https://africanjournalofbiomedicalresearch.com/index.php/AJBR
Afr. J. Biomed. Res. Vol. 27(4s) (November 2024); 1929-1934
Research Article

Beta-Sitosterol and Stigmasterol as Synergestic Anticancer Potential in Breast Cancer Isolated from Euphorbiaceae Family Plants

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Abstract:
This study explores the potential anticancer properties of phytosterols like beta-sitosterol and stigmasterol isolated from *Euphorbia Pulcherrima* leaves and *Ricinus communis* seed with synergestic effect. Mechanism of Beta-sitosterol suppresses angiogenesis and metastasis and induces apoptosis and cell cycle arrest. It also inhibits cell proliferation, migration, and invasion.While Stigmasterol suppresses the NF-κB and PI3K/Akt signalling pathways while inducing autophagy and death. Additionally both exhibit wound healing activity, antibacterial, antiulcer, anticonvulsant, antioxidant, and anti-inflammatory properties. Both phytosterols have been shown to be effective against various breast cancer cell. The *in-vitro* anticancer activity of β-sitosterol and stigmasterol, as well as their combination shows synergistic effect against the breast cancer cell lines MCF-7 (hormone receptor-positive) Inhibit cell proliferation and induce apoptosis and MDA-MB-231 (hormone receptor-negative) Suppress cell migration and invasion.along with their percentage inhibition using the MTT at several concentration ranges between
6.12 µg/ml and 100 µg/ml. At a dosage of 100 µg/ml, its combination exhibits the maximum percent inhibition. A bliss independence model was used to examine the synergistic action of β- sitosterol and stigmasterol. Because of their low toxicity and natural origin, they are interesting to candidates for combination therapy with other anticancer agents. These findings suggest that phytosterols may be effective against multiple breast cancer subtypes, To completely comprehend their causes and treatment approaches, more investigation is required.

Keywords: β-Sitosterol, Stigmosterol, synergism, Breast cancer.

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Received: 29/10/2024 *Accepted: 15/11/2024*

DOI: https://doi.org/10.53555/AJBR.v27i4S.3966

Introduction:

One of the main risks to human health and survival is tumor, which has a high rate of recurrence and mortality. [1] Among women, breast cancer is the second most common cause of death due to cancer. One of the most prevalent malignancies in the world is breast cancer [2]. Over the past 20 years, research on the disease has significantly advanced our knowledge of it, leading to the development of less harmful and more effective treatments. Breast tissue tumors, usually from the lobules that provide milk to the ducts or the inner lining of the milk ducts. [3] "Early" breast cancer refers to cancer that is detected and amenable to surgical removal. On the other hand, cancer cells that go undiagnosed can grow and eventually kill a person [4]

The underlying biological mechanisms are now being considered when making treatment options, which in the past were mostly centered on the anatomic extent of the disease. [5] Natural substances called phytosterols can be found in nuts, seeds, and vegetable oils, or they can be added to consumer food products. The incidence and prognosis of certain cancers are inversely correlated with the consumption of phytosterols.[6] Analogs of cholesterol, phytosterols or plant sterols are present in plant cells. More than 250 different phytosterols have been found, in addition to sitosterol, stigmasterol, and campesterol.[7] phytosterols suggested that they might prevent our diets from absorbing cholesterol by acting as a steric barrier. [8] By lowering intestinal cholesterol absorption, phytosterol ingestion lowers blood LDL cholesterol levels and lowers the risk of cardiovascular disease.The possible impacts and mechanisms of action of phytosterols on cancer types. A significant amount of phytosterols reduce the growth of stomach, ovarian, lung, and breast cancers. A variety of modes of action appear to be employed by phytosterols, including the stimulation of malignant cell death and the prevention of carcinogen formation, cancer-cell proliferation, angiogenesis, invasion, and metastasis [9] The Euphorbiaceae family is a significant one that includes a variety of therapeutic plants.[10] A phytochemical analysis of the Euphorbiaceae family revealed the presence of glycoproteins, lectins, phorbol esters, diterpenes, triterpenes, sterols, and saponins[11].Usually therapeutic plants with high levels of naturally occurring phytoestrogens, or chemicals that resemble estrogen. These have been demonstrated to have a variety of impacts on people, such as the prevention of malignancies [12] and the enhancement of brain function [13].It was proposed that eating foods high in phytoestrogens could lower the risk of contracting breast cancer [14] While the MCF-7 breast cancer cell line's in vitro proliferation is inhibited by dietary phytoestrogen ingestion [15] One recognized phytosterol with estrogen-mimicking properties is β-sitosterol [16.] BS has angiogenic, antioxidant, antimicrobial, angiogenic, antioxidant, immunomodulatory, antidiabetic, anti-inflammatory, anticancer, and antinociceptive properties [17]. Primarily through promoting apoptosis, triggering cell cycle arrest, controlling oxidative stress, increasing metabolic reprogramming, preventing invasion and metastasis, adjusting immunology and inflammation [18]

Stigmasterol is an unsaturated phytosterol belonging to the class of tetracyclic triterpenes. It is one of the most common plant sterols, found in a variety of natural sources, including vegetable fats or oils from many plants.stigmasterol has been examined via in vitro and in vivo assays and molecular docking for its various biological activities on different metabolic disorders. The findings indicate potent pharmacological effects such as anticancer, anti-osteoarthritis, anti-inflammatory, anti-diabetic, immunomodulatory, antiparasitic, antifungal, antibacterial, antioxidant, and neuroprotective properties [19] stigmasterol has become a unique compound due to its diverse pharmacological properties [20]

Nowadays, the mainstays of medical care are targeted medicines and chemotherapeutic drugs. Because tumors may be innately (denovo) resistant to the medications, patients may not always react to these treatments. Moreover, those who show initial response to treatment are prone to develop resistance over time, which could lead to treatment failure or a recurrence of the disease. Multiple chemotherapeutic drugs with unique pharmacological mechanisms are utilized in combination therapy to improve clinical results. most widely used reference models, which provide a mathematical foundation for accurately evaluating drug interactions in combination models, are used to assess synergism in drug combinations. most often used reference models, which provide a mathematical framework to accurately evaluate drug interactions in combinationmodels and assess synergism in medication combinations. [21]

These techniques evaluate a beneficial interaction effect based on the actions of each individual medicine in a combination. There are four primary tactics that are effect-based.Response additivity, highest single agent, combination subthresholding, and bliss independence model [22] Assuming that medications act on distinct sites of action, this model suggests that both pharmaceuticals used in combination drugs act independently and do not interfere with one another. However, this model makes the assumption that the observed impact is a result of both medications. When the calculation index for this method is less than, greater than, or equal to 1, respectively, it indicates synergy, antagonistic,additivity [21, 22].

Material and method

The plant of *Euphorbia pulcherrima* plant and seed of *Ricinus communis* seed were collected from the Nature Garden -Akluj and plant were authenticated at the Botanical Survey of India Pune (Maharashtra). . The cancer cell line MCF-7 and MDA-MB-231 was purchase from the National Centre for Cell Science (NCCS) Pune.The cells were cultured at a temperature of 37°C within a humidified atmosphere containing 5% CO_2 after confluence level achieving 80-90%, the cells were used in assays. In a Soxhlet extractor, the plant *Ricinus communis* seed powder and leaves of *Euphorbia Pulcherrima* (powder) was progressively extracted with water, ethanol, and petroleum ether.

The filtrate was collected and then evaporated under reduced pressure and dry. Extract was fractionated with the help of coloum chromatography and isolate the compound from Ether extract of E. Pulcherrima (L) leaves and *Ricinus communis* seeds by using solvent system such as Hexane: ethyl acetate in ratio 7: 3 and Pet. ether: Ethyl acetate: Acetonitrile were in 8.2:1.8:0.1 ratio and their confirmation was done by TLC and various spectral data like HPTLC,FTIR,MASS,NMR etc.

Material and Method:

MTT assay

MCF-7 and MDA-MB-231 cell lines were chosen for *in vitro* anticancer potential screening. The cell viability was determined using MTT assay. Cells were incubated at a concentration of 1 × 104cells/ml in culture medium for 24 h at 37°C and 5% CO_2. Cells were seeded at a concentration (70µl) 104 cells/well in 100 µl culture medium and 100µl Betasitosterol and Stigmasterol and its combination compounds (6.10, 12.5, 25, 50, 100 µg/ml) into micro plates respectively (tissue culture grade, and 96 wells). Control wells were incubated with DMSO (0.2% in PBS) and cell line. All samples were incubated in triplicate. Controls were maintained to determine the control cell survival and the percentage of live cells after culture. Cell cultures were incubated for 24 h at 37°C and 5% CO_2 in CO_2 incubator (Thermo scientific BB150). After incubation, the medium was completely removed and Added 20 µl of MTT reagent (5mg/min PBS). After addition of MTT, cells incubated for 4 hrs at 37 ^{0}C in CO_2 incubator. Observed the wells for formazan crystal formation under microscope. The yellowish MTT was reduced todark coloured formazan by viable cells only. After removing the medium completely. Added 200µl of DMSO (kept for 10 min) and incubate at 37^{0}C (wrapped with aluminium foil). Triplicate samples were analyzed by measuring the absorbance of each sample by a Elisa microplate reader (Benesphera E21) at a wavelength of 570 nm [23,24]

Bliss independence for synergism

The Bliss independence model is widely used to analyses drug combination data when screening for drug combinations i.e, Antagonism, Additivity and Synergy [25]

BLISS formula

CI = EA+EB-EAEB/EAB

Where,

EAB = EA+EB (1-EA)

EA-Effect of Beta, EB-Effect of Stigma CI-Combination index

While the Synergestic effect indicated as CI<1, Antagonism as CI>1 and Additive effect as CI=1.[26]

Result

MTT assay Percent inhibition against MCF-7

Table no. 1 Summary of MTT assay Percent inhibition against MCF-7

Concentration (µg/ml)	6.12	12.5	25	50	100
Betasitosterol (% Inhibition)	53.67	63.96	69.94	75.88	77.33
Stigmasterol (% Inhibition)	18.24	29.18	40.41	52.6	60.57
Combination (% Inhibition)	41.52	49.82	76.8	86.78	89.67

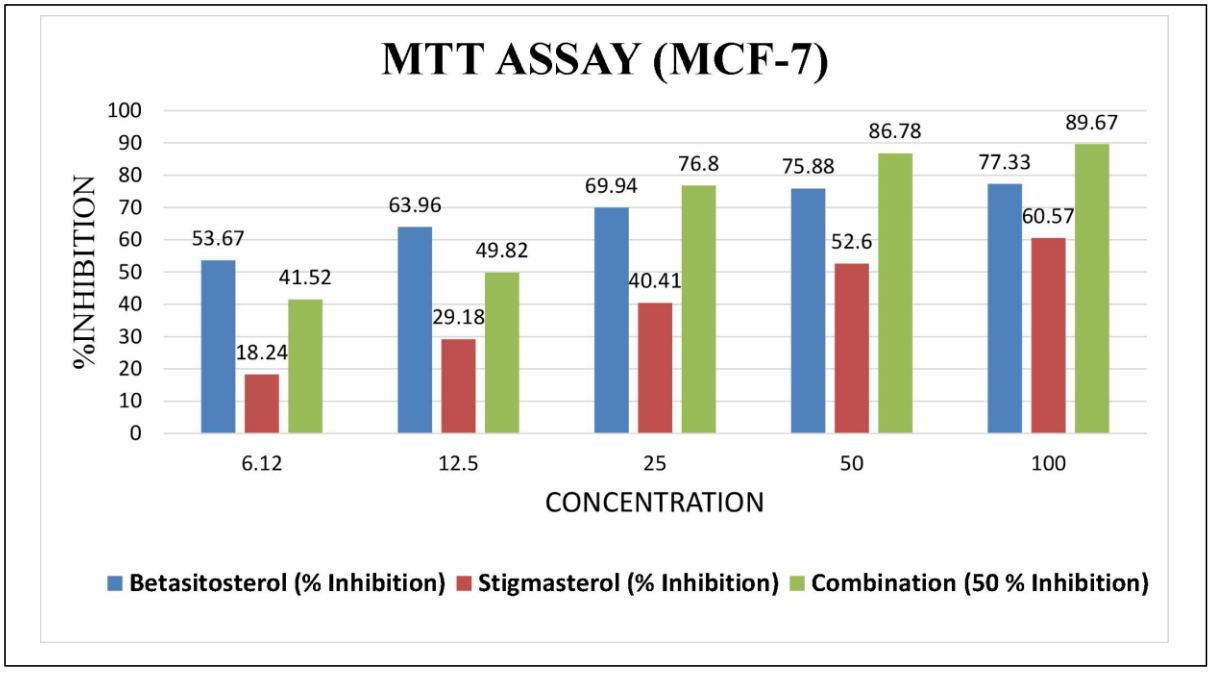

Fig.no.1 Comparative data of MTT assay (MCF-7 cell line)

MTT assay Percent inhibition against MBA-MB-231

Table no. 2 Summary of MTT assay Percent inhibition against MBA-MB-231

Concentration (µg/ml)	6.12	12.5	25	50	100
Betasitosterol (% Inhibition)	51.59	61.66	69.7	74.99	75.82
Stigmasterol (% Inhibition)	10.93	24.58	34.91	52.15	62.21
Combination (% Inhibition)	41.14	44.5	77.15	86.51	89.07

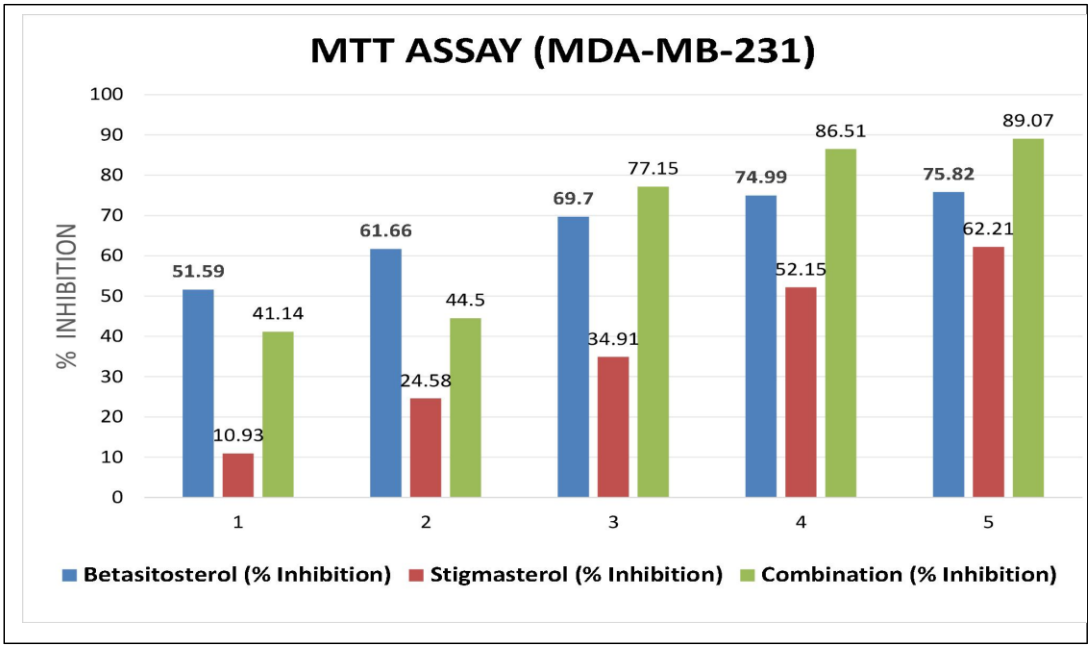

Fig.no.2 Comparative data of MTT assay (MDA-MB-231 cell line)

Bliss independence (MTT assay MCF-7)

Table no. 3 Bliss independence Expected and Observed effect (MTT assayMCF-7)

Concentration (µg/ml)	Expected effect	Observed effect	Combination index (CI)
6.1	35.95	41.52	0.2
12.5	46.57	49.82	0.3
25	55.17	76.8	0.3
50	64.24	86.78	0.4
100	68.94	89.67	0.4

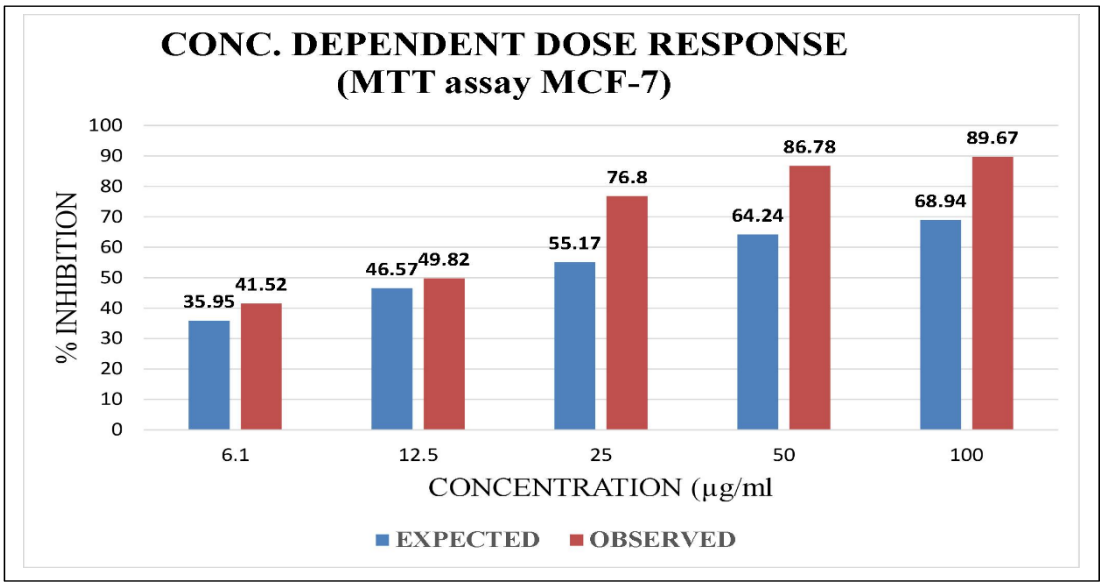

Fig.no.3 Concentration dependent dose response (MTT assay using MCF-7)

Bliss independence (MTT assay MDA-MB-231)

Table no. 4 Bliss independence Expected and Observed effect (MTT assayMDA-MB-231)

Concentration (µg/ml)	Expected effect	Observed effect	Combination index (CI)
6.1	36.72	41.14	0.2
12.5	43.12	44.5	0.2
25	52.3	77.15	0.3
50	63.56	86.51	0.3
100	69.01	89.07	0.4

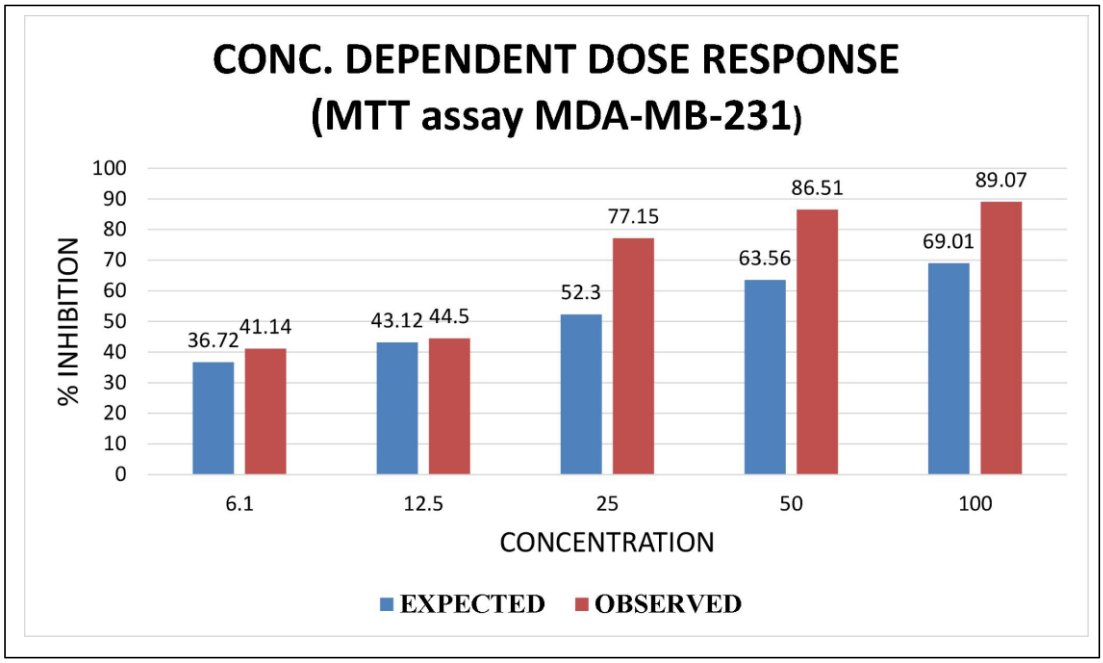

Fig.no.4 Concentration dependent dose response (MTT assay usingMDA-MB-231)

In vitro anticancer cell line studies of βesitosterol and stigmasterol were carried out using the MCF-7 and MDA-MB-231 cell lines. For estimation of % inhibition, the MTT assay method were implemented. During the investigation of the MTT assay for the MCF-7 cell line, stigmasterol and their combination in a 1:1 ratio were screened between concentrations of 6.12 µg/ml to100 µg/ml. Betasitosterol at 100 µg/ml shows 77.33% inhibition, stigmasterol at 100 µg/ml shows 60.57% inhibition, while a combination of both drugs at 100 µg/ml shows 89.67% inhibition. On the other hand, the MTT assay for the MDA-MB-231 cell line Betasitosterol at 100 µg/ml shows 75.82 % inhibition, while at 100 µg/ml shows 62.21% inhibition, while a combination of both drugs at 100 µg/ml shows 89.07% inhibition.

Conclusion

βesitosterol, stigmasterol, and its combination in a 1:1 ratio for its in vitro breast cancer potential was carried out using the MCF-7 and MDA-MB-231 cell lines. Findings suggested that at100 µg/ml it shows the highest percent inhibition. This screening is done by MTT assay. Moreover, synergism between β-sitosterol, and stigmasterol was investigated using the dose-dependent Bliss independence model, and the finding concludes that β-sitosterol, and stigmasterol in combination 1:1 ratio show remarkable anti-cancer potential.

Acknowledgement

The authors acknowledge College of Pharmacy, Akluj for providing laboratory facilities and support.

Conflict of interest – There is no conflict of interest declared by authors

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