

A STUDY ON IN VITRO ANTI BREASTCANCER ACTIVITY OF CRUDE ETHANOL AND ACETONE PIGMENT EXTRACTS OF *MICROCOCCLUS LUTEUS* BY MTT ASSAY AND ANALYSIS OF PIGMENT BY THINLAYER CHROMATOGRAPHY

*R.Ushasri¹ & C.Gods Will Shalomi²

J.B.A.S.COLLEGE FOR WOMEN, K.B.DASAN ROAD, TEYNAMPET, CHENNAI-18

*Corresponding Author Email: rushasri@gmail.com

ABSTRACT

Breast cancer is cancer that develops from breast tissue. Signs of breast cancer may include a lump in the breast, a change in breast shape, dimpling of the skin, fluid. *Micrococcus luteus* is gram positive cocci in tetrads characterized by yellow colour pigmentation found in mucous membranes such as the nasal cavities, the upper respiratory tract, and the lining of the mouth. The aim of our study was to study on in vitro anti breast cancer activity of crude ethanol and acetone pigment extracts of *Micrococcus luteus* isolated from soil by MTT assay and TLC. *Micrococcus luteus* was isolated, characterized and identified from soil and subcultured on TSA and was inoculated in to LB broth and incubated for 72 hrs at room temp in rotary shaker. The broth was centrifuged and filtered. The crude ethanol and acetone pigment extracts were prepared. The MCF-7 cell line was used for cytotoxic study by MTT assay. The results for an anti breast cancer activity of crude ethanol and acetone pigment extracts were found to be 48% and 51%. It was concluded that in vitro anticancer activity of crude acetone pigment extract was found to be higher than crude ethanol pigment extract.

KEY WORDS

MCF-7 Cell line, MTT assay, Tryptone soya agar, DMSO

INTRODUCTION

Breast cancer is cancer that develops from breast tissue. Signs of breast cancer may include a lump in the breast, a change in breast shape, dimpling of the skin, fluid coming from the nipple, or a red scaly patch of skin. In those with distant spread of the disease, there may be bone pain, swollen lymph nodes, shortness of breath, or yellow skin. Risk factors for developing breast cancer include obesity, lack of physical exercise, drinking alcohol, hormone replacement therapy during menopause, ionizing radiation, early age at first menstruation, and having children late or not at all^[1]. About 5–10% of cases are due to genes inherited from a person's

parents, including BRCA1 and BRCA2 among others. Breast cancer most commonly develops in cells from the lining of milk ducts and the lobules that supply the ducts with milk^[2]. Cancers developing from the ducts are known as ductal carcinomas, while those developing from lobules are known as lobular carcinomas. In addition; there are more than 18 other sub-types of breast cancer^[3]. Some cancers develop from pre-invasive lesions such as ductal carcinoma in situ.

The balance of benefits versus harms of breast cancer screening is controversial. A 2013 Cochrane review stated that it is unclear if mammographic screening does more good or

harm. A 2009 review for the US Preventive Services Task Force found evidence of benefit in those 40 to 70 years of age, and the organization recommends screening every two years in women 50 to 74 years old. The medications tamoxifen or raloxifene may be used in an effort to prevent breast cancer in those who are at high risk of developing it. Surgical removal of both breasts is another useful preventative measure in some high risk women. In those who have been diagnosed with cancer, a number of treatments may be used, including surgery, radiation therapy.

Breast reconstruction may take place at the time of surgery or at a later date. In those in whom the cancer has spread to other parts of the body, treatments are mostly aimed at improving quality of life and comfort. The first noticeable symptom of breast cancer is typically a lump that feels different from the rest of the breast tissue. More than 80% of breast cancer cases are discovered when the woman feels a lump. The earliest breast cancers are detected by a mammogram. Lumps found in lymph nodes located in the armpits can also indicate breast cancer.

Indications of breast cancer other than a lump may include thickening different from the other breast tissue, one breast becoming larger or lower, a nipple changing position or shape or becoming inverted, skin puckering or dimpling, a rash on or around a nipple, discharge from nipple/s, constant pain in part of the breast or armpit, and swelling beneath the armpit or around the collarbone. Pain is an unreliable tool in determining the presence or absence of breast cancer, but may be indicative of other breast health issues.

Micrococcus luteus (*M. luteus*), is a Gram-positive bacteria, 0.05 to 3.5 microns in diameter, that is most commonly found in mucous membranes such as the nasal cavities, the upper respiratory

tract, and the lining of the mouth. The bacteria are also found in dust, soil and the air that we breathe, and is nonsporeforming. Gram-positive Spheres, 0.9 to 1.8 pm in diameter, occurring in clumps, tetrads, or packets and in irregular clusters of tetrads once regarded as non-pathogenic.

M. luteus (formerly *Micrococcus lysodeikticus*) is of historical interest for the part it played in Fleming's discovery of lysozyme, to which it is exquisitely sensitive. This bacterium, which is often used for educational studies, produces bright yellow colonies on nutrient agar. *M. luteus* (NCTC2665, "Fleming strain") has one of the smallest genomes of free-living actinobacteria^[4]. It has just one circular chromosome of 2,501,097 base pairs, which is predicted to encode 2,403 proteins. The genome encodes only four sigma factors and 14 response regulators, a finding that may reflect its adaptation to a very narrow ecological niche (mammalian skin). *M. luteus* is very sensitive to beta-lactam antibiotics, and this may reflect the presence of a reduced set of penicillin-binding proteins and the absence of a *wbIC* gene, which has an important role in the antibiotic resistance in other actinobacteria. *M. luteus* has relatively few genes concerned with carbohydrate transport and metabolism, and its inability to use glucose as a sole carbon source may be because it lacks a gene encoding glucokinase. *M. LUTEUS* seems to be able to metabolize glycogen only via trehalose and to make trehalose only via glycogen. Colour is a fundamental component and is probably one of the first qualities manifested by our senses. Since, the synthetic colours are toxic, it is essential to produce coloured pigments from natural resources. Natural pigments are extracted not only from fruits, vegetables, roots but also from microorganisms and are often called biocolours^[5]. Microbial pigments pose no

seasonal production problems but shows high productivity. *Micrococcus luteus* was originally isolated by Alexander Fleming in 1929 as *Micrococcus lysodeikticus*. It is very capable of survival under stress conditions such as low temperature, starvation.

MATERIALS AND METHODS:

MATERIALS:

Soil sample, Glass funnels, Whatman filter paper, Sterile conical flasks, Sterile test tubes. Inoculation loop, Laminar hood, Inverted microscope, Sterile pipettes, Sterile tips, TLC plates, Rabbit plasma, Organic solvents, Rotary shaker, Spectrophotometer, Microtitre plates, MCF-7 cell line, Minimum essential medium, Fetal bovine serum, MTT dye, DMSO

METHODS:

Soil sample was collected in sterile conical flask, serially diluted using sterile saline and plated on to sterile Tryptone soya agar. The plates were incubated for 24 hrs at 37^{0c}. The plates were observed for pigmented colonies ^[6]. The pigmented colonies were characterized and identified by morphological, biochemical and specific tests ^[7]. The pigmented bacterium was identified as *Micrococcus luteus* based on biochemical and specific tests and compared with standard MTCC strain ^[8]. The organism was subcultured and inoculated in to LB broth in rotary shaker for 72 hrs. The greenish yellow pigment was extracted by centrifugation ^[9]. The supernatant was collected and filtered using sterile whatmann filter ^[10]. The crude extract was prepared using ethanol and acetone under reduced pressure ^[11]. The MCF-7 cell line was purchased from NCCS, Pune, subcultured using Minimum essential medium with 10% FBS in co² incubator. The cytotoxicity assay was carried out by MTT assay ^{[11] [12]}.

Microculture tetrazolium (MTT) assay: The monolayer cell culture was trypsinized and the cell count was adjusted to 1ml using medium. To each well of 96 well microtitre plates, 0.1ml of diluted cell suspension was added ^[13]. After 72 hour, the sample solution in wells was flicked off and 50µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37^o c in 5% Co2 incubator. The supernatant was removed, 50 µl of Dimethyl sulphur oxide was added, and the plates were gently shaken to solubilize the formed formazan ^[14]. The absorbance was measured using a micro plate reader at a wavelength of 490- 570 nm.

The percentage growth inhibition was calculated using following formula,

$$\% \text{cell inhibition} = 100 - \left\{ \frac{(At - Ab)}{(Ac - Ab)} \right\} \times 100$$

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac= Absorbance value of control.

Thin layer chromatography

Thin-layer chromatography (TLC) is the simplest and cheapest method of detecting constituents since the method is easy to run, reproducible and requires little equipment (Marston et al., 1997). TLC is an important method for the isolation, purification and confirmation of natural products. Compared with other chromatographic methods, TLC is often considered to be deficient in reproducibility and accuracy, but some distinctive attributes of this tool should be considered: low cost analysis, high-throughput screening of samples, minimal sample preparation, whole sample integrity, disposable stationary phase. Thin Layer Chromatography (TLC) is a solid-liquid type in which the two phases are a solid (stationary phase) and a liquid (moving phase). Solids most commonly used in chromatography are silica gel (SiO₂ x H₂O) and alumina (Al₂O₃ x H₂O). In our experiments thin layer chromatography (usually 5 µl of a 100 mg crude

extract/ml solution) is loaded on Merck TLC F254 or manual silica gel glass plates using different mobile phases. The mobile phase flows over the surface, usually driven by surface tension forces and the solutes are eluted across the surface and are separated. After development, the plate is dried and the surface subjected to one of a number of different techniques that render the solutes visible as spots on the plate. Thus the separation appears as a number of (more or less) circular colored spots on the plate spreading from the point of injection to the position of the solvent front. The relative position of the spot is a characteristic of the specific substance and the diameter and intensity of the spot is related to the amount of material present in the spot. The relative position is calculated using the formula.

$R_f = \text{Distance travelled by solute} / \text{Distance travelled by solvent}$.

RESULTS:

Gram staining:

[a] Gram positive cocci in tetrads.

Cultural characters:

[b] Yellow pigmented colonies were found on Nutrient agar.

MTT assay:

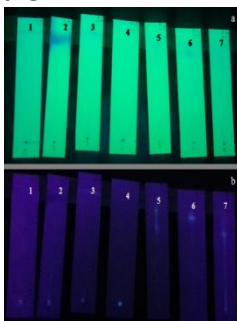
The percentage of cytotoxicity was calculated and the IC₅₀ Value for Breast cancer for ethanol and acetone pigment extract at 50µl, 100µl and 150µl concentrations were found to be 41%,45% ,48% and 45%,48% and 51% respectively.



Figure- [a] Gram positive cocci in tetrads



Figure-[b] Yellow pigmented colonies on Nutrient agar



[Fig-c] TLC Image

TLC Analysis

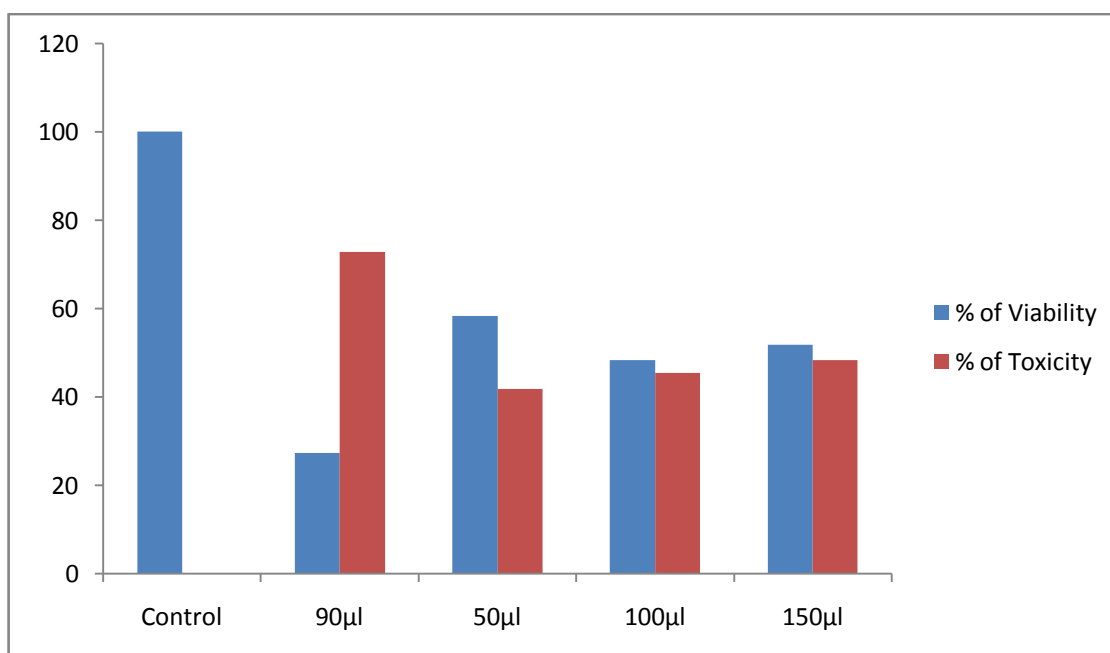
UV-Short-254 nm

UV-Long-365 nm

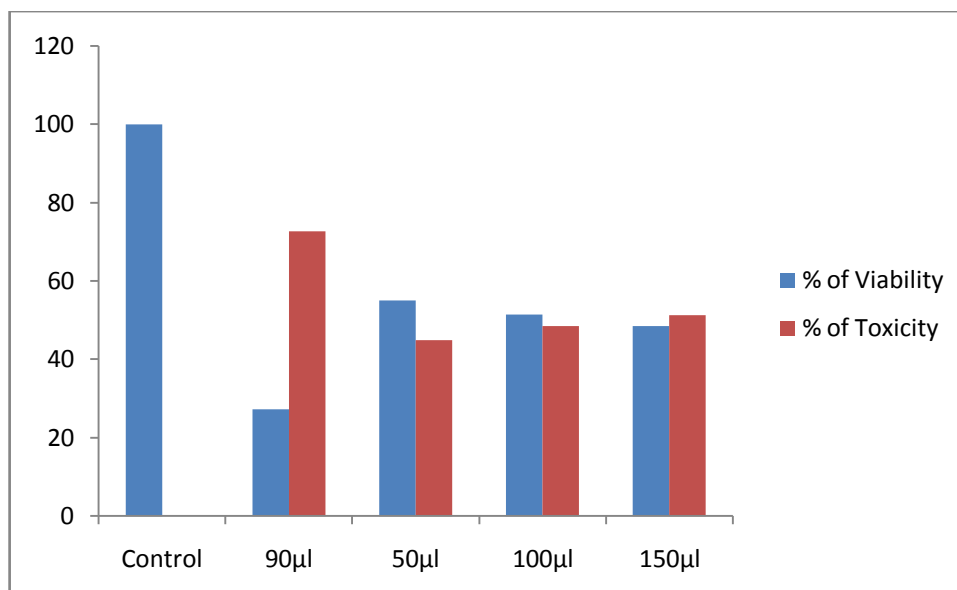
SOLVENT	RF RATIO
Toluene: Methanol	7:3
Toluene: Methanol	5:5
Chloroform: Methanol	7:3
Chloroform: Methanol	9:1
Toluene: Isopropanol	7:3
Toluene: Isopropanol	5:2
Acetone: Water	5:3
Acetone: Water	9:1

Table-[a]- TLC report

<i>Micrococcus luteus</i>	<i>S.aureus</i>
Gram positive cocci in pairs or tetrads	Gram positive cocci in clusters.
Yellow orange colonies on nutrient agar	Golden yellow colonies on Nutrient agar
Urease negative	Urease positive
Bacitracin sensitive	Bacitracin Positive
Coagulase negative	Coagulase positive



[a]Bar chart- MTT report - Ethanol



[b]Bar chart- MTT report -Acetone

DISCUSSION:

The percentage of cytotoxicity was calculated and the IC₅₀ Value for Breast cancer cell line by ethanol and acetone pigment extracts at 50µl, 100µl and 150µl concentrations were found to be 41%,45% ,48% and 45%,48% and 51% respectively^[15].

CONCLUSION:

Micrococcus luteus, Gram positive cocci appears in pairs or tetrads, pigmented bacterium capable of producing yellowish green pigment. The pigment extracted using ethanol and acetone extracts was found to be potential against Breast cancer. MCF-7 cell line was bought from NCCS, Pune and subcultured. The % of cytotoxicity was determined by MTT assay^[14] The % of inhibition for Breast cancer cell line by crude acetone pigment extract was found to be higher (51%) than ethanol pigment extract (48%)^[15] Thin layer chromatographic studies revealed that the RF value is greater for Toluene: isopropanol and Acetone: Water in UV short and long wave length range.

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***Corresponding Author:**

R.USHASRI

rushasri@gmail.com