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Asarone Possesses Antiproliferative Potential in Breast Cancer Cell Line (MCF-7) Through Via Apoptosis and Inflammatory-Mediated Signaling Pathways

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Abstract

Breast cancer is a significant global health challenge, requiring continuous exploration of new treatments. Asarone, a bioactive compound from the Acorus genus, shows promising anticancer properties but its effects on breast cancer cells are underexplored. This study investigates asarone's anticancer potential against breast cancer cell lines using in vitro and in silico approaches. Asarone's antioxidant activity was evaluated using DPPH radical scavenging assays, revealing a dose-dependent (25.56, 32.18, 47.73, 54.83 and 66.74%) effect on free radicals. MTT assays showed a dose-dependent decrease in cell viability, indicating asarone's cytotoxicity towards breast cancer cells. mRNA expression analysis showed that targeting apoptosis regulators such as Bax (1, 1.3, 1.52 fold change upregualtion) and Bad (1, 1.4, and 1.6 fold upregulation) gene expression demonstrated that asarone induces apoptosis via the intrinsic pathway. Additionally, asarone inhibited Akt mNRA (1, 0.6, and 0.4 fold change down regulation), caspase-3 (1, 1.4, and 1.7 upregulation) and cytochrome-c mRNA (1, 1.2 and 1,54 fold change upregulation) suggesting interference with key cancer progression pathways. Molecular docking studies predicted favorable binding interactions between asarone and crucial proteins involved in apoptosis and cell survival, including Bax, Bad, cytochrome c, caspase 3, and Akt. These findings collectively highlight the multifaceted anticancer mechanisms of asarone against breast cancer cells. This study underscores the potential of asarone as a natural therapeutic agent for breast cancer, offering avenues for further exploration in translational research and clinical trials. The current study significantly advances our understanding of asarone's anticancer properties, offering promising directions for developing new and effective breast cancer therapies.

Keywords: *Asarone, Apoptosis, Breast Cancer, Health and well-being, Inflammation, Public Health.*

Introduction

Breast cancer is still a major global health concern, and various treatment approaches are always being investigated to maximize therapeutic benefit and reduce adverse effects. Although it affects both men and women equally, breast cancer is one of the most frequent malignancies worldwide. Important information about the incidence, prevalence, risk factors, and death rates related to breast cancer may be found in epidemiological research [1]. Globally, incidence rates differ greatly, with industrialized nations showing greater rates. Variations in occurrence are caused by a number of factors, including age, gender, ethnicity, genetic susceptibility, hormonal factors, lifestyle, and environmental effects. Breast cancer is most commonly

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diagnosed in women over 50, however it can strike younger women as well. Age is a key risk factor for the disease. Inheritance of breast cancer in the family, genetic mutations (e.g., BRCA1 and BRCA2), hormonal variables (e.g., early menstruation, late menopause, hormonehormone replacement treatment), alcoholism, obesity, physical inactivity, and variables related to reproduction (such as nuliparity and late age at first births).

Many industrialized nations have seen a decline in breast cancer mortality rates, which may be linked to early identification through screening programs, advancements in treatment methods (including radiation therapy, chemotherapy, surgery, and hormone therapy), as well as better supportive care. Survivability results are still influenced by differences in socioeconomic status, knowledge levels, and access to healthcare resources.In addition to providing insight into the incidence of breast cancer, epidemiology research works to improve early diagnosis and treatment results by lowering risk factors and developing screening protocols, public health policies, and treatments. Continuous monitoring and investigation are necessary to handle the changing issues brought about by breast cancer and to work towards its prevention and control on a global scale [2,3].

Natural substances have drawn interest in this context due to their potential to supplement or replace traditional treatments. A significant bioactive ingredient from the Acorus genus, asarone, has shown encouraging anticancer effects in several cancer types. Its precise effects on breast cancer cells haven't been well researched, though. Therefore, examining Asarone's anticancer potential in breast cancer cell lines using both in vitro and in silico methods offers a fascinating direction for future research. Furthermore, the underlying processes responsible for its anticancer effect can be clarified through mechanistic research. In addition to these experimental techniques, in silico techniques provide information on the molecular interactions between Asarone and important targets connected to the advancement of breast cancer [4-6].

The potential of asarone to increase the combined effectiveness of traditional chemotherapy medications has been studied. Asarone and chemotherapeutic drugs have been demonstrated in preclinical research to have synergistic effects; this suggests that asarone may play a part in overcoming drug resistance and enhancing treatment results. Asarone's anticancer effects in breast cancer are still being fully understood, but initial research suggests that it has a role in controlling important molecular targets such cell cycle proteins, apoptosis regulators, and signaling pathways like PI3K/Akt and MAPK [7]. Asarone's potential to cure neurodegenerative diseases including Alzheimer's disease has been explored due to its neuroprotective qualities. Research has indicated that it can improve cognitive performance and memory, lessen neuroinflammation, and shield neurons from harm in preclinical settings. Asarone reduces inflammation by preventing the release of mediators and cytokines that promote inflammation. These characteristics point to its potential for the treatment of inflammatory diseases such inflammatory bowel disease and arthritis.

The antioxidant activity of asarone aids in the reduction of oxidative stress and the neutralization of free radicals. Its therapeutic potential in a number of oxidative damage-related ailments, such as age-related disorders and cardiovascular diseases, may be aided by its antioxidant activity. Research has indicated that asarone has antibacterial effects against a broad variety of microbes, such as fungi, bacteria, and parasites. These results point to its potential for use as an all-natural antibacterial agent to treat infectious illnesses. Studies on Asarone's effects on anxiety and sedation have produced encouraging findings in animal models of anxiety and sleeplessness. Its regulation of the central nervous system's

neurotransmitter systems is responsible for these effects.Studies reveal that asarone has hepatoprotective qualities, shielding the liver against a range of pollutants and substances. Its potential for the prevention and treatment of liver disorders such hepatitis and liver cirrhosis is suggested by this hepatoprotective action [8]. All things considered, these investigations demonstrate the variety of pharmacological actions of asarone and its possible medicinal uses in a range of medical disorders. Nevertheless, more study, particularly clinical trials, is needed to fully understand its safety, efficacy, and mechanisms of action in humans. This study was aimed to find out whether asarone acts on breast cancer cell line using an invitro and in silico approach.

Materials And Methods

In vitro Analysis

Antioxidant Activity

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity

Scavenging of DPPH radical was assessed by the method of [9]. Briefly, DPPH solution (1.0 ml) was added to 1mg/ml of Asarone at different concentrations (100, 200, 300, 400, and 500 µg/ml). The mixture was kept at room temperature for 50 min and the activity was measured at 517 nm. Ascorbic acid at various concentrations (100, 200, 300, 400, and 500 µg/ml) was used as standard. The capability to scavenge the DPPH radical was calculated using the following formula:

DPPH radicals scavenged (%) = (Control OD − Sample OD/Control OD) × 100.

Procurement and Culture of Human Breast Cancer Cell Line (MCF-7)

The MCF-7 cell line was acquired from The National Centre for Cell Science (NCCS), located in Pune, India, and cultivated following the prescribed cell culture protocols. In summary, the breast cancer cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) under standard conditions of 37°C temperature and 5% CO_2 atmosphere. Culturing was carried out at 37°C in a humidified atmosphere containing 5% CO2 to provide optimal growth conditions for the A549 cells. This optimal culture environment facilitated the robust growth and maintenance of the MCF-7 Breast cancer cell line, ensuring its viability and suitability for subsequent experimental procedures.

Cell Viability Assay

MCF-7 cells were seeded at a density of 5x10*5 cells/well in 96-well plates and allowed to attach to the well overnight. After incubation, cultured cells were stimulated with various concentrations of Asarone in triplicate and incubated at 37˚C in a 5% humidified CO_2 incubator for 24 h [10]. Subsequently, 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well, and incubation was continued for a further 4 h at 37℃. To dissolve the formazan formed from MTT, the cells were resuspended in 200 µl dimethyl sulfoxide (DMSO), and the optical density (OD) of the solution was determined using a spectrometer at a wavelength of 570 nm. The experiments were repeated 3 times, independently. The mean optical density (OD) ± SD for each group of replicates was calculated. The entire procedure was repeated 3 times. The mean optical (OD) ±SD for each group replicates were calculated. The inhibitory rate of cell growth was calculated using Growth inhibition %.

% Growth inhibition = (1 - OD extract treated)/OD negative control x 100

Gene Expression Analysis by Real Time-PCR

Gene expression levels were examined using real-time PCR. The total RNA was isolated by using TriR Reagent (Sigma). Total RNA (2 µg) from each sample was reverse transcribed using a commercial Superscript III first strand cDNA

synthesis kit (Invitrogen, USA) according to the manufacturer's protocol. Real time-PCR was carried out in a MX3000p PCR system (Stratagene, Europe). Reaction was performed using MESA Green PCR master mix (It contains all the PCR components along with SYBR green dye) Eurogentec, USA. The specificity of the amplification product was determined by melting curve analysis for each primer pairs. The data were analyzed by comparative CT method and the fold change is calculated by 2−CT method using CFX Manager Version 2.1 (Bio-Rad, USA).

Molecular Docking Analysis

The binding interactions of a compound with specific target proteins were investigated using the molecular docking software PyRx. Crystal structures of these target proteins were retrieved from the Protein Data Bank (PDB). Throughout the docking process, a grid box measuring 90 Å × 90 Å × 90 Å, with a grid spacing of 0.45 Å, was employed to facilitate accurate ligand-protein interaction prediction. Docking calculations were carried out utilizing the Lamarckian genetic algorithm (LGA), with 100 genetic algorithm runs executed to ensure comprehensive exploration of the conformational space. Post-docking analysis was conducted to identify and characterize high-pose interactions between the compound of interest (Asarone) and the apoptosis-regulating target proteins. Additionally, the binding affinities of the ligand towards the receptors were meticulously evaluated to elucidate the binding mode. The resulting 3D structures of the ligand-protein complexes from the docking simulations were visualized and analyzed using BIOVIA Discovery Studio, enabling a detailed examination of the molecular interactions and the binding conformations. This comprehensive approach provides insights into the potential mechanisms of action and therapeutic relevance of the compound in modulating apoptotic pathways through its interactions with specific protein targets.

Statistical Analysis

The data presented are represented as the means ± standard deviation (SD) derived from three independent experiments conducted in triplicate. Statistical analysis was carried out using one-way ANOVA, with a significance level set at $p<0.05$ to indicate statistically significant results.

Results

Antioxidant Activity of Asarone (dpph Radical Scavenging)

An extensively used technique for assessing the antioxidant activity of substances, including natural products like asarone, is the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. In this experiment, the stable free radical DPPH, which has a distinct purple hue, combines with antioxidants by giving electrons or hydrogen atoms, causing the DPPH solution's colour to change from purple to yellow. The studied compound's antioxidant activity is correlated with the degree of discolouration. Table 1 & Figure 1 indicate that the asarone extract exhibits dose-dependent DPPH radical scavenging activity, as evidenced by the increase in percentage inhibition with increasing concentrations of the extract. The mean percentage inhibition of DPPH radicals by the asarone extract ranges from 25.51% at a concentration of 100 µg/ml to 66.74% at a concentration of 500 µg/ml. These findings demonstrate the potent antioxidant activity of the asarone extract, with higher concentrations showing greater efficacy in scavenging DPPH radicals. The results support the potential therapeutic application of asarone as a natural antioxidant agent for combating oxidative stress-related disorders.

Table 1. Showing Percentage Inhibition of DPPH Radical Scavenging Activity of Compound Asarone

Conc (µg/ml)	Mean (Std % inhibition)	Mean (drug % inhibition)
100	42.18	25.51
200	53.76	32.18
300	64.85	43.77
400	78.81	54.83
500	87.74	66.74

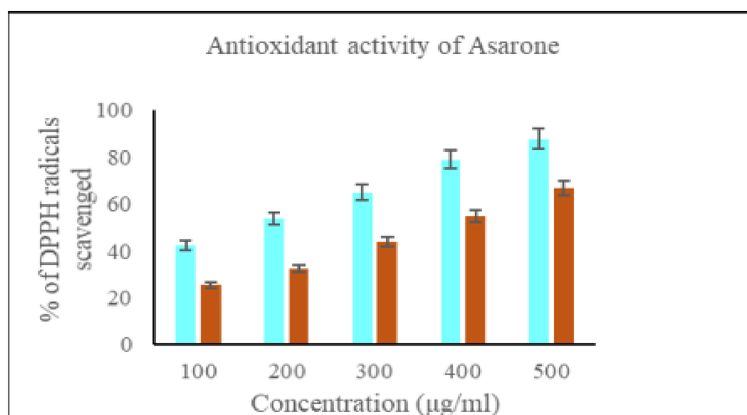

Figure 1. DPPH Radical Scavenging Activity of Asarone and Standard Drug from 100 to 500 µg/ml

Antiproliferative Effects of Asarone in MCF-7 Cells by MTT

Fig.3 illustrates the outcomes of a cell viability assay conducted with different concentrations (100 to 500µ) of the Asarone. As depicted in figure 2, there is a noticeable decrease in cell viability with increasing concentrations of the asarone. This decline in cell viability indicates the potential anti-cancer properties of Asarone. The findings suggest that higher concentrations of the asarone correlate with reduced cell viability, highlighting its potential effectiveness in inhibiting cancer cell growth. This observation underscores the significance of further exploring the therapeutic potential of Asarone as a natural anti-cancer agent.

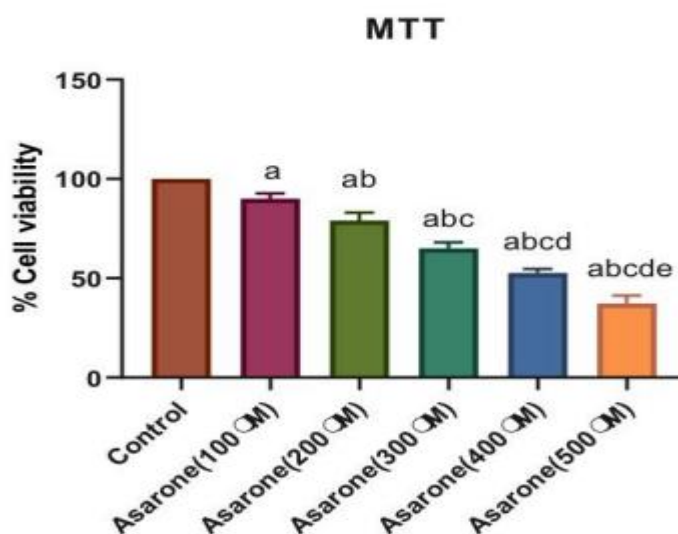

Figure 2. Effect of Asarone on the Cell Viability in Human Breast Cancer Cells (MCF-7 cells)

Effect of Asarone on Bax, Bad and Cytochrome C mRNA Expression in MCF-7 Cells

Figure 3a shows the impact of varying concentrations of asarone on Bax mRNA expression. The data indicate a significant upregulation of Bax mRNA in response to asarone treatment, suggesting that asarone induces apoptosis through the intrinsic pathway by promoting the expression of this pro-apoptotic protein. Figure 3b displays the effect of asarone on Bad mRNA expression. Similar to Bax, Bad mRNA levels were significantly elevated in a dose-dependent manner upon asarone treatment. This further supports the role of asarone in promoting apoptosis, as Bad is another key pro-apoptotic protein involved in the intrinsic apoptotic pathway. Figure 3c demonstrates the effect of asarone on Cytochrome C mRNA expression. The results show an increase in Cytochrome C mRNA levels following asarone treatment, reinforcing the notion that asarone triggers the intrinsic apoptotic pathway, as Cytochrome C release is a crucial step in this process. Overall, these results highlight the potential of asarone to induce apoptosis in MCF-7 breast cancer cells by upregulating key pro-apoptotic genes, including Bax, Bad, and Cytochrome C. This provides mechanistic insights into asarone's anticancer effects and supports its potential as a therapeutic agent for breast cancer treatment (Figure 3a-c).

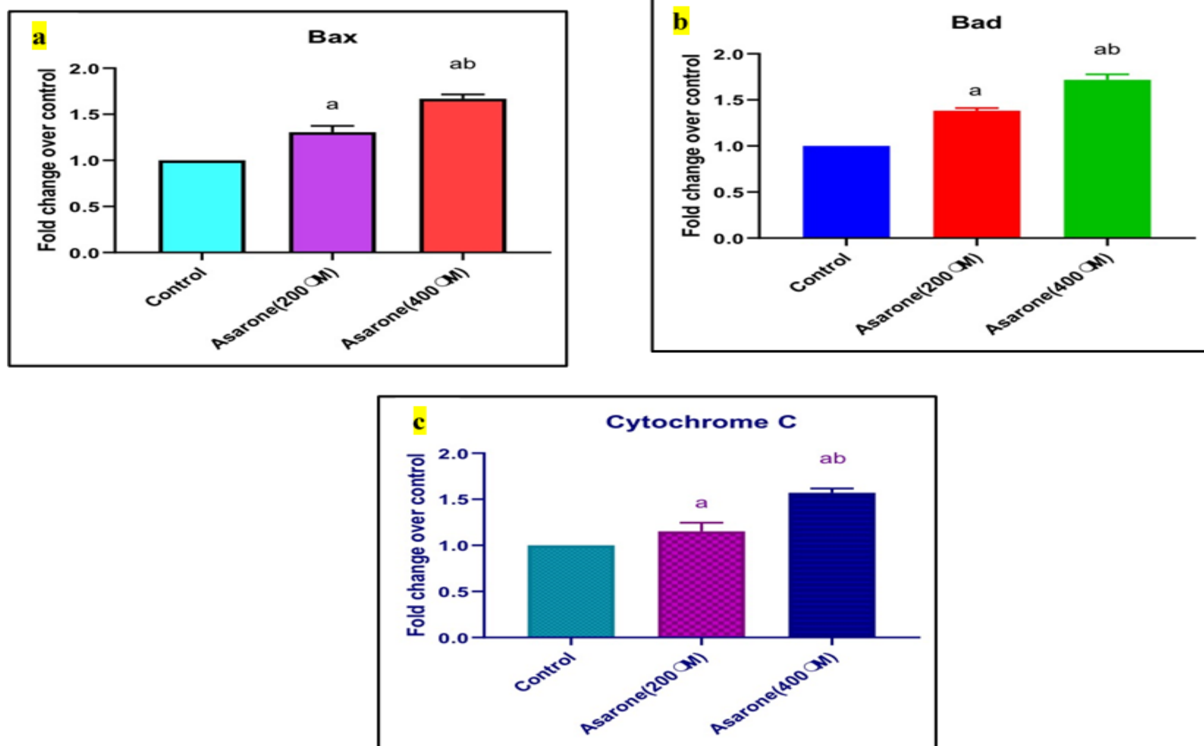

Figure 3a-c. Effect of Asarone on Bax mRNA (Fig 3a), Bad mRNA (Fig.3b) and Cytochrome C mRNA (Fig.3c) Expression in MCF-7 Cells

Effect of Asarone on Caspase-3 and Akt mRNA Expression in Human Breast Cancer (MCF-7) Cells

Figure 4a shows the impact of asarone on caspase-3 mRNA expression in MCF-7 cells. The data indicate a significant increase in caspase-3 mRNA levels in response to asarone treatment. Caspase-3 is a critical executioner in the apoptosis process, and its upregulation suggests that asarone promotes apoptosis by enhancing the expression of this essential protease. Figure 4b presents the effect of asarone on Akt mRNA expression. The results demonstrate a marked decrease in Akt mRNA

levels following asarone treatment. Akt kinase is involved in promoting cell survival and proliferation; therefore, its downregulation by asarone indicates that the compound interferes with key signaling pathways that are crucial for cancer cell growth and survival. These findings suggest that asarone induces apoptosis in MCF-7 breast cancer cells by upregulating caspase-3 and downregulating Akt mRNA expression. This dual action highlights asarone's potential as a therapeutic agent, capable of promoting cancer cell death while inhibiting survival pathways.

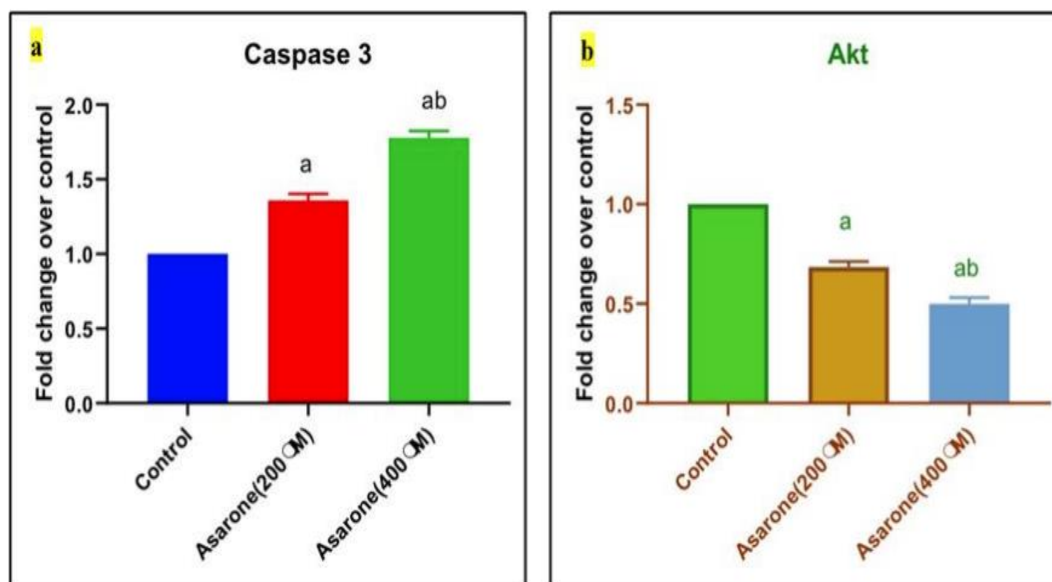

Figure 4 a &b. Effect of Asarone on Caspase-3 and Akt mRNA Expression in MCF-7 Cells

Molecular Interaction of Asarone with Apoptotic Signaling Molecules by Docking Methods

The binding energy of Asarone with Bax was found to be -5.0 kcal/mol. Bax is a pro-apoptotic protein, and its interaction with Asarone suggests a moderate affinity, which could imply potential modulation of apoptotic pathways. Asarone exhibited a binding energy of -5.0 kcal/mol with Bad, another pro-apoptotic protein. This result indicates a similar binding affinity to that observed with Bax, suggesting that Asarone may influence apoptosis through interaction with multiple targets. The interaction of Asarone with Cytochrome c resulted in a binding energy of -4.3 kcal/mol. Cytochrome c plays a critical role in the intrinsic pathway of apoptosis, and the observed binding energy suggests a weaker interaction compared to other targets. The binding energy of Asarone with Caspase 3 was -4.8 kcal/mol. Caspase 3 is a key executioner protease in the apoptotic cascade. The moderate binding affinity indicates potential inhibition or modulation of Caspase 3 activity by Asarone. Asarone showed a binding energy of -5.3 kcal/mol with Akt, a protein kinase involved in cell survival and proliferation pathways. This relatively stronger binding affinity suggests that Asarone may influence cell survival signaling pathways. The interaction of Asarone with IL6, an inflammatory cytokine, resulted in a binding energy of -4.9 kcal/mol. This binding energy suggests a moderate interaction, which could imply potential anti-inflammatory effects of Asarone. Asarone exhibited the strongest binding affinity with TNFα, with a binding energy of -5.9 kcal/mol. TNFα is a key mediator of inflammation and apoptosis. The strong interaction suggests that Asarone may have significant anti-inflammatory and apoptotic regulatory properties (Table 2 & Figure 5 a-f).

Table 2. Illustrate Molecular Docking Analysis of Asarone Ligand with Bax, Bad, Cytochrome c, Caspase3, Akt, IL6 Targets and Performed using PyRx Software

S.No	Ligand	Protein	Binding energy (kcal/mol)
1	Asarone (636822)	Bax	-5.0
2		Bad	-5.0
3		Cytochrome c	-4.3
4		Caspase 3	-4.8
5		Akt	-5.3
6		IL6	-4.9
7		TNFα	-5.9

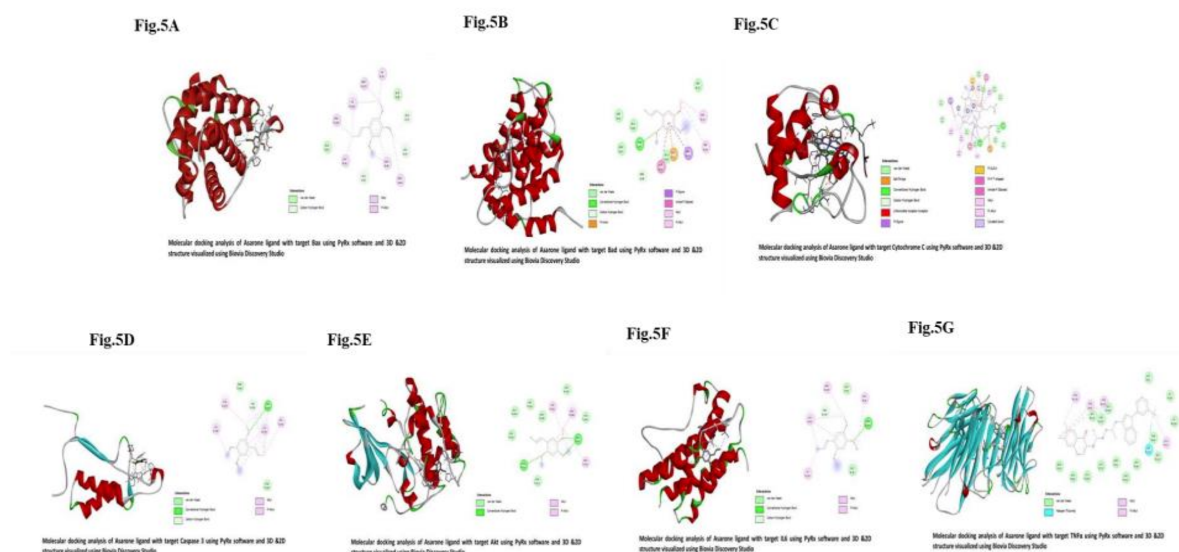

Figure 5A-G. Illustrate Molecular Docking Analysis of Asarone with Bax (Fig. 5A), Bad (Fig. 5B), Cytochrome c (Fig. 5C), Caspase3 (Fig. 5D), Akt (Fig. 5E), IL6 (Fig. 5F) and TNF-α (Fig. 5G)

Discussion

The findings of a study looking into asarone's potential as an anticancer agent efficacy in *invitro* assays. The favorable outcome would suggest that asarone significantly damages breast cancer cells in vitro. Apoptosis assay results, MTT results, and molecular studies may reveal a dose-dependent reduction in cell viability, an increase in apoptosis, and changes to important molecular targets linked to the advancement of cancer. Mechanistic Insights: The molecular processes that underlie asarone's anticancer actions can be the subject of discussion. Asarone, for instance, may trigger apoptosis via the intrinsic pathway by encouraging the release of mitochondrial cytochrome c and the activation of caspases [11,12]. Furthermore, asarone may prevent the growth of cancer cells by influencing signalling pathways that are known to support cancer cells survival and proliferation, like PI3K/Akt and MAPK. Synergistic Benefits and Combination Therapies: If relevant, the favourable outcome might point to asarone's possible synergistic benefits when combined with targeted therapies or traditional chemotherapy medications. Combination therapies may increase effectiveness while reducing side effects, opening up new possibilities for individualized cancer treatment.The positive outcome may have consequences for translational research, which might be discussed. These may include

the need for additional preclinical investigations, formulation or delivery technique optimization, and eventually the transition to clinical trials. Future in vivo research to confirm the effectiveness and safety of asarone in animal models may be designed with input from in vitro investigations. [13]. Limitations and Future Directions: Please note that this study has limitations. For example, it only used one cell line. Tthe necessity of more mechanistic research to completely comprehend asarone's anticancer actions. Further exploration into the medicinal potential of asarone can be guided by suggesting future research directions, such as examining its pharmacokinetics in animal models or examining its effects in conjunction with other natural substances [14-16]. Overall, the results of this study highlight the multifaceted therapeutic potential of Asarone. Its strong antioxidant activity, coupled with its ability to induce apoptosis and inhibit cell survival pathways in breast cancer cells, underscores its promise as a natural anti-cancer agent. The molecular docking analysis further supports these findings, providing insights into the specific interactions between Asarone and key apoptotic and inflammatory proteins [17-20]. Further in-depth studies, including in vivo experiments and clinical trials, are warranted to fully elucidate the therapeutic potential of Asarone and its application in cancer treatment.

Conclusion

The results of this investigation indicate, in summary, that asarone shows encouraging anticancer potential against cells that cause breast cancer. Asarone showed dose-dependent cytotoxicity, apoptosis induction, and modification of important molecular targets linked to the advancement of cancer through in vitro tests. These findings shed important light on the mechanisms behind asarone's anticancer actions, which include its capacity to stimulate caspases, increase mitochondrial cytochrome c release, and obstruct pathways involved in cell survival such PI3K/Akt. The successful conclusion of this study emphasizes asarone's medicinal potential as a natural substance for the treatment of breast cancer. To forward its development towards therapeutic applications, more preclinical research is necessary, including in vivo investigations to confirm its safety and efficacy. Furthermore, investigating synergistic effects with targeted therapies or traditional chemotherapeutic medications may increase asarone's therapeutic efficacy and offer fresh approaches to combination therapy. Overall, the results shown highlight the importance of asarone as a topic for more oncology study and advance our knowledge of its potential as an anticancer drug. Prospects for the creation of innovative and successful therapeutic treatments for breast cancer and other cancers seem promising if the molecular processes and translational applications of asarone are further investigated.

Conflict of Interest

The authors hereby declare that there is no conflict of interest in this study.

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