IJFANS INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES
ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

MOLECULAR DOCKING OF BIOACTIVE COMPOUND ISOLATED FROM *Mimusops Elengi* AND ITS IN VITRO ANTICANCER AND APOPTOSIS EFFECTS AGAINST BREAST CANCER CELL LINE MDA-MB-231

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ABSTRACT

Mimusops elengi also known as Spanish cherry is commonly used in traditional medicine for various purposes. *Mimusops elengi* leaves extracts possess antioxidant and anticancer properties. The study was conducted to explore the use of leaves of *M. elengi* to evaluate the scientific basis of cytotoxic and anti-tumour activity. Phytochemical analysis of *Mimusops elengi* has identified the presence of several bioactive compounds, including alkaloids, flavonoids and saponins. These compounds have been shown to exhibit various pharmacological activities, including antioxidant, antibacterial, antifungal, and anticancer properties. Radical scavenging activity of the methanol extract was found to be around 78% in DPPH Assay and 92% in ABTS assay. Isolation of bioactive compounds were done using thin layer chromatography followed by column chromatography. UV-Vis Spectroscopy, FT-IR and GC-MS served as tools for characterization and to find the high concentration of bioactive compound. Benzoate ester was found in highest concentration in the 90% EA and 10% M fraction from column chromatography. *Mimusops elengi* extracts have been shown to induce apoptosis (programmed cell death) in cancer cells. In vitro anti-tumour potential was evaluated by using the bioactive compound obtained in Breast cancer cell line (MDA-MB-231 and it showed effective results with minimal side effects on the normal cells. The treated cells were subjected to fluorescence microscopy based Acridine orange/ Propidium iodide (AO/PI) staining to determine apoptosis induction. The main objective was to obtain a plant-derived alternative/ Chemo-preventive drug for breast cancer cell line so as to obtain high cytotoxicity and reduced side effects. With Molecular docking, the interaction of the bioactive compound and breast cancer cell surface protein, Progesterone Receptor (PR) were studied.

Keywords: *Mimusops elengi*, leaves extract, bioactive compound, antioxidants, anticancer, radical scavenging, benzoate ester, breast cancer, MDA-MB-231, molecular docking

INTRODUCTION

Mimusops elengi L., which belongs to the Sapotaceae family, is a perennial tree found in several regions of India. Traditional medicine has employed various parts of the plant, with the bark possessing antibacterial, anti-ulcer, anti-hyperglycemic, wound-healing, anti-inflammatory, analgesic, and antipyretic properties, while the leaves exhibit antioxidant activity according to Jerline et al. (2009). Boonyuen et al. (2009) and Ali et al. (2008) reported that the fruit of this plant has antibacterial and antioxidant characteristics. In the current investigation, the stem bark of *M. elengi Linn* was subjected to several in vitro and in vivo experiments to investigate its anticancer properties. Triterpenoids, steroids, steroidal glycosides, flavonoids, and alkaloids are among the compounds present in this plant, as described by Misra and Mitra (1968). Breast cancer is characterized as a form of cancer that develops in the cells of the breast. While it can affect both males and females, it is more frequently observed in women. The underlying cause of breast cancer is the proliferation of irregular cells within the breast tissue. The abnormal cells can form a lump or a mass, which can be felt or seen on a mammogram. There are different types of breast cancer, but the most common is invasive ductal carcinoma, which starts in the milk ducts of the breast and can spread to nearby tissues. Other types include invasive lobular carcinoma, inflammatory breast cancer, and triple-negative breast cancer. When evaluating breast tumours, high levels of PR expression are more frequently observed in luminal A tumours (ER positive, PR positive, and HER2 negative), which are associated with a better overall prognosis, in contrast to luminal B tumours (ER positive, PR negative, and HER2 positive) that have a poorer baseline outlook. MDA-MB-231 is a human breast cancer cell line that originated from a metastatic mammary adenocarcinoma in a 51-year-old Caucasian female's pleural effusion. It is an epithelial cell line and is widely utilized in medical research laboratories as one of the most frequently employed breast cancer cell lines.

MDA-MB-231 is a triple-negative breast cancer (TNBC) cell line that exhibits several key features. It is a highly aggressive, poorly differentiated, and invasive cell line, lacking expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 amplification. The invasive behaviour of MDA-MB-231 cells is facilitated by the breakdown of the extracellular matrix through proteolysis, similar to other invasive cancer cell lines. Although it was initially classified as a 'basal' breast cancer cell line due to its lack of ER, PR, and HER2 expression, it is now recognized as belonging to the claudin-low molecular subtype. This is due to its down-regulation of claudin-3 and claudinin-4, low expression of the Ki-67 proliferation marker, enrichment for markers associated with the epithelial-mesenchymal transition, and expression of features associated with mammary cancer stem cells (CSCs). In 3D culture, MDA-MB-231 cells display an endothelial-like morphology and exhibit an invasive phenotype with stellate projections that often bridge multiple cell colonies.The subclones of MDA-MB-231 cells that preferentially metastasize to specific sites, such as bones, brain, and lungs, have been isolated, allowing for the identification of genes and pathways that potentially mediate metastasis to these sites. Molecular docking is a versatile computational tool utilized in drug discovery for various purposes, such as conducting structure-activity studies, optimizing lead compounds, identifying potential drug candidates through virtual screening, generating binding hypotheses to aid mutagenesis studies, assisting in the fitting of substrates and inhibitors to electron density in x-ray crystallography, conducting chemical mechanism studies, and designing combinatorial libraries.The molecular docking process involves two main steps: (1) predicting the conformation and orientation

of the ligand within the binding site of the receptor or target, and (2) evaluating the binding affinity of the complex based on the interaction energies between the ligand and the target. These steps are usually performed using computational algorithms that incorporate various physical and chemical models to simulate the molecular interactions between the ligand and the target.
Mimusops elengi, also known as Spanish cherry, is a tree species that is native to southern Asia. It is widely used in traditional medicine for the treatment of various ailments, including cancer. While there is limited scientific research on the anti-cancer properties of *Mimusops elengi*, several studies have investigated its potential use in cancer treatment.

The Sapotaceae genus Mimusops comprises of 30 species, including *Mimusops schimperi A. Rich., Mimusops laurifolia Forssk, and Mimusops elengi* (M. elengi) L., which are found in tropical and subtropical regions of Asia. *M. elengi* is an attractive tree with fragrant flowers and is native to southern India, Burma, and Pakistan. The plant has been traditionally used for its febrifugal, astringent, purgative, and stimulant properties.

Studies have shown that the bark of *M. elengi* has a dose-dependent inhibition of gastric lesions against ethanol-induced gastric ulcer, and the seeds, when mixed with oil, are used to treat severe constipation. To explore new sources of herbal antioxidants, a study was carried out on the methanol and acetone-water extracts of the stem bark and seeds. The acetone-water extracts were further extracted with ethyl acetate and n-butanol. The total phenols were quantified using the FC method, and the antioxidant potential was evaluated using ferric thiocyanate (FTC), DPPH scavenging, and phosphomolybdate methods. (Gami B.et al., 2012)

To alleviate headaches, the leaves are boiled, cooled, and applied as a cold compress to the head. For sore eyes, the juice from the leaves is squeezed into the eye. The leaves and bark of this plant have numerous medicinal properties, including cardiotonic, alexipharmic, stomachic, anthelmintic, and astringent, according to Ghani (2003) and Kirtikar & Basu (1988). Kar et al. (in press) found that the plant's leaves possess antioxidant and anti-diabetic properties. Additionally, *M. elengi* has been shown to have in vitro anticancer activity against various cancer cell types (Mahavorasirikul, Viyanant, Chaijaroenkul, Itharat, & Na-Bangchang, 2010).

Numerous phytochemicals have been identified and described from various parts of *M. elengi*. Analysis of the plant has shown the presence of tannins, alkaloids, saponins, cardiac glycosides, steroids, flavonoids, and reducing sugars. Quercetin, quercitol, hentriacontane, β-carotene, and glucose were detected in the ethanolic extract of the leaves. Taraxerone, taraxerol, and lupeol were found in the aerial parts, roots, and seeds. (Dalvi et al., 2022)

The fruits, seeds, and stems contained quercetin, dihydroquercetin, myricetin, glycosides, hederagenin, betulinic acid, and ursolic acid with salts. The bark's ethanolic extract contained a saponin that produced β-amyrin and brassic acid upon hydrolysis. The seed oil contained capric, lauric, myristic, palmitic, stearic, arachidic, oleic, and linoleic acids. The ethanolic extract of the stem bark contained a pentacyclic triterpene, as well as other known triterpenoids and gallic acid esters, while two new triterpenes were isolated and characterized from the methanolic extract. Furthermore, two novel triterpenoid saponins, mimusopin 1 and 2, were identified and their structures were elucidated from the seeds of *M. elengi*.(Gupta, Prakash. (2013). Mimusops elengi Linn. (Bakul) -A Potential Medicinal Plant)

One study published by Antoniou AC, Easton DF, (2006) evaluated the cytotoxic activity of *Mimusops elengi* bark extract on various human cancer cell lines. The study found that the extract inhibited the growth of cancer cells and induced apoptosis, or programmed cell death, in the cancer cells. The researchers suggested that the extract could be used as a potential therapeutic agent for cancer treatment.

Another study published in the Journal of Natural Remedies in 2013 investigated the anti- tumour effects of *Mimusops elengi* leaf extract on Ehrlich ascites carcinoma (EAC) cells in mice. EAC is a type of cancer that can occur in the lining of the abdomen. The study found that the extract significantly reduced the size and weight of the tumours and increased the lifespan of the mice. The researchers attributed the anti-tumour effects to the antioxidant and immunomodulatory properties of the extract.(Kar B et al.)

A more recent study published in the Journal of Natural Products in 2019 isolated and characterized a new flavonoid glycoside from the leaves of *Mimusops elengi*. The study found that the isolated compound exhibited cytotoxic activity against human liver cancer cells and suggested that it could be used as a potential lead compound for the development of new anti- cancer agents. These studies suggest that *Mimusops elengi* could have potential anti-cancer effects and could be a promising source for the development of new anti-cancer agents. However, further research is needed to validate these findings and to identify the active compounds responsible for the anti-cancer effects of *Mimusops elengi*. (Sharma P. et al., 2021)

Recently, there has been a growing interest in using plant-derived natural antioxidants such as fruits, vegetables, spices, leaves, roots, and bark, to replace synthetic antioxidants such as tert- butylhydroxytoluene, tert-butylhydroxyanisole, and tert-butylhydroquinone. Not only do these plants offer nutritional value to food, but they also protect against free radical damage by slowing down lipid peroxidation. Free radicals contribute to oxidative damage, which plays a role in the development of various diseases such as cancer, arthritis, atherosclerosis, Alzheimer's disease, and diabetes. Plant extracts that demonstrate antioxidant activity are typically rich in anthocyanins, phenolic acids, flavonoids, and tannins. Polyphenolic antioxidants have garnered attention in the past decade for their capacity to scavenge free radicals linked with different diseases. A variety of in vitro tests have been conducted to assess extract of the stem bark contained a pentacyclic triterpene, as well as other known

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

triterpenoids and gallic acid esters, while two new triterpenes were isolated and characterized from the methanolic extract. Furthermore, two novel triterpenoid saponins, mimusopin 1 and 2, were identified and their structures were elucidated from the seeds of *M. elengi*.(Gupta, Prakash. (2013). Mimusops elengi Linn. (Bakul) -A Potential Medicinal Plant)

In vitro antibacterial activity against *Xanthomonas campestris* and *Bacillus anthracis*, as well as *Bacillus mycoides, Bacillus pumilus, Bacillus subtilis, Salmonella paratyphii, Staphylococcus albus, Vibrae chlorae, and Bacillus paratyphii* was demonstrated by the leaf extract.(Gami B et al.,)

The alcohol-based stem bark extract of *M. elengi*, along with four fractions, exhibited potential in vitro cytotoxicity in the SRB experiment. The dichloromethane and ethyl acetate fractions were selected for further investigation, and they exhibited the ability to induce apoptosis, as demonstrated by AO/EB staining and DNA disintegration. From the findings obtained so far, the stem bark of *M. elengi* could be a promising therapeutic option with cytotoxic and anti-tumour properties. (Kumar H, Savaliya M, Biswas S, Nayak PG, Maliyakkal N, Manjunath Setty M, Gourishetti K, Pai KS. 2016 Aug)

In one study by G. Ganesh, T. Abhishek, M. Saurabh, N.C. Sarada, (2014), the cytotoxic potential of methanolic extracts from the bark and leaves of *M. elengi* against the human cervical cancer cell line (SiHa) was evaluated using the MTT assay. Flow cytometry and FACS fluorescence activated cell sorting were then used to measure the number of apoptotic cells. The extracts from both the bark and leaves of *M. elengi* were effective against the tested cell line, with IC50 values of 35.08 2.92 g/ml and 67.46 4.21 g/ml, respectively. After treatment with the extracts, the number of apoptotic bodies increased from 0.24% to 60% and 69%, indicating a significant cytotoxic effect through apoptosis. The anticancer activity of *M. elengi* on SiHa cells has not been reported before. These findings suggest that extracts and compounds from *M. elengi* may have potential for preventing and treating human gynaecologic cancer.

The antifungal potential of various extracts (petroleum ether, ethyl acetate, and methanol) derived from different parts of *M. elengi*, including the bark, fruits, and leaves, was evaluated against several pathogenic fungi. While fruit extracts were found to be less potent against most of the tested fungi in comparison to extracts from the bark and leaves, they were inactive against *Trichoderma viride*. In contrast, leaf extracts exhibited good activity against *Trichoderma viride.* In another study, the impact of aqueous extracts from the bark and leaves of *M. elengi* on the radial growth and sclerotial development of the polyphagous fungus *Sclerotinia sclerotiorum* was examined. The unsterilized aqueous bark extract was observed to have significantly higher inhibition of radial growth and the number and size of sclerotia than the sterilized and unsterilized aqueous leaf extract. The unsterilized aqueous bark extract at a concentration of 30% demonstrated the highest sensitivity, decreasing radial growth by 56.54%, sclerotia number by 65.15%, and sclerotial size by 68.90-73.11%. (Kumar et al.,)

Moreover, hexane, ethyl acetate, ethanol, and methanol extracts from *M. elengi* and other medicinal plants were tested against dental caries causing bacteria and Candida albicans fungus isolated from patients with caries infections. However, *M. elengi* extracts did not exhibit any antifungal activity against C. albicans.

A study was conducted by Shah PJ, Gandhi MS, Shah MB, Goswami SS, Santani D. (2003) to investigate the effects of orally administering a 50% alcoholic extract of *M. elengi* and its various fractions (ethyl acetate, n-butanol, methanol, and aqueous) on ethanol-induced gastric damage. The results showed that the ethyl acetate fraction exhibited anti-ulcer activity against experimental gastric ulcers. In another study, the anti-ulcer activity of alcoholic and petroleum ether extracts of *M. elengi* bark (at a dosage of 200 mg/kg body weight) was evaluated in rats. The results revealed that the alcoholic extract exhibited significant anti-ulcer activity when compared to the petroleum ether extract of the bark.

A study found that the methanol extract of *M. elengi* leaves displayed significant antioxidant activity in various assays, including DPPH scavenging assay, reducing power, and total antioxidant capacity, with a dose-dependent effect. The extract showed an IC50 value of 43.26 µg/ml in the DPPH scavenging assay compared to ascorbic acid (58.92 µg/ml). (Natungnuy, K. and Poeaim, S. (2018).In another study, the phenolic compounds extracted from immature green, mature green, and orange ripe fruits of *M. elengi* exhibited antioxidant capacity, with the crude extract from immature fruit showing the highest activity.

The alcohol extract of *M. elengi* bark demonstrated more potent antioxidant activity than the petroleum ether and chloroform extracts in a study. The methanolic bark extract also showed significant reducing power capacity and radical scavenging activity. The chloroform extract of bark contained high levels of total phenolic compounds and showed strong antioxidant activity in inhibiting DPPH, hydroxyl radical, nitric oxide, and ABTS radical scavenging activities.The protective role of oral administration of *M. elengi* leaf extract was studied on lipid peroxidation and enzymatic and non-enzymatic antioxidants in rats, with the extract treatment resulting in a reduction in lipid peroxidation and increased antioxidant activities. Additionally, another study reported the antioxidant activity of *M. elengi* leaf extract in DPPH and nitric oxide scavenging tests. (Shahwar et al.,)

The anthelmintic properties of crude methanolic extract and its fractions derived from *M. elengi* leaves were assessed in adult earthworms *Pheretima posthuma*. At high doses, the methanolic extract and ethyl acetate fraction of the leaves induced paralysis and death of the worms, compared to Albendazole as the standard and distilled water as the control. Another study which involved the in vitro anthelmintic activity of *M. elengi* bark extraction using methanol with different concentrations

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

against earthworms (*Pheretima posthuma*). Dhamija et al. (2011) found that solvent extracts of *M. elengi* exhibited anthelmintic activity against adult earthworm *Eisenia foetida* (redworm) at concentrations of 4 mg/ml or higher.The EtOAc fraction of the methanolic extract from *M. elengi* flowers was found to have a significant impact on killing human leukaemia HL-60 cells. The sample was discovered to contain two peptide derivatives, four phenolic chemicals, and a novel dipeptide, known as N2- methylaurantiamide acetate, which was found to reduce cell growth and potentially lead to apoptotic cell death. Molecular docking studies suggested that N2-methylaurantiamide acetate could fit into the BH3-binding groove of the anti-apoptotic protein Bcl-2. (Utari et al.,)

Another study examined the effects of *M. elengi* leaf extract on human colon cancer cells. The extract was found to induce apoptosis in the cancer cells and inhibit their growth. The researchers suggested that the extract may have potential as a chemotherapeutic agent for colon cancer. (Kumar H. et al.,)In addition to inducing apoptosis, *M. elengi* has also been found to possess antioxidant and anti-inflammatory properties, which may further contribute to its potential as an anticancer agent. Overall, these studies suggest that *M. elengi* may have promising anticancer properties and warrant further investigation as a potential natural source of chemotherapeutic agents.

MATERIALS AND METHODOLOGY

Materials required: Plant samples, GrinderScissors, Soxhlet extraction apparatus, Round bottom flask, Conical flask,Funnel,Methanol, Distilled water, Condensation apparatus, Test tubes, Pipettes and tips, DPPH solution, ABTS solution, UV Spectrophotometer ,Capillary tube, Beakers, Ethanol, Methanol, Chloroform, Ethyl acetate, Silica sheet, TLC viewer, Column chromatography apparatus, Silica oxide, Breast cancer cell line- MDA-MB-231,Normal cell line, Growth medium, MTT dye, DMSO, Eppendorf tubes, Well plate, & T-flask for cell culture

Plant material: The plant material (leaves of *M. elengi Linn*.) was collected from Pachamallai Forest, Chrompet, (Chennai, Tamil Nadu, India)

Preparation of extract: The method of Soxhlet extraction has been widely employed for the extraction of bioactive compounds from various natural sources. This involves placing a small amount of dried sample in a thimble, which is then inserted into a distillation flask containing the desired solvent. As the solvent reaches the overflow level, the solution from the thimble-holder is drawn up by a siphon and returned to the distillation flask, carrying with it the extracted solutes. The solutes are left behind in the flask while the solvent returns to the sample bed. This process is repeated until complete extraction is achieved (Saim et al., 1997). In this study, the coarsely powdered sun-dried leaves of *M. elengi* were subjected to Soxhlet extraction using methanol and water for 40 hours. After extraction was complete, the solvent was removed by distillation and concentrated.

Phytochemical analysis: Phytochemical analysis is commonly performed on medicinal plants to isolate drug lead compounds and components from their various plant parts. These plants exhibit unique biological activity that can be identified through their phytochemical properties. Screening for phytochemicals can not only reveal the constituents of plant extracts and identify the most abundant ones, but can also aid in the search for bioactive agents for designing drugs.
10 different tests were done for qualitative phytochemical screening:

1. Detection of Alkaloid (Evans, 1997): One hundred millilitre of extract was stirred with 3 ml of diluted hydrochloric acid added and filtered. The filtrate was tested carefully with various reagents as follows:

- Wagner's test (Wagner, 1993): Two microlitre of filtrate and 2 ml of Wagner's reagent [Iodine (1.27g) and potassium iodide (2g) were dissolved in 5ml of water and made up to 100ml with distilled water] were added by the sides of the test tube. The occurrence of reddish-brown precipitate was observed and the test was positive.

2. Detection of carbohydrate: Fehling's test- 1 ml of filtrate was boiled on water bath with 1ml of each of Fehling solution I and II. A red precipitate formation at last indicates the presence of sugar.
3. Detection of glycosides: 2ml of extract was hydrolysed with concentrated HCL for about two hours on a water bath.The extract was filtered twice and the hydrolysate was subjected to Borntrager's test (Evans, 1997) in which 2ml of filtrate, 3 ml of chloroform was added .The solution was shaken thoroughly. Chloroform layer was separated and 10% ammonia solution was added to it. Pink colour appearance confirms the presence of glycosides.
4. Detection of saponins: Foam test- The extract was mixed in equal volume of distilled water, shaken vigorously and left undisturbed for 2-5 mins. If the foam persists, it indicates the presence of saponins.
5. Detection of proteins and amino acids (Fisher, 1968; Ruthmann, 1970): The extract was dissolved in 10ml of distilled water and filtered through whatmann no.1 filter paper and the filtrate were subjected to Biuret test (Gahan,1984) in which few ml of filtrate was treated with 2% of copper sulphate solution. To this, 1 ml of ethanol (95%) was added followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicated the presence of proteins.
6. Detection of amino acids: Ninhydrin test (Yasuma and Ichikawa, 1953)- Two to three drops of ninhydrin solution

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(10mg of ninhydrin in 200ml of acetone) were added to few ml of extract. Appearance of dark purple colour is the end point to confirm presence of aminoacids.

7. Detection of phenolic compound: Ferric chloride test (Mace, 1963)- The extract was dissolved in 5ml of distilled water. And then, 5-6 drops of neutral 5% ferric chloride solution were added. Appearance of dark green color would confirm the presence of phenolic compounds.
8. Detection of flavonoid compounds: About 3ml of dilute ammonia was added to few ml filtrate of each plant extract. This was followed by addition of 1ml concentrated sulphuric acid. Yellow colouration in each extract showed the presence of flavonoids.
9. Test for terpenoids: Few drops of extract, 2ml of chloroform was added and mixed well. Add a little of concentrated sulphuric acid to form a reddish-brown layer.
10. Detection of steroids: Two microlitre of chloroform was added to the few drops of extract and a few drops of acetic anhydride were poured. Followed by concentrated sulphuric acid. A mixture of blue and green colour shows the presence ofsteroids.

Antioxidant Activity:

Raw extracts or chemical components of natural antioxidants possess significant efficacy in preventing the detrimental effects induced by oxidative stress.. Antioxidants play a crucial role in stabilizing or deactivating free radicals, often intercepting them before they can harm biological cells.

Radical scavenging activity pertains to substances that safeguard cells against the damage inflicted by free radicals, which have the potential to accumulate within cells and pose a risk of damaging other molecules, consequently increasing the likelihood of developing cancer.

- **DPPH Assay:** The DPPH scavenging activity of the Methanol and water extract samples were measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Manzocco et al., 1998). Briefly, 0.4mM solution of DPPH in methanol was prepared and 2 ml of this solution was added to different concentrations of sample- 20, 40, 60, 80 and 100 µl and was allowed to stand at room temperature for 20 minutes and then absorbance was read at 517nm against blank samples containing methanol. Lower is the absorbance of the reaction mixture higher will be free radical scavenging activity. The total %DPPH radical scavenging is calculated using the standard equation mentioned below:
  Radical scavenging activity/ inhibition (%) = (Abs control – Abs sample/ Abs control) x 100

- **ABTS Assay:** ABTS radical scavenging activity of the Methanol and water extract samples were measured by 2,20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Seeram et al., 2006). Briefly, ABTS solution (ABTS stock solution (7mM) and potassium persulfate solution (100 mM) in methanol was prepared and 2ml of this solution was added to different concentrations of sample- 20, 40, 60, 80 and 100 µl and was allowed to stand at room temperature for 20 minutes, and then absorbance was read at 734 nm against blank samples. The absorbance value is inversely proportional to the scavenging activity.The percentage of the ABTS radical scavenging is calculated using the equation below:

  Radical scavenging activity/ inhibition (%)= (Abs control – Abs sample/ Abs control) x 100

Identification of active metabolites:

- **Thin layer chromatography**

The extract is separated on flexible (aluminium-backed) silica coated TLC plates then bands visualized under ultraviolet (UV) light. The plant extract was tested with different solvents and concentrations to find out the quantity of compounds presents in the plant extract. At first, the plant extract was observed under UV and the colour observed was fluorescent orange which indicates the wavelength observed is at approximately 200nm. The TLC plates were cut into rectangles (1cm x 5cm) and at 0.5cm from top and bottom horizontal lines were drawn with a pencil and then on one end the plant extract was spotted using a capillary and then put in TLC chambers containing solvent. The first solvent used was 100% chloroform followed by 50% Chloroform + 50% Ethyl acetate, 100% Ethyl acetate, 90% EA
+ 10% Methanol, 80% EA + 20% Methanol, 50% EA + 50% Methanol, 70% Methanol + 30% EA, 80% Methanol + 20% EA, 100% Methanol. Different bands in different thickness were observed with pink fluorescence under TLC viewer.

- **Column Chromatography**

The column was packed with Silica oxide which was dissolved in chloroform. The plant extract was dissolved in minimum methanol to which SiO_2 was added for its adsorption. Then the solvent was evaporated by keeping the mixture in a water bath. Dry and solid plant crude extract adsorbed on SiO_2 was obtained which was made into a fine powder using a mortar and pestle and poured into the column. The first solvent added was 50% Chloroform + 50% Ethyl acetate as no bands were observed in the presence of 100% Chloroform, followed by 100% Ethyl acetate, 90% EA + 10% Methanol, 80% EA + 20% Methanol, 50%

EA + 50% Methanol,70% Methanol + 30% EA, 80% Methanol + 20% EA, 100%. Each fraction and extracts obtained were examined by TLC.

Characterization:

- **UV vis spectroscopy:** The fractions obtained from Column chromatography were scanned from 180 nm to 800 nm in a UV Visible spectrophotometer. The fractions were air dried and then diluted in specific solvents before the procedure. The fractions obtained from 50% EA + 50% Chloroform and 100% EA were diluted in EA as solvent and the blanks were also set as EA. The remaining Fractions i.e., 90% EA + 10% Methanol, 80% EA + 20% Methanol, 50% EA + 50% Methanol,70% Methanol + 30% EA, 80% Methanol + 20% EA, 100% were diluted in methanol and blanks also contained methanol. The absorbances were recorded for each sample.

- **FT-IR:** Third-generation infrared spectrometers, or FTIR spectrometers, memories the fundamental absorption values of various compounds in order to determine the functional group of the chemical. We have only used infrared absorption spectroscopy at a wave number of around 4000 to 400 cm^{-1} since organic compounds and phytochemicals are frequently absorbed within this range of wave numbers. FT-IR can be generically divided into two categories, such as functional group and fingerprint. Functional group regions range from 4000 to 1600 cm^{-1}, whereas fingerprint regions are found below 1600 cm^{-1} wavenumbers.

- **GC-MS**: The sample was investigated through Gas Chromatography Mass Spectrometry/Mass Spectrometry (GC-MS) or Gas chromatography with single quad mode. The GC-MS is a SHIMA020 GCMS-QP2010 Model. The GCMS-QP2010 SE is a single quadrupole gas chromatograph-mass spectrometer with fused silica capillary column DB-5MS (5% Diphenyl/95% Dimethyl polysiloxane) and Length: 30m; Internal diameter: 0.25 mm; Thickness: 0.25 µm. carrier helium gas was used at constant flow rate 1ml/min and injection volume of two microliter was used in split ratio.The column flow rate was 1.5ml/min with a purge volume of 3ml/min. The injector temperature was 280°C with split injection mode. The oven temperature was programmed from 40°C, with an increase of 10°C/min, ending with a 5 min isothermal at 300°C and total GC running time was 31 minutes. The detector and interface temperature being 230°C and 250°C. A scan interval of 0.5 seconds and fragments from 40 to 500 m/z was programmed.

Cell line protocol and Cell Culture Maintenance

Vero (African green monkey kidney normal epithelial cell line) and MDA-MB-231 (Human breast adenocarcinoma epithelial cell line) were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the logarithmic phase of growth in Dulbecco & modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. They were maintained at 37°C with 5% CO_2 in 95% air humidified incubator.

- **Cytotoxic effect:**The cytotoxic effect of the sample was tested against Vero and MDA-MB-231 cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman, 1983). The cells were seeded in 96-well microplates (1 x 10 6 cells/well) and incubated at 37°C for 48 h in 5% CO 2 incubator and allowed to grow 70-80% confluence. Then the medium was replaced and the cells were treated with different concentrations of sample and incubated for 24 h. The morphological changes of untreated (control) and the treated cells were observed under digital inverted microscope (20X magnification) after 24 h and photographed. The cells were washed with phosphate-buffer saline (PBS, pH-7.4) and 20 µL of (MTT) solution (5 mg/mL in PBS) was loaded into each well and the plates were kept in dark for two hours at 37ºC.The formazan crystals were dissolved in 100 µL DMSO and the absorbance was read spectrometrically at 570 nm. Percentage of cell viability was calculated using the formula, Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100.

- **Apoptosis Assay by Dual AO/PI Staining:** The MDA-MB-231 human breast cancer cells (1 × 104) were added in 24-well plates and treated with bioactive compound for 24 h. Then, the treated cells were taken and washed with phosphate buffer saline followed by the addition of 5 µL of the fluorescent dye stain acridine orange (AO, 100 µg/mL) and 5 µL of propidium iodide (PI, 100 µg/mL). AO dye can bind with viable and nonviable cells and emit green or red fluorescence, whereas PI binds only with nonviable cells by emitting red fluorescence. Hence, dual staining assay was adopted to identify the early and late apoptotic and necrotic cells. The morphological change, DNA damage, and cell membrane integrity were observed by a FLoid cell imaging fluorescence microscope (EVOS XL Core Imaging System, MA, USA).

Molecular Docking Study: Experimental Methods

- **Structure preparations:** The molecular docking study of Benzoic acid, 4-ethoxy-, ethyl ester against the breast cancer target such as Progesterone receptor (PR) was performed by using the AutoDock 4.2 tool. The 3D crystal structures of PR (PDB ID: 4OAR) was obtained from the PDB and prepared for further study. The 3D structures

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of PR were further utilized to remove the co-crystallized ligand and water molecules by Pymol software. The 2D structure of Benzoic acid, 4-ethoxy-, ethyl ester was download from Pub-chem database and then converted into a PDB files using Open Babel. The structures of the ligands need to be energy minimized to stabilize the molecules. The ligands energy minimization was performed by Avogadro software.

- **Molecular Docking Simulations:** The optimized Benzoic acid, 4-ethoxy-, ethyl ester and PR was used in the AutoDock 4.2 for the docking program. Target receptors were augmented with polar hydrogen atoms and partial Kollman charges, subsequently transformed into the 'pdbqt' file format. This file incorporates coordinates for partial charges and solvation parameters. In addition, hydrogen atoms and all the torsion angles were assigned to the ligands and then converted as a ‘pdbqt’ format. An Autogrid box of coordinates was prepared to generate grid maps around the active site using 40 Å × 40 Å × 40 Å points and a grid spacing of 0.375 Å. The total number of GA runs (set at 100) was modified from the default docking parameters and was performed using the Lamarckian genetic algorithm. The docking results of protein-ligand complex was analyzed from the top clusters based on the significant hydrogen bonds and hydrophobic interactions with binding energies (kcal/mol), least root mean square deviation (RMSD) and inhibition concentrations.

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RESULTS

- **Phytochemical analysis:** It was found that Alkaloids, Proteins and Steroids were present in low concentration whereas all the other phytochemicals were present in moderate concentration. No phytochemical was present in high concentration.

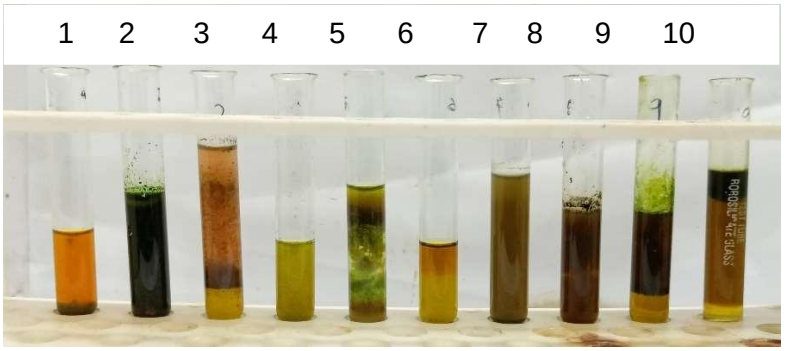

Figure 1: Phytochemical analysis

Table:1 Presence of phytochemicals:

S. No	Phytochemicals	Result
1	Alkaloids	+
2	Carbohydrate	++
3	Glycosides	++
4	Saponins	++
5	Proteins	+
6	Amino Acids	++
7	Phenolic compounds	++
8	Flavonoids	++
9	Terpenoids	++
10	Steroid	+

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- **Antioxidant analysis**

Radical scavenging activity was observed using DPPH assay and ABTS Assay. Both Methanol and Water extract were tested for antioxidant analysis. It can be stated that with increase in concentration of plant extract, antioxidant activity also increased directly. There was much increase in antioxidant activity of Methanol extract than Water extract. Between DPPH and ABTS, high antioxidant activity was observed in ABTS Assay for Methanol extract. We can infer that high number of samples contain high potential of antioxidant activity.

- **DPPH Assay**: Degree of discoloration from deep purple to yellow indicates scavenging potential.

(a) Methanol extract

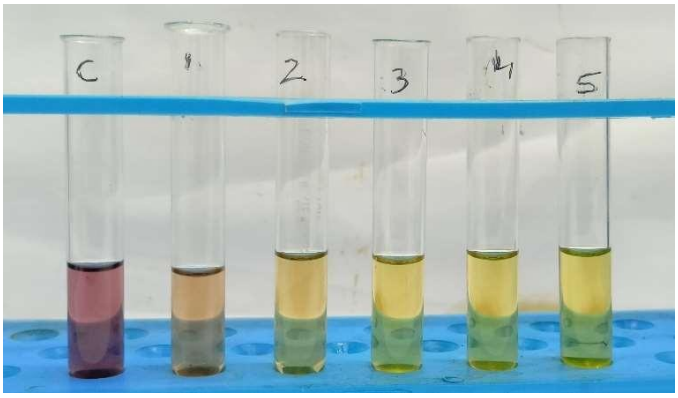

Figure 2: DPPH Assay- Methanol extract

Table 2: DPPH Assay for Methanol Extract

ıtrations (µl)	**Absorbance**	**ical scavenging Activity (%)**
Control	0.899	0
20	0.792	11.9
40	0.652	27.47
60	0.437	51.39
80	0.236	73.74
100	0.199	77.86

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(b) Water extract

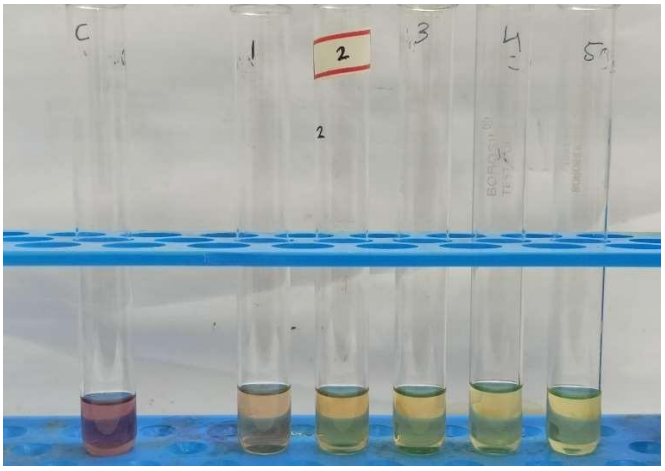

Figure 3: DPPH Assay- Water extract

Table 3: DPPH Assay for Water extract

Concentrations (µl)	Absorbance	Radical scavenging Activity (%)
Control	0.841	0
20	0.761	9.51
40	0.686	18.43
60	0.611	27.34
80	0.483	42.56
100	0.364	56.71

- **ABTS Assay**: Degree of discoloration from blue to light green indicates scavenging potential.

(a) Methanol extract

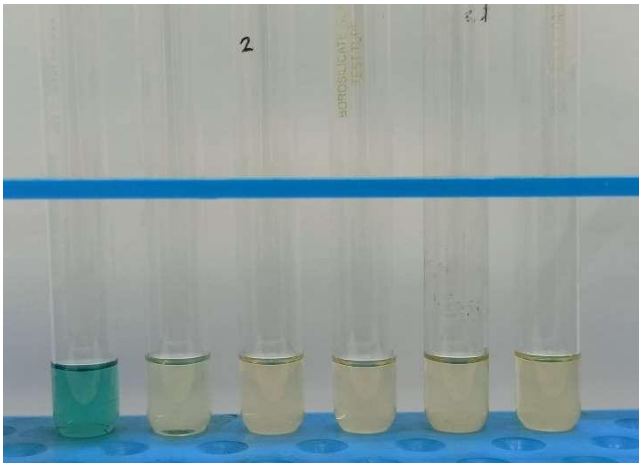

Figure 4: ABTS Assay- Methanol extract

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Table 4: ABTS Assay for Methanol extract

Concentrations (µl)	Absorbance	Radical scavenging Activity (%)
Control	0.963	0
20	0.213	77.88
40	0.178	81.51
60	0.121	87.43
80	0.094	90.23
100	0.073	92.41

(b) Water extract

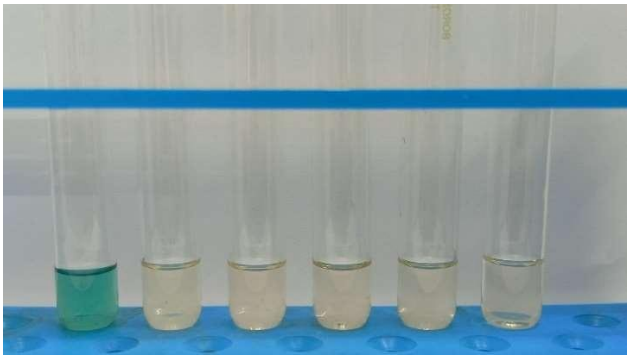

Figure 5: ABTS Assay- Water extract

Table 5: ABTS Assay for Water extract

Concentrations (µl)	Absorbance	Radical scavenging Activity (%)
Control	0.873	0
20	0.811	7.1
40	0.771	11.68
60	0.713	18.32
80	0.683	21.76
100	0.608	30.35

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Characterization:

After TLC and Column Chromatography, the fractions obtained from different concentrations were characterized using UV-vis Spectroscopy, Fourier-Transform Infrared Spectroscopy and Gas Chromatography- Mass Spectroscopy.

- **UV-vis spectroscopy**: The given fractions of different concentrations were observed under UV-vis Spectroscopy and following peaks were observed which indicated the number and quantity of bioactive compounds present in the fractions at different wavelengths.

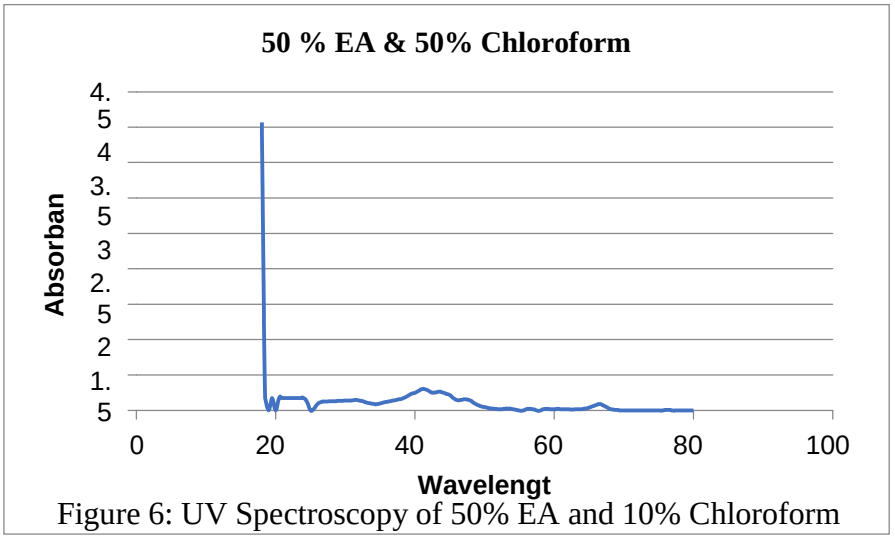

Figure 6: UV Spectroscopy of 50% EA and 10% Chloroform

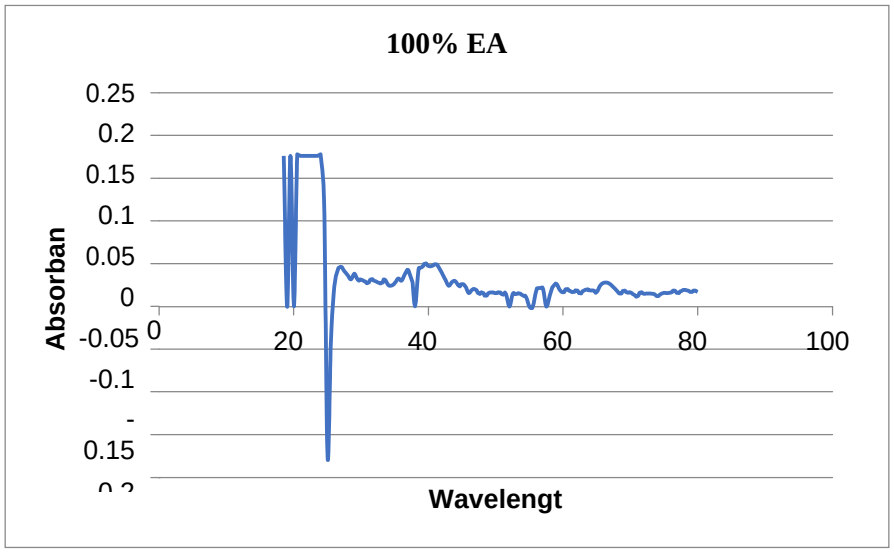

Figure 7: UV Spectroscopy of 100% EA

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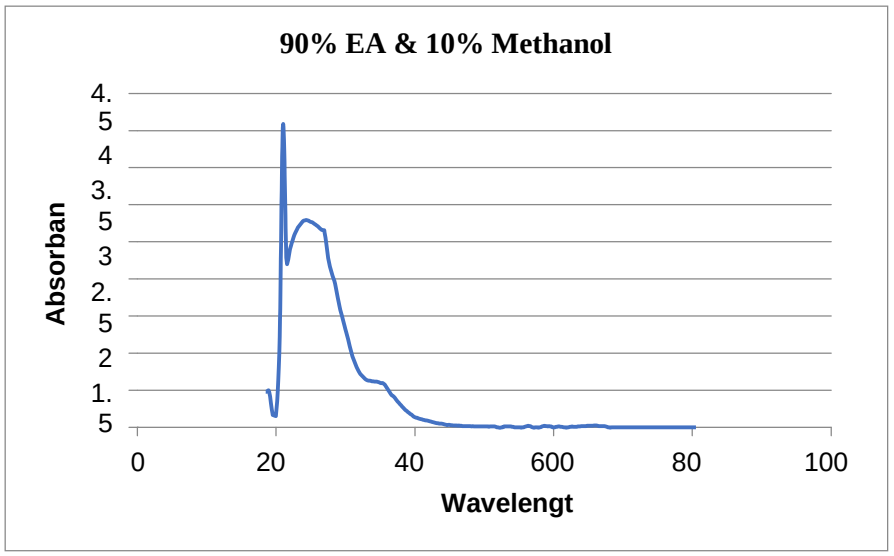

Figure 8: UV Spectroscopy of 90% EA & 10% Methanol

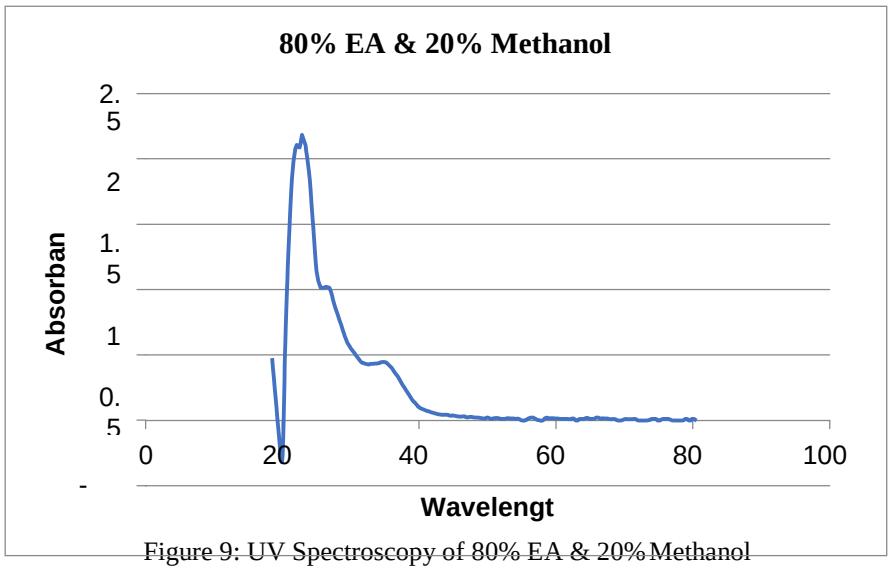

Figure 9: UV Spectroscopy of 80% EA & 20% Methanol

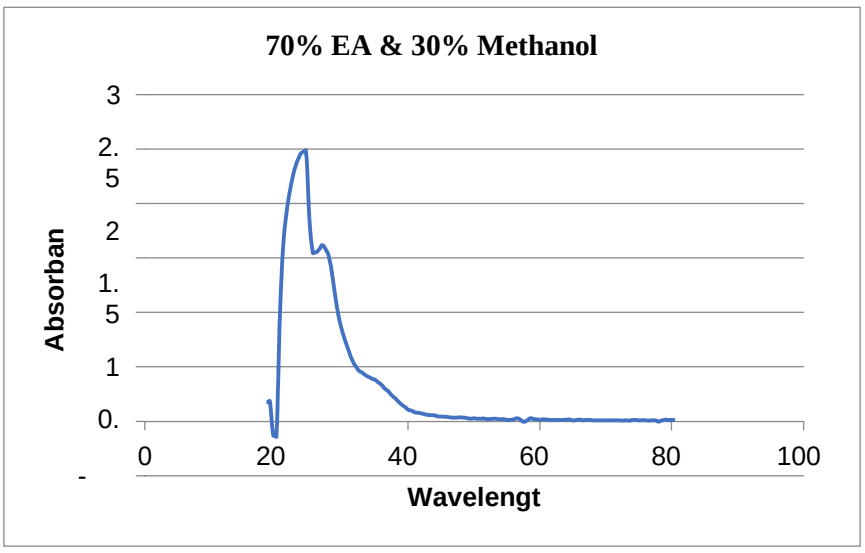

Figure 10: UV Spectroscopy of 70% EA & 30% Methanol

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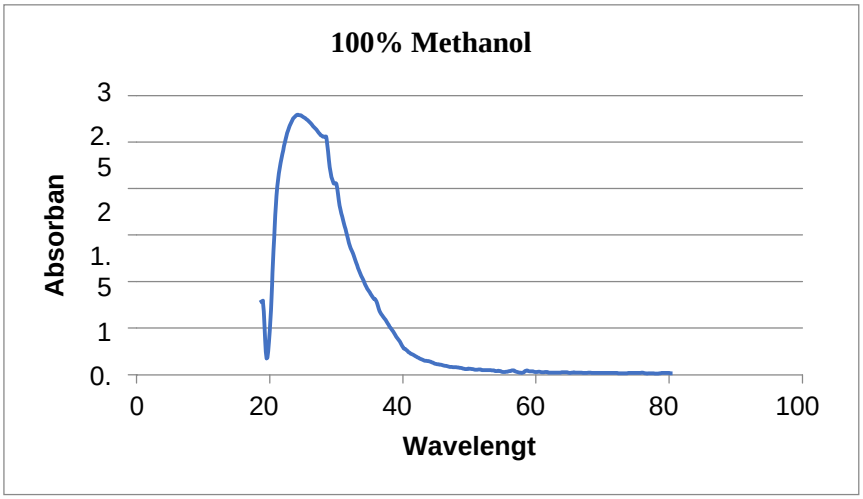

Figure 11: UV Spectroscopy of 100% Methanol

- **Fourier-Transform Infrared Radiation**: The fraction having 90% EA and 10% Methanol was tested for FTIR analysis since it showed presence of various functional groups. The FTIR graph is divided into two parts:

- 1600 - 4000 cm^{-1} functional group region
- <1600 cm^{-1} finger print region.

In functional group region 5 major peaks were observed whereas the finger print region had 4 major peaks. The following functional groups were observed: O-H stretch was observed between 3680- 3308 cm^{-1}, C-H stretch between 2974- 2834 cm^{-1}, C=O stretch was observed between 2834- 1706 cm^{-1}.
In the fingerprint region, the functional groups observed were: C=C at 1592 $cm^{-1,}$ C-O group at 1015 cm^{-1} and C-Br at 634 $cm^{-1.}$

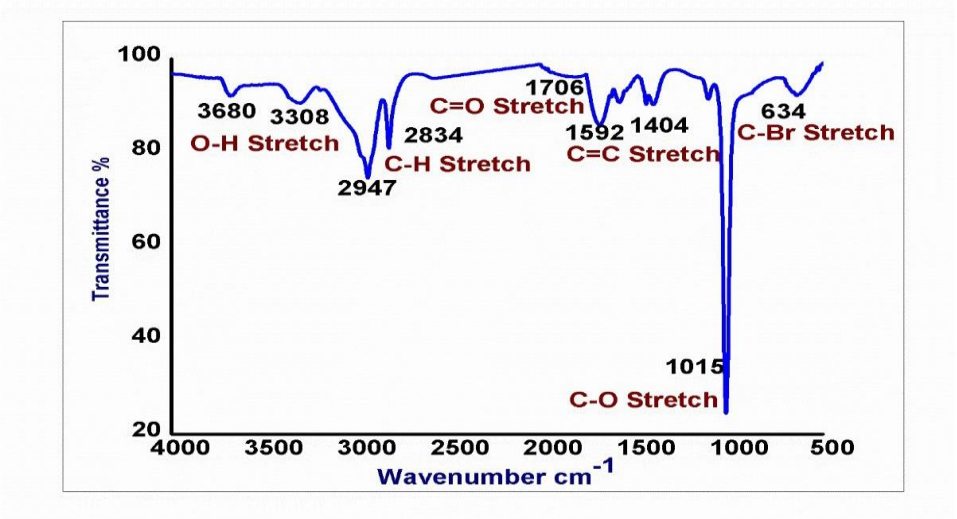

Figure 12: FTIR analysis of 90% EA and 10% Methanol

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- **Gas chromatography- Mass Spectroscopy**: The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. It indicated presence of many bioactive compounds out of which only few were in high concentration. Benzoic acid, 4-ethoxy-, ethyl ester was the highest in concentration with 14.12% of area coverage with a retention time of 13.719 minutes. Hence, it was selected as the bioactive drug for breast cancer.

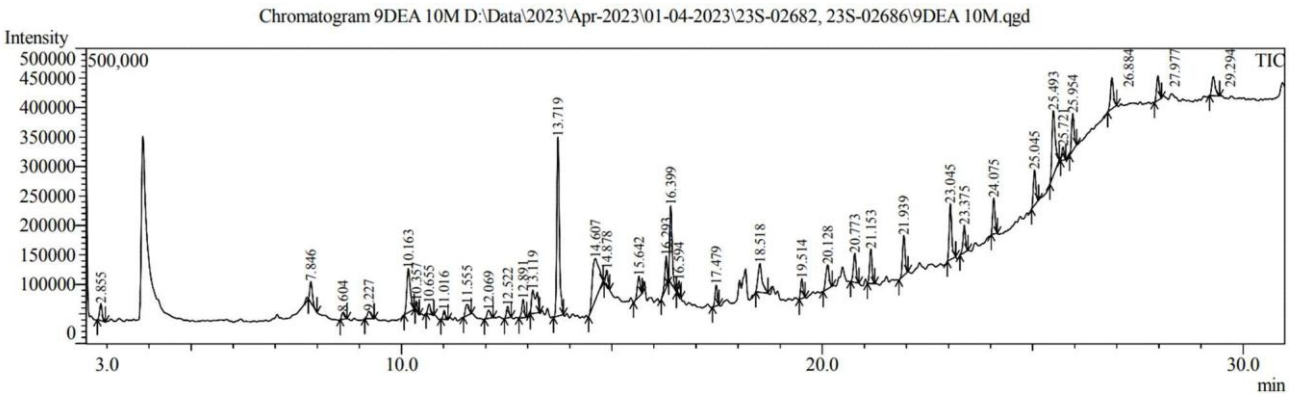

Figure 13: GCMS analysis of 90% EA and 10% Methanol

Cytotoxicity and Cell Viability: MTT Assay

(a)Vero cell line: The fraction 90EA & 10 M containing Benzoic acid, 4-ethoxy-, ethyl ester was loaded in the Vero cells in increasing concentration of 20 µl, 40 µl, 60 µl, 80 µl, and 100 µl to observe the cytotoxicity and side effects. There are negligible side effects as the cell viability decreased only 9% approximately. The cells were observed under inverted microscope.

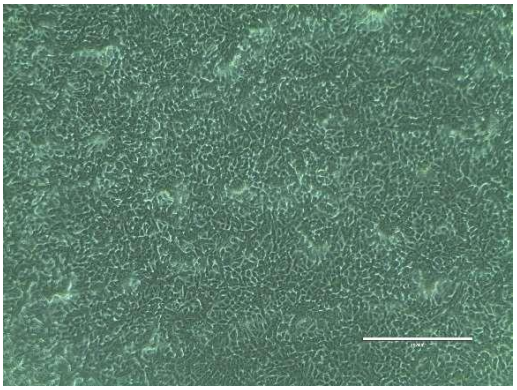

Figure 14: Vero Cells – Control

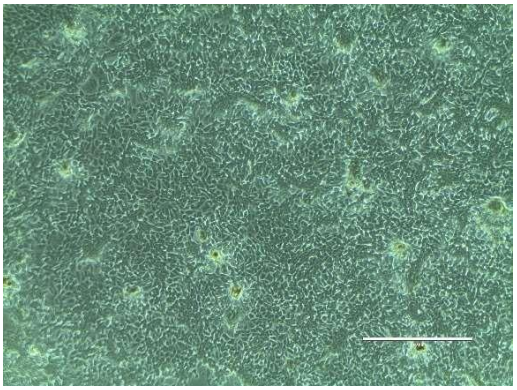

Figure 15: Vero Cells – 20 µl

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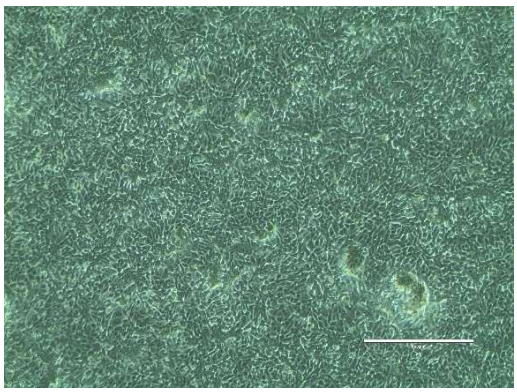

Figure 16: Vero Cells – 40 µl

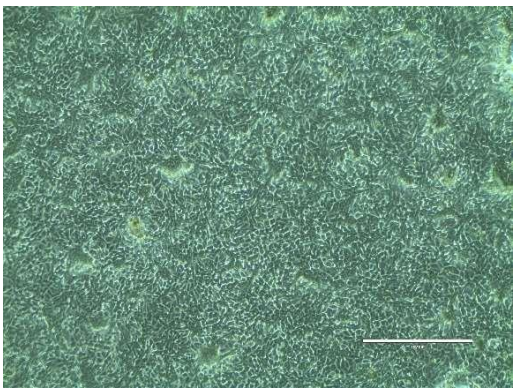

Figure 17: Vero Cells – 60 µl

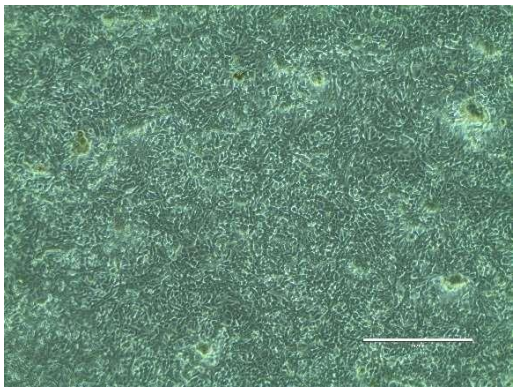

Figure 18: Vero Cells – 80 µl

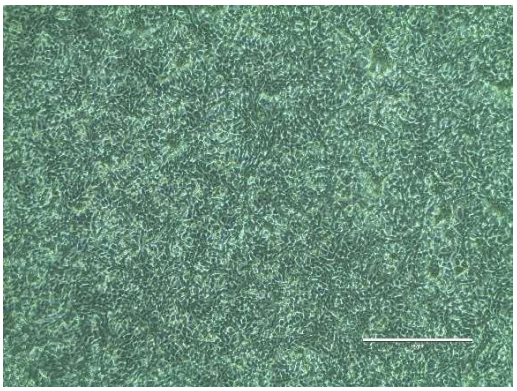

Figure 19: Vero Cells – 100 µl

(b)**MDA-MB-231 cell line**: The fraction 90EA & 10 M containing Benzoic acid, 4-ethoxy-, ethyl ester was loaded in the breast cancer cell line in increasing concentration of 20 µl, 40 µl, 60 µl, 80 µl, and 100 µl to observe the cytotoxicity level. It can be observed that the cell viability has decreased by 85% and there are visible morphological changes in the cell shape as the cells have shrunken which indicates that the bioactive compound Benzoic acid, 4-ethoxy-, ethyl ester is effective against breast cancer. The cells were observed under inverted microscope.

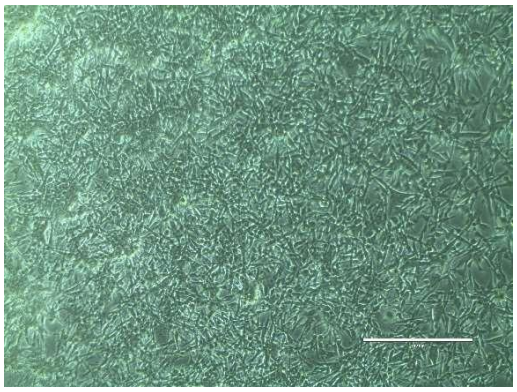

Figure 20: Breast cancer cells- control

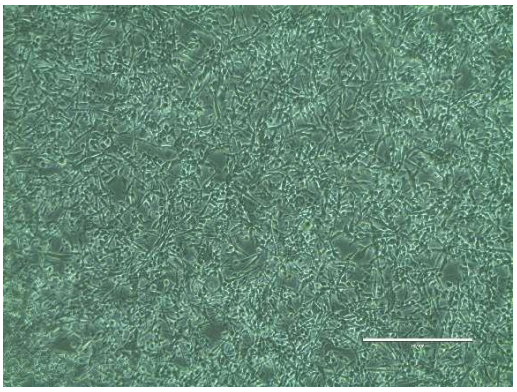

Figure 21: Breast cancer cells- 20 µl

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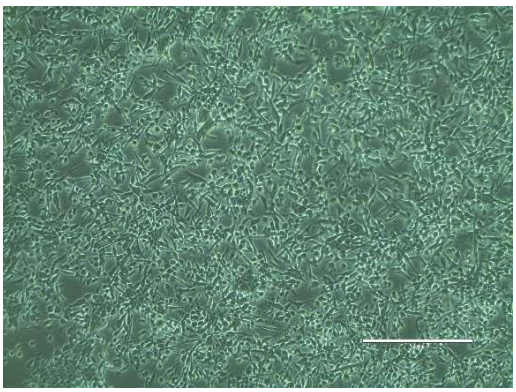

Figure 22: Breast cancer cells- 40 µl

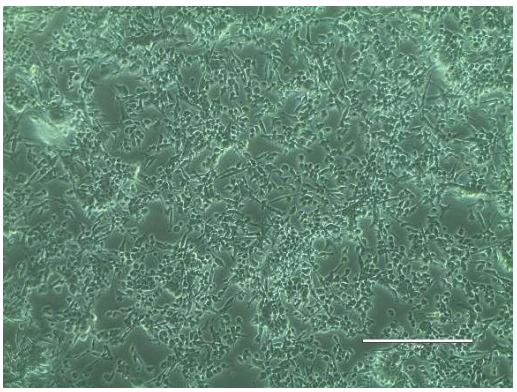

Figure 23: Breast cancer cells- 60 µl

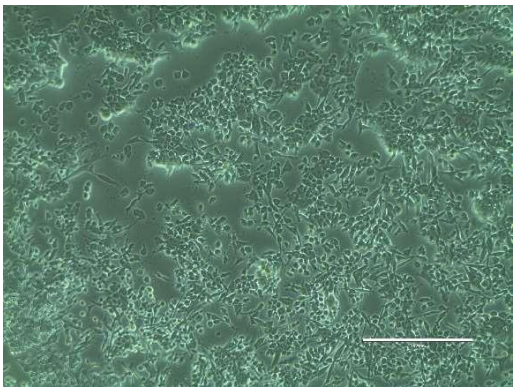

Figure 24: Breast cancer cells- 80 µl

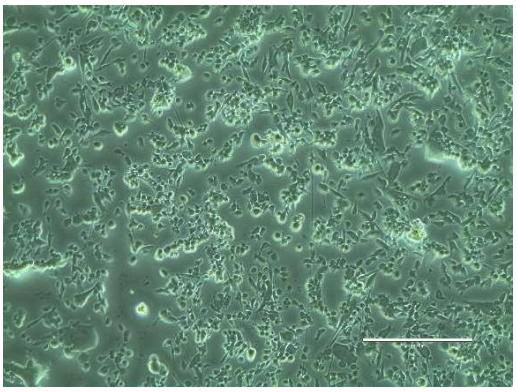

Figure 25: Breast cancer cells- 100 µl

MTT Assay: The cells treated with MTT dye were observed under Multi-well Spectrophotometer and absorbance was measured.

Table 6: MTT Assay for Vero cell line

Concentrations (µL)	Absorbance		Average	Cell Viability (%)
	I	II		
Control	0.876	0.883	0.8795	100
20	0.861	0.867	0.864	98.23763502
40	0.85	0.856	0.853	96.98692439
60	0.838	0.824	0.831	94.48550313
80	0.822	0.811	0.8165	92.83683911
100	0.8	0.803	0.8015	91.13132462

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Table 7: MTT Assay for MDA-MB-231 Cell line

ntrations (µL)	Absorbance		Average	Cell Viability (%)
	I	II		
Control	0.589	0.576	0.5825	100
20	0.467	0.461	0.464	79.65665236
40	0.346	0.338	0.342	58.71244635
60	0.21	0.217	0.2135	36.65236052
80	0.154	0.143	0.1485	25.49356223
100	0.098	0.088	0.093	15.96566524

Apoptosis Analysis: Apoptosis is a tightly controlled form of cell suicide that is known to be the main mechanism for cells to die in response to cytotoxicity. It is characterised by a number of phenotypic and cellular changes, including membrane blebbing, chromatin condensation, DNA fragmentation, and cleavage of essential proteins. The results revealed a rise in early apoptotic cells, which had a light green nucleus, and late apoptotic cells, which had vivid orange patches of condensed chromatin, which set them apart from necrotic cells with orange-reddish fluorescence. Because of the lack of membrane integrity caused by the bioactive compound, necrotic cells are luminous red.

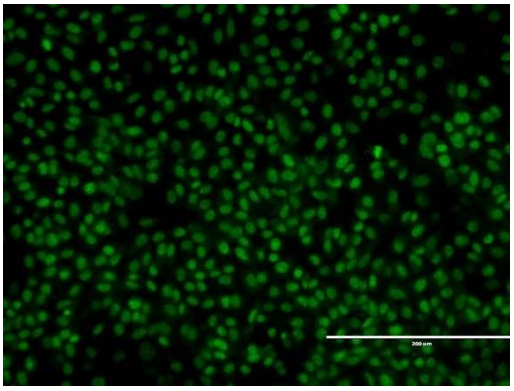

Figure 26: Control cell stained with AO

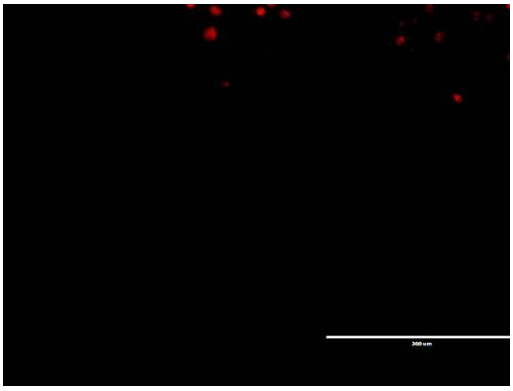

Figure 27: Control cell stained with PI

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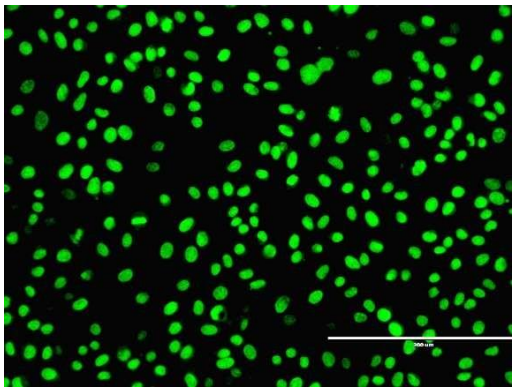

Figure 28: Treated cell stained with AO

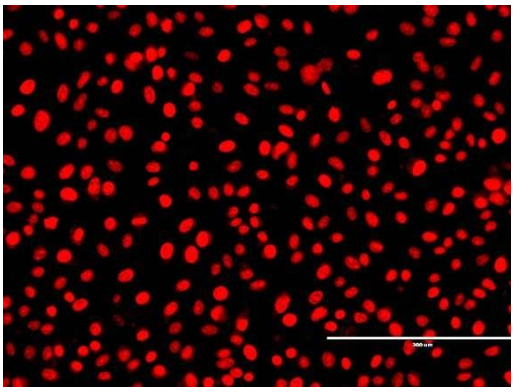

Figure 29: Treated cell stained with PI

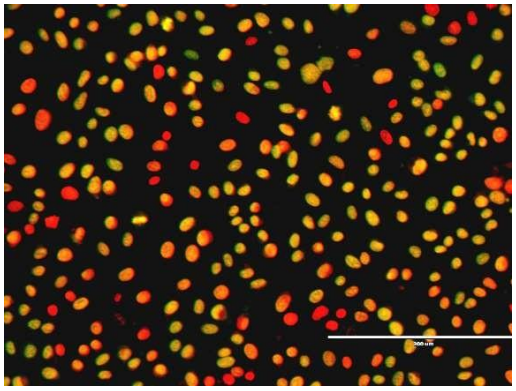

Figure 30: Treated cell stained with AO and PI

Molecular docking: Molecular docking was done between Benzoic acid, 4-ethoxy-, ethyl ester which acted as the ligand and Progesterone Receptor (PR) which acted as the target protein. PR is regulated by estrogen and estrogen receptor and leads to Breast cancer when highly expressed.There are 15 active residues in the PR binding pocket. Two hydrogen bonds were formed between Benzoic acid, 4-ethoxy-, ethyl ester and two of the active residues in PR. One of the bonds is formed between amine group of Arginine and carboxyl oxygen and another being between amine group of Glycine and carboxyl oxygen with same binding energy of -5.87 kcal/mol. Out of the 100 conformations, the given conformation is the best pose. Therefore, it can be said that Benzoic acid, 4-ethoxy, ethyl ester can be successfully integrated into PR for the overall anticancer and apoptotic effect.

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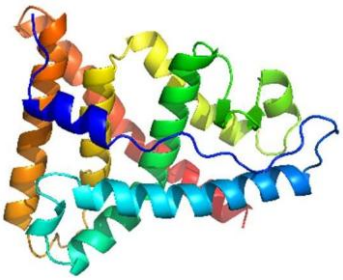

Figure 31: Structure of PR

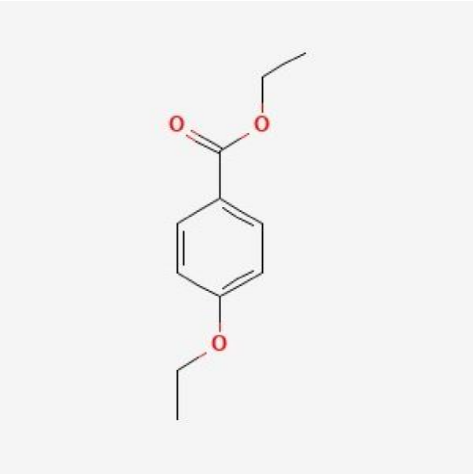

Figure 32: Molecular structure of Benzoate ester

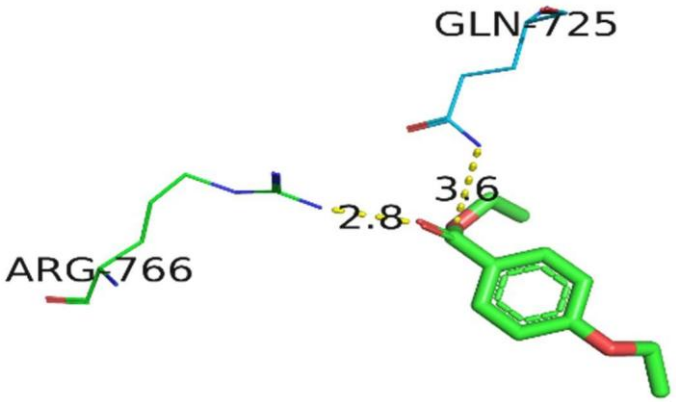

Figure 33: Docked structure of Benzoate ester with PR

Table 8: Properties of Benzoate ester

S. No	Property	
1	PubChem CID	90232
2	Molecular Formula	C11H14O3
3	Molecular weight	194.23

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Table 9: Active Binding Sites of PR

S. No	Active Binding Sites
1	ARG-766
2	ASN-719
3	CYS- 891
4	GLN-725
5	GLU- 723
6	GLY- 722
7	LEU- 715
8	LEU- 797
9	LEU-721
10	MET- 795
11	MET- 801
12	PHE-778
13	PHE-794
14	THR-894
15	TRP- 755

Table 10: Hydrogen bonds formed between residues

S. No	Binding energy (kcal/mol)	H bond interaction (D-H…A)	Distance (A^o)
1	-5.87	GLN-725 (N-H…O)	3.6
2		ARG-766 (N-H…O)	2.8

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DISCUSSION

Breast cancer is the most common type of cancer and the second leading cause of death among women aged 45 to 55. Approximately one in eight women will develop breast cancer, which is typically treated with total tissue excision, chemotherapy, radiation, and hormone therapy. The disease is characterized by uncontrolled growth of epithelial cells originating in the breast ducts or lobules. Breast cancer encompasses early, non-invasive breast cancer, such as ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS), primary invasive breast cancer that has spread to the surrounding breast tissue, and advanced or metastatic breast cancer that has spread to the lymph nodes or other parts of the body. Although synthetic drugs are effective in treating this condition, they can have adverse effects on normal cells, leading to various other diseases and associated side effects. As a result, there is a great demand for plant-derived drugs and medicines to prevent and manage cancer metastasis. Chemoprevention is the best way to integrate biocompatible plant-derived drugs as a breast cancer treatment. *Mimusopselengi* has many therapeutic properties such as anti-inflammatory, anti-bacterial, anti-ulcer, anti- hyperglycemic, antioxidant, wound-healing, analgesic, and antipyretic properties. In the study conducted *M.elengi* showed excellent antioxidant property against DPPH and ABTS assay being 77.86% and 92.41% respectively for methanol extracts. Benzoic acid, 4-ethoxy-, ethyl ester, the bioactive compound isolated from the leaves extract of *M.elengi* which proved to be effective against breast cancer cell line MDA-MB-231. When administered, this compound significantly reduced the viability of cancer cells by 85%, causing them to shrink in size, while leaving normal cells unharmed with negligible side effects or morphological changes.Apoptosis was analysed using AO/PI staining method. Molecular docking of Benzoate ester and Progesterone receptor was successful and produced a stable conformation by making two hydrogen bonds with Arginine and Glycine of PR amino acid chain. This suggests that benzoate ester can be administered for hormone-related breast cancer. PR is a transcription factor that regulates gene expression by binding to specific DNA sequences, modulating the expression of various genes involved in cellular differentiation and proliferation. Dysregulation of PR signalling has been implicated in various reproductive disorders, endometriosis, and breast cancer. PR is a common target for hormonal therapies for breast cancer, as blocking its activity can slow or stop the growth of breast cancer cells.

Based on the research conducted, it can be inferred that *Mimusops elengi* has potential as an effective agent in the treatment of breast cancer as well as other types of cancer. Besides its anticancer properties, it is already widely used as a traditional medicine and possesses other medicinal properties.

ISSN PRINT 2319 1775 Online 2320 7876
 UGC CARE Listed (Group -I) Journal Volume 11, Iss 10, 2022

SUMMARY AND CONCLUSION:

Mimusops elengi, also known as Spanish cherry, bullet wood, and tanbark tree, is a small to large evergreen tree that is distributed throughout India, Southeast Asia, and parts of Africa. The tree can grow up to 40 meters in height, with a straight trunk and a dense, roundedcrown. Various parts of the *Mimusops elengi* plant have been reportedly used in traditional medicine for various purposes. The bark has antibacterial, anti-ulcer, anti-hyperglycemic, wound- healing, anti-inflammatory, analgesic, and antipyretic properties, while the leaves have antioxidant activity. The fruit of the plant also contains antibacterial and antioxidant properties. *Mimusops elengi* is also of interest to researchers due to its potential therapeutic properties, including its potential as an anti-cancer agent. Studies have shown that the plant contains a number of triterpenoids, steroids, steroidal glycosides, flavonoids, and alkaloids, which may contribute to its pharmacological effects.

The methanolic extract of *Mimusops elengi* leaves contains several pharmacologically beneficial phytochemicals and other substances, including benzoate ester, which has been found to be effective against breast cancer cells. To isolate the bioactive compound, column chromatography was used followed by characterization using UV-vis spectroscopy, FTIR, and GC-MS. The bioactive compound was then administered to the MDA-MB-231 breast cancer cell line and produced impressive results. As the concentration of the bioactive compound increased, the cancer cells shrank and lead to apoptosis. The observed cytotoxicity for the cancer cell line was 85%, while that for the Vero cell line was only 9%, and side effects were negligible.

From successful molecular docking Benzoate ester was found to form two hydrogen bonds with Arginine and Glycine of the Progesterone receptor (PR) amino acid chain, indicating that it may be useful in treating hormone-related breast cancer. PR is a transcription factor that plays a role in the regulation of gene expression by binding to specific DNA sequences and modulating the expression of genes involved in cellular differentiation and proliferation. Abnormal PR signalling has been linked to reproductive disorders such as infertility, endometriosis, and breast cancer, making it a common target for hormonal therapies in breast cancer treatment.

The results of this research suggest that *Mimusops elengi* could be an effective chemo- preventive agent in the treatment of breast cancer and other cancers, in addition to its use as a traditional medicine and possession of other medicinal properties.

ISSN PRINT 2319 1775 Online 2320 7876
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