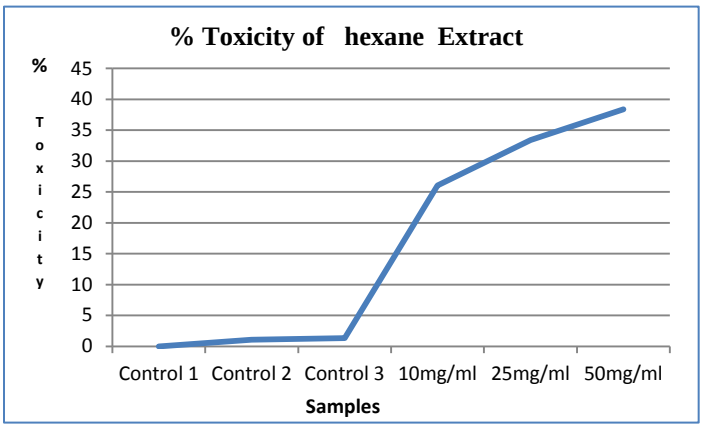
Sample	Control 1	Control 2	Control 3	10 mg/ml	25 mg/ml	50 mg/ml
% Cell Viability	100	98.91	98.69	73.96	66.59	61.60



Graph 5: cell viability of hexane extract

Table 12: % Compound Toxicity of hexane solvent extract at 570nm

Sample	Control 1	Control 2	Control 3	10 mg/ml	25 mg/ml	50 mg/ml
% Compound Toxicity	0	1.08	1.30	26.03	33.40	38.39



Graph 6: % Toxicity of hexane extract

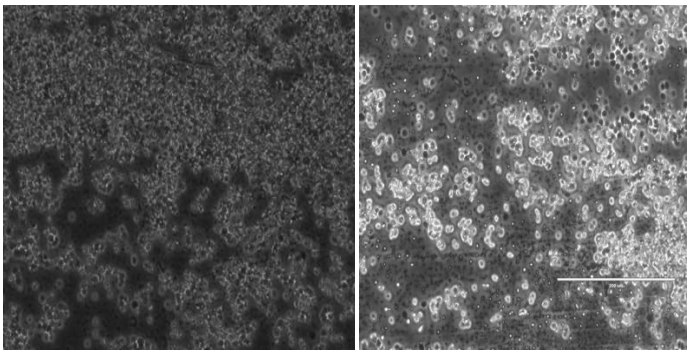


Figure 22: Normal MCF7- cells, Trypsinization of MCF -7 cells (left→right)

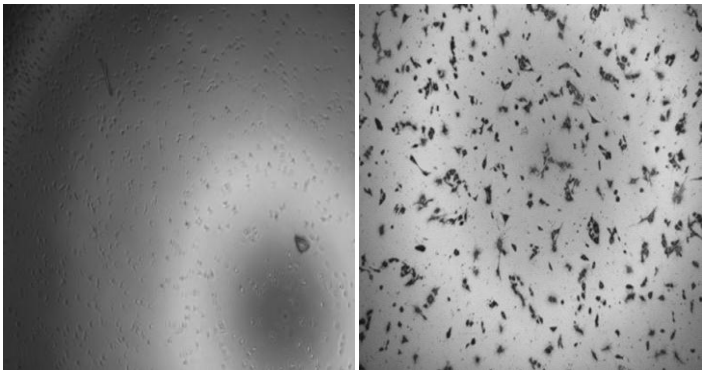


Figure 23: Drug treatment to MCF-7 cells, Crystal formation of MCF -7 cells (left→right)

IV. DISCUSSION

Phytochemical analysis was performed in 4 solvents. The results are in table 2. Methanol extract has the more active compounds.

Phytochemical analysis shows that most of the phytochemicals got dissolved in hexane and methanol followed by ethyl acetate and petroleum ether. A particular photochemical has its own affinity to a particular solvent. In the above result hexane has high affinity towards alkaloids and flavanoids and low affinity towards aminoacids. Ethyl acetate has high affinity towards aminoacids and good affinity towards alkaloids and saponins. Methanol has a good affinity towards alkaloids, phenols and flavanoids and low affinity towards saponins and aminoacids. Petroleum ether has a good affinity towards alkaloids and low affinity towards flavonoids. The phytochemical constituent which is common in all the 4 solvents are alkaloids.

Antioxidant activity was performed and results are in table 3. Graphs are plotted and graphs 1, 2, 3 and 4 shows the results in different solvents. Antioxidant activity shows that methanol extract has high free radical scavenging activity which can be noticed in graph 1.Antioxidant activity was performed for the extracts from 4 solvents. Absorbance value is highest for methanol extract followed by hexane, ethyl acetate and petroleum ether. This shows high antioxidation capacity for methanol extract. Higher the absorbance values, higher the antioxidation capacity.

Antimicrobial activity was performed and is shown in figure 20. Results are presented in tables 4, 5, 6 and 7 shows the results of methanol, hexane, petroleum ether and ethyl acetate extracts respectively. Anti-microbial activity for the extracts of methanol extract showed better results than to that of hexane, ethyl acetate and petroleum ether. A highest value of zone of inhibition was found in methanol extract against *E.coli.*

MTT Assay was performed and is given in tables 9, 10 and 11 shows the results. Graphs 5 and 6 shows cell viability and extract toxicity respectively, over breast cancer cells. We can notice that as the compound concentration increases from 10mg/ml to 50mg/ml, there is a reduction in percentage of cell viability of MCF-7 cells which indicate that the hexane extract has potent compounds that can hinder the growth of breast cancer cells. In contrast, with reference to compound toxicity, as concentration increases, the potential of toxicity rises, showing that the plant extract is toxic to breast cancer cells.

V. CONCLUSION

Medicinal plants constitute an effective source of both traditional and modern medicines. About 80% of rural population depends upon the herbal medicine for their primary health care. Medicinal plants are plants containing inherent active ingredients and produce bioactive compounds which act on different system of animals and man and interfere in the metabolisms of microbes infecting them. The bioactive compounds play an important role in regulating host microbe interaction in favor of the host. The medicinal properties of plants could be antioxidant, antimicrobial, anti-inflammatory based on the phytochemicals in them.

In the present work, phytochemical work shows that the most of important plant phytochemicals were effectively dissolved in methanol and hexane followed by ethylacetate and petroleum ether.

Antioxidant work shows that the maximum antioxidation capacity is with methanol extracts followed by hexane, ethylacetate and petroleum ether. Hence for antimicrobial activity methanol and hexane have been analyzed as these two solvents shown best results. Methanol has shown highest value of zone of inhibition against *E.coli* followed by *Bacillus*. Hexane has shown highest value of zone of inhibition against *E.coli* followed by *Streptococcus*.

MTT assay which was performed using extracts of hexane extract. The percentage toxicity values of hexane extract were increasing from point to point. This states that hexane extract has potent capacity in killing the breast cancer cells. Hence for further drug designing and isolation of active compounds for breast cancer methanol extracts can be preferred.

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