INTRODUCTION

Introduction

Cancer: A global concern

Cancer poses a significant global concern due to its hallmark feature of uncontrolled cell division and proliferation of abnormal cells, disrupting the fundamental principles of cellular behaviour governing multicellular organisms' development and maintenance. A crucial aspect of comprehending cancer's development and progression lies in investigating the biological variances between normal and cancerous cells. In 2020 alone, cancer accounted for approximately 10 million deaths worldwide (Arnold et al., 2022). Notably, breast cancer stood out as the most prevalent type, with 2.26 million reported cases (Arnold et al., 2022).

Metastasis, the process wherein cancer cells migrate from the primary tumour to other parts of the body, leading to the formation of secondary tumours, represents a critical challenge in cancer treatment (Lambert et al., 2016; Gerstberger et al.,2023). Understanding the intricate mechanisms driving metastasis is paramount for devising successful approaches to treat and control cancer. Addressing the complexities of cancer metastasis remains a significant hurdle in oncology (Lambert et al., 2016). However, recent breakthroughs in unravelling the complex molecular and cellular mechanisms underlying cancer progression offer promising avenues for developing targeted therapies and personalized treatment strategies. With the disease's increasing heterogeneity and complexity, there is a constant demand for identifying and designing new drugs targeting cancer cells. Hence, ensuring cancer drug safety and efficacy remains critical and essential (Gerstberger et al.,2023).

Breast cancer: Global Incidences and Prognosis

Breast cancer emerges as a prevalent malignancy globally, with its incidence on the rise (Arnold et al., 2022). In 2020 alone, global diagnoses of breast cancer reached 2.3 million

cases, resulting in 685,000 fatalities (ME et al.,2024). By the conclusion of 2020, the worldwide population encompassed 7.8 million women who had received a breast cancer diagnosis within the preceding five years, cementing its status as the most widespread cancer type globally (ME et al.,2024). This disease affects women of all ages post-puberty, with higher rates observed in older age groups (ME et al.,2024).

According to the World Health Organization, reducing breast cancer mortality globally hinges on three key pillars: early detection, accurate diagnosis, and prompt treatment (ME et al.,2024).

Breast cancer: National Incidences and Prognosis

The breast cancer scenario in India, as presented by the National Centre for Disease Informatics and Research (NCDIR) and the Indian Council of Medical Research (ICMR) in 2020, reveals that breast cancer ranks as the second-highest incidence among all types of cancer affecting women (Sathishkumar et al., 2022). Projections suggest that by 2025, the overall burden of breast cancer cases will reach 232,832 new cases (Sathishkumar et al., 2022). In India, the prevalence of breast cancer is swiftly escalating, with an estimated 0.26 million new cases and a projected 0.15 million deaths by 2040 (Dar & Sharma, 2018).

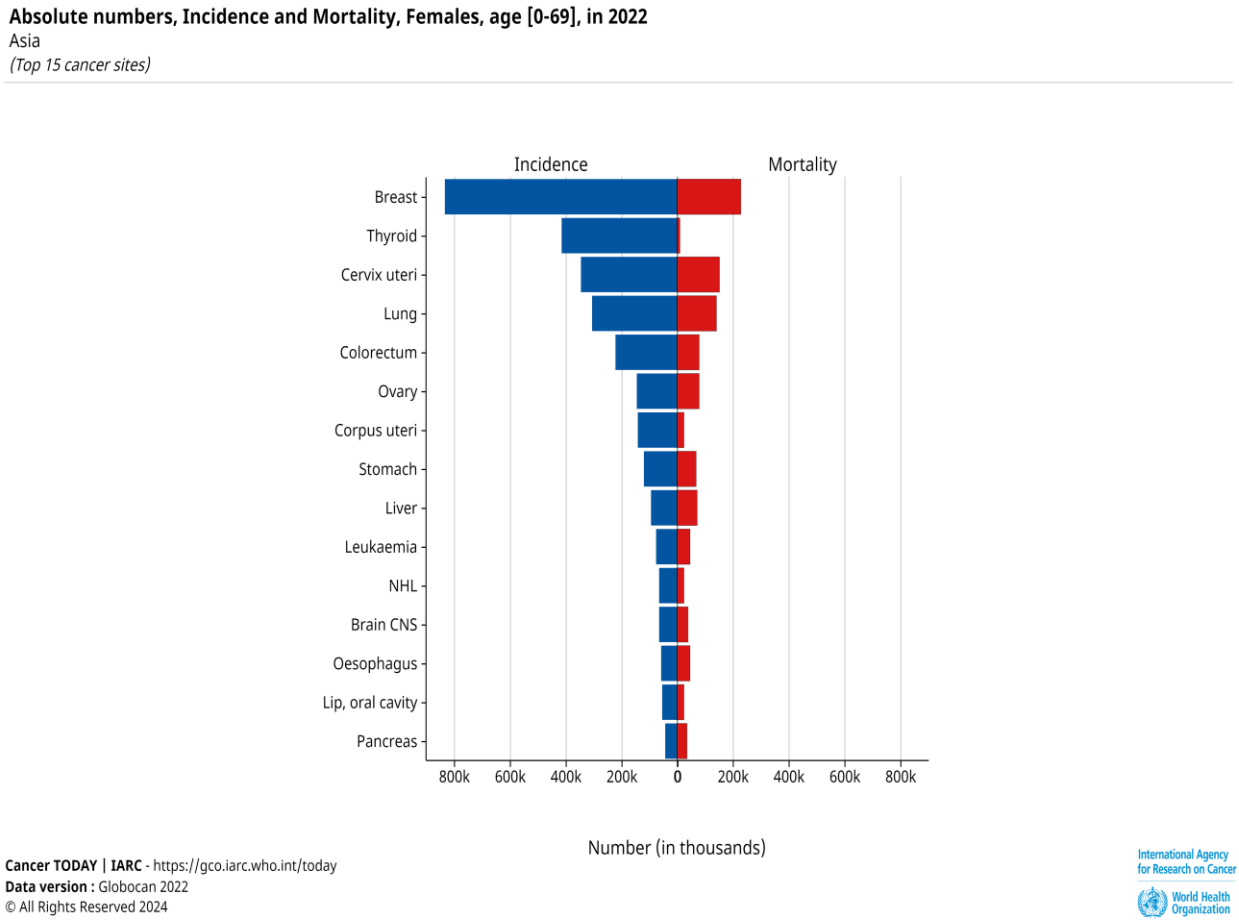


Figure 1: Estimated number of cancer cases in 2022 of all ages (GLOBOCAN,2022) in Asia

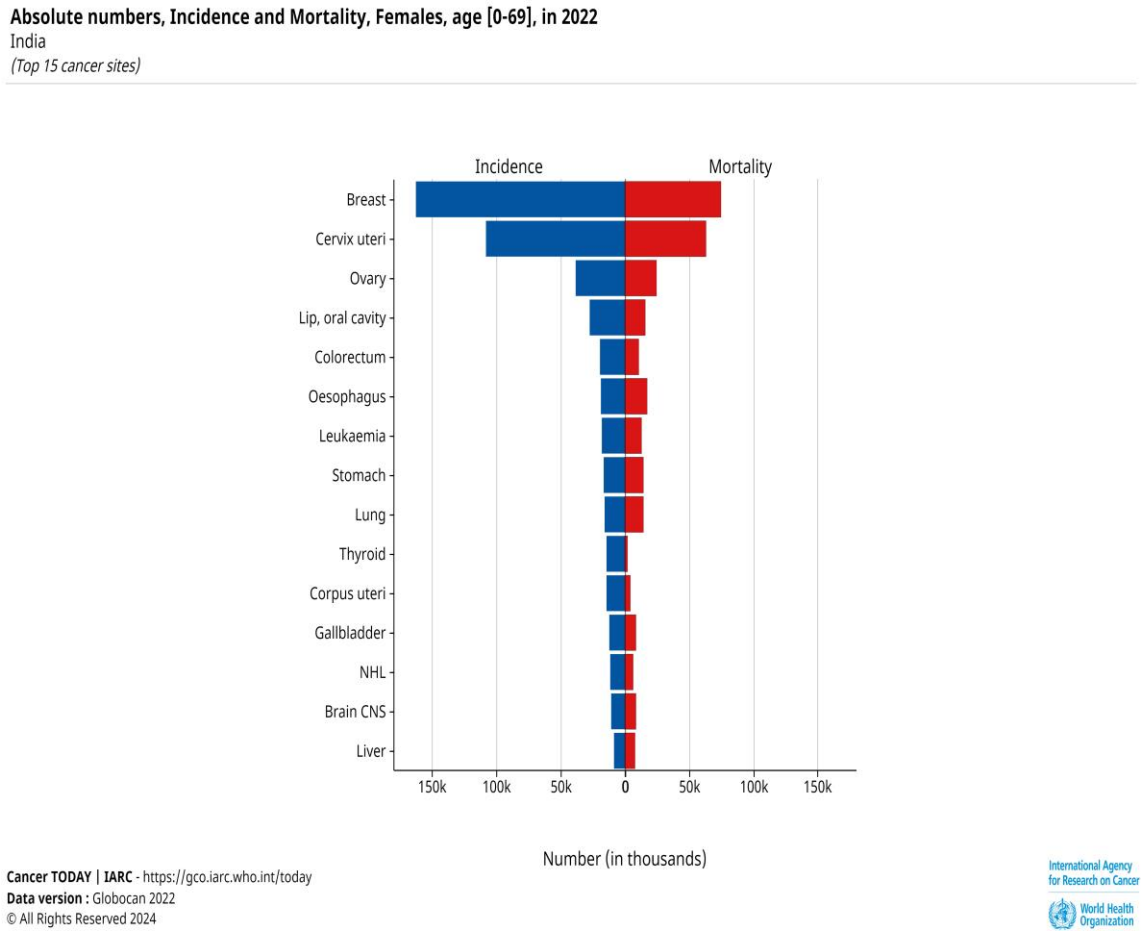


Figure 2: Estimated number of major types of new cancer cases in 2022 (GLOBOCAN,2022) in India

Breast cancer - Molecular classification of its sub types

Breast cancer presents as a genetically and clinically diverse disease with various subtypes. The classification of these subtypes has evolved over time with advancements in techniques and understanding of the complexity of the disease. The most accepted classification of breast cancer is based on an immuno-histochemical perspective, focusing on the expression of hormone receptors such as oestrogen (ER), progesterone (PR), and human epidermal growth factor (HER2) (Tsang & Gary, 2020). Consequently, four main subtypes of breast cancer are widely recognised: luminal A, luminal B, HER2-positive, and triple-negative (Table 1).

Table 1 : Molecular subtypes of breast cancer		
Luminal A	This subtype is characterized by the presence of estrogen and/or progesterone receptors and low levels of HER2 expression. Luminal A tumors typically have a lower proliferation rate and a better prognosis compared to other subtypes	Prat,2015, Tarighati et al.,2023
Luminal B	Luminal B tumors also express estrogen and/or progesterone receptors but have higher levels of proliferation markers and may show overexpression of HER2. Luminal B tumors are generally associated with a worse prognosis compared to Luminal A tumors	
HER2-enriched	This subtype is characterized by overexpression of the HER2/neu gene. HER2-enriched tumors tend to be more aggressive, but targeted therapies such as trastuzumab (Herceptin) have significantly improved outcomes for patients with this subtype	Slamon,2001; Tarighati et al.,2023
Triple-Negative Breast Cancer (TNBC)	TNBC lacks expression of estrogen and progesterone receptors and does not overexpress HER2. This subtype tends to be more aggressive and is associated with a poorer prognosis. Since TNBC does not express hormone receptors or HER2, it is less responsive to hormone-based or HER2-targeted therapies	Dent,2007; Tarighati et al.,2023
Basal-like	This subtype is often used interchangeably with triple-negative breast cancer (TNBC). Basal-like tumors share characteristics with the basal cells of the breast and are often associated with a more aggressive course	Perou,2000; Tarighati et al.,2023

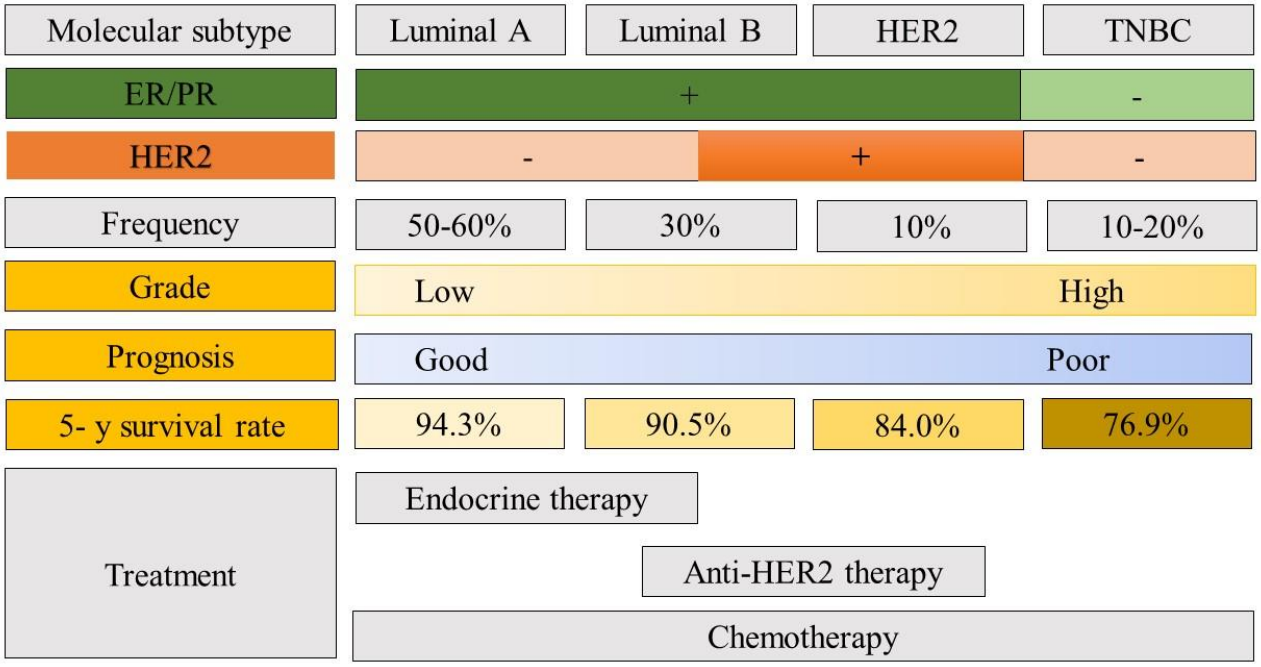


Figure 3: Breast cancer molecular subtypes characteristics. Image curtsy: Burguin *et al*.,2021

It is crucial to acknowledge that while molecular subtypes play a significant role in guiding treatment decisions and predicting prognosis, they represent just one facet of a comprehensive approach to breast cancer classification. Ongoing advancements in molecular profiling and genomic analysis continually enhance our comprehension of breast cancer subtypes, facilitating the development of more personalized and targeted treatment approaches (Pinilla et al., 2022). Furthermore, ongoing research efforts may result in further refinement of existing subtypes or the discovery of novel ones.

Breast cancer - Risk factors

Two primary factors significantly elevate the risk of breast cancer development: gender and age. Breast cancer predominantly affects women, with the risk increasing notably with age, particularly after menopause (Shang and Xu, 2022). Family history and genetic predisposition also play substantial roles in increasing susceptibility to breast cancer (Liu et

al., 2021). Early onset of menstruation (before age 12) and late occurrence of menopause (after age 55) are associated with heightened vulnerability to breast cancer (Shang and Xu, 2022). Additionally, nulliparity, giving birth after age 30, and not breastfeeding is considered risk factors. Hormone replacement therapy, particularly with estrogen and progesterone, may further elevate the risk, especially with long-term use (Shang and Xu, 2022). Prolonged heavy alcohol consumption is also linked to an increased likelihood of developing breast cancer later in life.

Exposure to radiation during early life stages can heighten the risk of breast cancer, as can factors related to hormones and reproduction, including late onset of menopause (Gregoire et al.,2022). Environmental pollutants commonly found in plastics may potentially increase the risk, along with certain occupational exposures involving carcinogens such as chemicals and radiation (Baj et al., 2022).

Current therapeutic strategy against breast cancer

Surgery stands as the primary approach for treating breast cancer, aiming to eradicate both the tumour and adjacent lymph nodes to achieve a cancer-free state. Options may include breast-conserving surgery, such as lumpectomy or mastectomy. Post-surgery radiation therapy is often employed to eliminate any residual cancer cells in the breast and/or adjacent lymph nodes (Machado et al.,2023). Additionally, it may be prescribed before surgical interventions to shrink tumours (Machado et al.,2023). Chemotherapy, a systemic treatment utilizing chemicals to eradicate cancer cells throughout the body, is typically employed when the disease has metastasized beyond the breast or when there is a high risk of cancer recurrence.

Hormone receptor-positive breast cancers, sensitive to hormones like oestrogen or progesterone, can be treated with hormone therapy, which inhibits hormone actions or

reduces their levels to minimize cancer recurrence. Some breast cancers overexpress specific proteins, such as HER2. Targeted therapies like Trastuzumab (Herceptin) focus on inhibiting cancer growth by targeting these proteins (Tsang and Gary ,2020). Immunotherapy, a recent approach, aims to enhance the body's immune system to recognize and combat cancerous cells (Barzaman et al.,2021).

Endocrine therapy, also known as hormonal therapy, is highly effective for cancers positive for oestrogen or progesterone receptors. Selective Oestrogen Receptor Modifiers (SERMs) like Tamoxifen and Selective Oestrogen Receptor Down regulators (SERDs) like Fulvestrant target oestrogen, inhibiting cancer cell proliferation and reducing recurrence (Al-Mahmood et al., 2018).

The introduction of biological medicines, targeted therapies, and gene therapy offers breast cancer patients the potential to reduce mortality and enhance their quality of life (Rodrigues et al.,2023).

Drawbacks in the current therapeutics for breast cancer

Breast cancer stands as the most prevalent malignancy among women and remains a leading cause of mortality worldwide. Extensive documentation reveals disruptions in multiple molecular pathways and simultaneous alterations in molecular markers. While surgery, chemotherapy, and radiotherapy offer therapeutic benefits, their associated adverse effects drive researchers and clinicians to seek alternative treatments (Trabulsy et al., 2019).

Radiation exposure can result in weariness, skin irritation, discolouration, or hyperpigmentation and elevate the risk of secondary malignancies (Dhankhar et al., 2010; Gromkowska-Kępka et al.,2021). Chemotherapy, targeting rapidly dividing cells, is often prescribed for metastatic cancers, post-treatment recurrences, or those with a high likelihood

of recurrence. Hormonal therapy may induce various adverse effects, including muscle and joint discomfort, hot flashes, vaginal dryness, increased susceptibility to osteoporosis and fractures, and elevated cholesterol levels (Marti-Soler et al., 2014). Tamoxifen, for instance, may lead to symptoms such as hot flashes, vaginal dryness, and abnormal vaginal discharge or bleeding. Monoclonal antibody medications like Trastuzumab and Pertuzumab, approved for HER2-positive breast cancer, carry a minimal risk of cardiovascular complications for patients receiving these treatments (Dent et al.,2021).

In the context of challenges in metastatic breast cancer treatment, a promising therapeutic avenue emerges with the utilization of traditional medicinal plants to target molecular markers altered during cancer.

Phytotherapeutic approach towards combating breast cancer

While traditional cancer remedies have existed for generations, the exploration of anticancer medications derived from plants has gained recent prominence due to their reduced side effects and sustained efficacy (Singh et al., 2016). Secondary metabolites found in medicinal plants contribute to their therapeutic effects, with documented abilities to inhibit cancer cell activity by impeding proliferation and promoting apoptotic cell death (Patel et al.,2020; Kowalczyk et al.,2020). Phytochemicals within these plants possess antioxidant and anticancer properties, capable of impeding the progression of carcinogenic cells without inducing toxicity in individuals (Singh et al., 2016). These natural compounds and their derivatives, distributed throughout the plant, exert diverse pharmacological effects and play crucial roles in halting cancer cell division through various pathways.

In addition to chemically synthesised anticancer agents, a plethora of anticancer compounds with distinct modes of action have been identified in literature, sourced from various plant species such as *Taxus brevifolia* (yielding Paclitaxel and Docetaxel),

Catharanthus roseus (providing Vinblastine and Vincristine), *Berberis amarensis* (producing Berbamine), and *Aglaia foveolata* (supplying Silvasterol) (Zhou et al.,2021; Cao et al.,2021; Mehdi et al.,2023; Upadhyay et al.,2020).

Major phyto-compounds identified against breast cancer

Utilising phyto-compounds presents a targeted approach, exhibiting specific toxicity towards cancer cells while sparing normal cells (Iqbal et al., 2018). This targeted strategy is crucial for mitigating the adverse effects and toxicity commonly associated with traditional chemotherapy, thereby enhancing the safety profile of cancer therapies. Phytochemicals found in medicinal plants harbour antioxidant and anticancer properties that can hinder cancer cell growth without eliciting toxicity. These natural compounds and their derivatives possess diverse pharmacological activities and significantly inhibit cancer cell proliferation through various mechanisms (Dehelean et al., 2021). Alongside chemically manufactured anticancer medications, a plethora of anticancer substances with distinct mechanisms of action have been sourced from diverse plant origins.

To date, extensive research has examined numerous plants and the phyto-compounds derived from them for their anticancer properties at global, national, and local levels. The findings from these evaluations are comprehensively presented in Table 2.

Table 2 : Ethanobotanical Data of Plants and their active molecules of interest				
Plant name	**Part used**	**Active molecule**	**Pathway (Mode of action)**	**Reference**
Allium wallichii	Whole plant	Steroids, terpenoids, flavonoids, reducing sugars and glycosides	Apoptosis	Bhandari J ,2017
Artemisia annua	Whole plant	Artemisinin	Inhibition of Qxidative stress , angiogenesis,	Efferth T,2017
Berberis vulgaris	Root, stem bark	Berberine, cannabisin	Antiangiogenic and anti tumor activity	Pierpaoli E *et al.*,2015
Colchicum autumnale	Leaves	Colchicine	Tumor targeted vascular disrupting agent activated by matrix metalloproteases	Bakar-Ateş et al.,2018
Curcuma longa	Root, rhizome	Curcumin	Mutagenesis, apoptosis, tumorigenesis, cell cycle regulation	Perrone D,2015
Ocimum sanctum	Leaves	Eugenol, orientin, vicenin	Apoptosis	Prithi R *et al.*, 2016
Solanum nigrum	Leaves	Solamargine, solasonine	Lysosomal mitochondiral death pathway	Al Sinani SS,2016
Vigna unguiculata	Seeds	Black-eyed-pea trypsin/ Chymotrypsin inhibitor	Mitochondrial impairment and oxidative damage	Mehdad A,2016

Review of the plants selected for the present study

The current study aimed to assess the effectiveness of four selected plants against breast cancer cell lines, chosen based on four distinct parameters (Barbosa et al., 2012):

1. Ethno-medicinal use: Selection was guided by the traditional medicinal use of the plants.
2. Existing literature reviews: Plant species were chosen based on previous studies and reviews highlighting their usage and efficacy.
3. Chemotaxonomic approach: The selection process involved considering the plants' taxonomy and chemical composition.
4. Regional availability: Plants were selected based on their availability for research purposes in the region.

Butea monosperma, Melia azedarach, Saraca asoca and Solanum virginianum were selected for their potential roles in apoptosis and metastasis, assessed through in vitro and in silico studies.

***Butea monosperma* (Lam.) Kuntze :**

Butea monosperma (Lam.) Kuntze (*Bm*), commonly known as the flame of the forest or Palasa in Sanskrit, is a medium-sized tree widely distributed across India. In the traditional Indian system of medicine, the flowers of B. monosperma hold various medicinal uses, including managing symptoms of stress, anxiety, cognition, hepatitis, and fertility (Burlia and Khadeb, 2007). The health benefits of B. monosperma are attributed to its bioactive components, such as eicosane, β-amyrin, vicenin-II, vitexin, and luteolin (Ammar et al., 2011). While previous studies have explored the anti-inflammatory potential of B. monosperma flowers (Rana et al., 2015; Zahra et al., 2024), there is a lack of sufficient literature regarding the anti-cancer potential of B. monosperma bark and leaves for breast cancer therapeutics.

***Melia azedarach* L.:**

Melia azedarach L. *(Ma)*, commonly known as the Chinaberry tree, possesses various pharmacological activities attributed to its leaves, fruits, bark, seeds, and roots. These activities include antifungal, anti-malarial, antibacterial, hepatoprotective, antioxidant, anti-fertility, anthelmintic, antipyretic, and cytotoxic properties (Sharma & Paul et al., 2013). Utilized extensively by indigenous and tribal communities in India, the entire plant, including leaves, stems, and roots, holds therapeutic value (Kar et al., 2022).

M. azedarach finds use in both Indian Ayurvedic and Arab Unani medicinal traditions, valued for its antioxidative, analgesic, anti-inflammatory, insecticidal, rodenticidal, antidiarrheal, deobstruent, diuretic, antidiabetic, cathartic, emetic, antirheumatic, and antihypertensive properties (Disha and Karthikeyan et al., 2024). The primary secondary metabolites in the plant parts are terpenoids and limonoids (tetranortriterpenoids), followed by steroids and flavonoids (Nivedha, 2019). Rich in polyphenols and flavonols, the leaves and bark of *M. azedarach* contribute significantly to its traditional medicinal uses (Mwamatope et al., 2020).

***Saraca asoca* (Roxb.)Willd.** :

Saraca asoca (Roxb.)Willd. *(Sa)* commonly known as Ashok or Ashokvriksha, is a widely recognized medicinal plant with various parts such as barks, leaves, flowers, and seeds, extensively employed in folk medicine for treating female reproductive system-related ailments (Bhalerao et al., 2014). The earliest documented reference to this tree dates back to ancient Ayurvedic treatises, including the Charka Samhita (100 A.D.), where Ashoka is recommended as a component in formulations for addressing gynaecological issues, particularly as an anodyne (Shivasankari et al., 2014). Notably, Ashokarista, a well-known preparation derived from the bark of this plant, is commercially available and widely utilized

for treating menstrual disorders. With its extensive traditional use in conventional medicine documented in ancient texts, *S. asoca* has garnered attention as a promising candidate for developing new medicinal products (Singh et al., 2015).

Traditionally, *Saraca asoca* is employed in treating various ailments, including gynaecological disorders, bacterial infections, parasitic worm infestations, hemorrhagic dysentery, uterine discomfort, skin diseases, cancer, circulatory, and cardiovascular abnormalities, among others (Rathod & Ghante, 2021). Each component of the *Saraca asoca* plant possesses therapeutic properties. The bark, leaves, and flowers contain an array of antioxidant compounds such as flavonoids, catechin, beta-sitosterol, and lignin glycosides (Borokar & Pansare, 2017), known for their role in stabilizing free radical molecules associated with cancer development.

***Solanum virginianum* L.**

Solanum virginianum L. *(Sv)*, also known as *Solanum xanthocarpum* Schrad. & H. Wendl., is a sprawling and densely thorny undershrub belonging to the Solanaceae family. Characterised by leaves with three different margin types—whole, lobed, or pinnatifid—and flowers arranged in lateral or terminal cymes, its fruit typically manifests as a spherical or elongated berry (Jagatheeswari, 2014). Commonly referred to as yellow-berried nightshade in English and kantakari in Sanskrit, this herb finds widespread usage in various medical systems, notably Ayurveda, and is a component of the traditional herbal formulation "Dashamula" used in ayurvedic medicines (Sanjay et al., 2009). Despite its rich folklore reference, systematic studies evaluating its anti-cancer properties are lacking, although it has been associated with numerous bioactivities, including hepato-protective, antibacterial, anthelmintic, and immune-stimulatory properties (Kushwaha et al., 2018; Konar et al., 2022).

All four plants lack systematic insight into their potential anti-breast cancer properties and the screening of phytochemicals responsible for their potent actions. Hence, the present study adopts an approach of preliminary anti-proliferative activity determination against breast cancer cell lines (MCF-7 and MDA-MB-231) for all four plants. Subsequently, species demonstrating the most promising activity will be selected for systematic and in-depth molecular analysis.

Rationale behind the cell-line used for *in-vitro* studies

MCF-7

MCF-7 cells have played a pivotal role in elucidating various aspects of breast cancer biology, including hormone function, cell signalling pathways, drug resistance mechanisms, and potential treatment strategies (Puthdee et al., 2020). Originating from a 69-year-old Caucasian woman with advanced metastatic breast cancer in 1970, MCF-7 cells are derived from epithelial tissue and express hormone receptors, notably oestrogen receptors and progesterone receptors (Soule et al., 1973; Horwitz et al., 1973). Their positive hormone receptor status, particularly the co-expression of oestrogen and progesterone receptors, makes them invaluable for investigating hormone-sensitive breast cancer (Puthdee et al., 2020).

Moreover, MCF-7 cells serve as a critical model for studying drug resistance mechanisms in breast cancer and exploring potential therapeutic interventions. Widely utilised in vitro for their versatility and availability in various variants, MCF-7 cells are particularly instrumental in the development and evaluation of chemotherapy drugs and in understanding the mechanisms underlying drug resistance (Patel et al., 2020).

MDA-MB-231

The MDA-MB-231 cell line stands as another extensively studied and utilised model in breast cancer research. Originating from a 51-year-old African American woman with

advanced breast cancer in 1973, these cells are derived from epithelial tissue and notably lack expression of oestrogen, progesterone, and HER2/neu receptors (Cailleau et al., 1978; Elstrodt et al., 2006).

MDA-MB-231 cells have played a critical role in elucidating the underlying mechanisms of cancer metastasis and the development of resistance to anti-cancer drugs in breast cancer (Hollestelle et al., 2007). Consequently, they serve as a prominent model system for investigating aggressive breast cancer characteristics, including metastasis, tumour invasion, epithelial-to-mesenchymal transition (EMT), and drug resistance.

Both MCF-7 and MDA-MB-231 cells are extensively utilised in academic and pharmaceutical research, underscoring their significance as fundamental resources for advancing our understanding of breast cancer biology and developing innovative therapeutic approaches.

Combinational therapy towards breast cancer

Combination therapy stands out as a novel strategy among various therapeutic approaches for addressing drug-resistant cancers, preventing tumor development, and augmenting overall therapeutic efficacy (Patel et al., 2020). Furthermore, identifying new therapeutic targets and developing innovative treatment approaches remains paramount for improving outcomes in patients with metastatic breast cancer. Various treatment modalities target cancer cells through diverse mechanisms, and combining these therapies can result in a potent synergy that enhances cancer treatment effectiveness. For instance, the combination of chemotherapy with targeted therapy, such as trastuzumab or lapatinib, has demonstrated improved response rates and outcomes compared to single-agent treatment (Chiou et al., 2018). Given that cancer cells can develop resistance to single-agent therapies over time, combining drugs with distinct mechanisms of action can overcome resistance more effectively (Aung et al., 2017).

This approach also reduces the likelihood of simultaneous resistance development to multiple drugs targeting different pathways, thereby optimising treatment outcomes. Moreover, combining therapies allows for the avoidance of high doses of individual drugs, minimising the risk of side effects and toxicity while maintaining or enhancing treatment effectiveness (Kowalczyk et al., 2020).

Enhancing the quality of life for breast cancer patients undergoing treatment remains paramount, considering the disease's complexity and the distinct responses of various subtypes to treatment. Combination therapy offers a promising approach to addressing the diverse nature of breast cancer by concurrently targeting multiple pathways or molecular targets (Patel et al., 2020). This comprehensive strategy enhances the likelihood of effectively treating the disease, irrespective of its specific subtype.

Recent studies utilising in vitro and in vivo analyses have explored various approaches to enhance anticancer properties by combining two or more natural substances or pharmaceutical medicines (Fantini M. et al., 2015; Aung et al., 2017). For instance, a recent study demonstrated the potential of combinatorial botanicals, such as green tea polyphenols and broccoli sprouts, with chemotherapeutics like tamoxifen to synergistically enhance anti-hormone therapy's efficacy in treating breast cancer (Chiou et al., 2018). Similarly, combining curcumin with 4-hydroxytamoxifen or genistein has shown improved inhibition of survival in endocrine-resistant breast cancer cells (P Kumar et al., 2000; Hemaiswarya & Doble, 2006). Additionally, compounds found in green tea have demonstrated the ability to enhance the delivery of doxorubicin into cancer cells while protecting the heart muscle from its adverse effects (Fujiki et al., 2014).

Furthermore, the concept of combination chemoprevention has gained recognition as a potential strategy for managing cancer, with studies exploring combinations of different phytochemicals or phytochemicals with conventional anticancer drugs (Talib et al., 2022;

Dassari et al., 2022). Notably, studies have demonstrated the synergistic effects of combining plant-derived compounds with conventional anticancer drugs, suggesting the potential for improved therapeutic efficacy (Hussain et al., 2015; Kowalczyk et al., 2020). This approach, utilising plant-derived compounds alongside coactivators such as hormones, growth factors, or synthetic drugs, holds promise for preventing carcinogenesis and reducing recurrence, thereby prolonging patient survival.

In general, combination therapy presents a diverse and evolving approach to breast cancer treatment, offering the potential for enhanced outcomes and better disease management. As research progresses in understanding the molecular mechanisms of breast cancer and identifying new therapeutic targets, the field of combination therapy is expected to advance, providing opportunities for improved and individualised treatments for patients. However, further studies are warranted to fully explore the potential of combination therapy and its effectiveness in treating breast cancer. Hence, the current study adopts a comprehensive combinational approach to identify potential intervention avenues.

Melatonin as a potentiator in combinational therapy against breast cancer

Melatonin is widely acknowledged as a chemical and a cell defender owing to its hematopoietic, antioxidative, and immunomodulatory properties (Mortezaee et al., 2019). Recent investigations have shed light on the potential advantages of melatonin in mitigating the adverse effects of chemotherapeutic drugs (Ma. Z et al., 2020). Acting as an immunomodulator and potent antioxidant, melatonin effectively mitigates cell death under conditions of oxidative stress (Espino et al., 2012; Hacışevki and Baba, 2018). Furthermore, melatonin intervenes in cellular apoptosis, inflammation, and oxidative processes, potentially enhancing the sensitivity of cancer cells to chemotherapy and radiation (Farhood et al., 2019).

Studies have demonstrated that melatonin exhibits anti-proliferative and anti-metastatic effects on breast cancer cell lines, restraining the viability and invasiveness of breast cancer mammospheres and influencing the expression of epithelial-mesenchymal transition (EMT)-related proteins (Martínez-Campa et al., 2024; Gurunathan et al., 2022; Maroufi et al., 2020). Its anti-invasive effect on breast cancer involves down-regulation of the p38 pathway and suppression of MMP-2 and -9 expression and activity (Hill et al., 2015). Moreover, melatonin decreases the proliferation and viability of cells and induces apoptosis in neoplastic mammary cells, with better efficacy in ER-positive tumours, which exhibit high expression of the melatonin receptor MT1 (Alonso-González et al., 2018). Additionally, melatonin displays anti-proliferative activity by stimulating apoptosis in breast cancer cells (MDA-MB-231) through simultaneous modulation of the COX-2/PGE2, p300/NF-κB, and PI3K/Akt signalling pathways and activation of the Apaf-1/caspase-dependent apoptotic pathway (Hasan et al., 2020). Alvarez-Garcia et al. have reported the anti-angiogenic effect of melatonin by downregulating VEGF expression in human breast cancer cells, thereby reducing VEGF levels around endothelial cells (Alvarez-García et al., 2013). The noteworthy synergistic effect of melatonin with other anticancer drugs has also been observed (Banerjee et al., 2021; Mafi et al., 2023). However, a common limitation across these studies is the use of higher dosages of treatment and prolonged exposure time. Hence, it is imperative to adopt a combination therapeutic approach when utilising melatonin in the treatment of breast cancer.

Molecular pathways involved in breast cancer therapeutics in present study

Breast cancer is a multifaceted disease characterized by abnormal cell growth in breast tissue, which can potentially lead to invasion of surrounding tissues and metastasis. Central to cancer development and progression is the evasion of programmed cell death, known as apoptosis. Breast cancer cells often exhibit dysregulation of signalling pathways involving

matrix metalloproteinases (MMPs) and interleukins (ILs), contributing to tumour growth, invasion, and metastasis.

Apoptotic pathway in breast cancer

Apoptosis, or programmed cell death, is a vital process that eliminates damaged or unwanted cells, serving as a defense mechanism against cancer. In breast cancer, dysregulation of the apoptotic pathway is prevalent, promoting increased cell survival and tumour progression. Key players in this pathway include pro-apoptotic proteins (e.g., Bax, Bak) and anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL). The delicate balance between these proteins dictates whether a cell undergoes apoptosis or survives. Alterations such as overexpression of anti-apoptotic proteins or mutations in pro-apoptotic proteins disrupt this balance, fostering cancer cell survival. Targeting the apoptotic pathway has emerged as a promising therapeutic strategy for breast cancer.

Caspases play a dual role in breast cancer progression and treatment. Malfunctioning caspases can promote tumour development, tissue invasion, and resistance to programmed cell death, contributing to cancer progression. Conversely, leveraging the apoptotic machinery mediated by caspases offers a compelling approach for therapeutic intervention. Caspase-3, responsible for executing programmed cell death, is often dysregulated in breast cancer, leading to negative prognosis and treatment resistance. Caspase-8 triggers apoptosis via death receptor-mediated signalling pathways, and its disruption enables cancer cells to evade programmed cell death. Interactions between Caspase-8 and other signalling pathways, such as NF-κB, facilitate breast cancer cell survival and dissemination. Caspase-9 serves as a crucial initiator caspase in the intrinsic apoptosis pathway, and mutations in Caspase-9 or its regulatory proteins impair apoptosis initiation in breast cancer cells. Additionally, Caspase-9 regulates cancer stem cells and metastatic potential in breast cancer models.

Combination therapies targeting multiple components of the apoptotic machinery, including Caspase-3, -8, and -9 pathways, show promise as therapeutic strategies for breast cancer. By simultaneously addressing various aspects of the apoptotic pathway, these combination treatments offer the potential for improved treatment outcomes and overcoming treatment resistance in breast cancer patients.

Matrix Metalloproteinases (MMPs) in breast cancer

MMPs, or matrix metalloproteinases, constitute a family of enzymes pivotal in breaking down components of the extracellular matrix, thereby facilitating processes like tissue remodeling, wound healing, and cell migration. In breast cancer, MMPs assume a critical role in tumor invasion and metastasis by fostering the degradation of the extracellular matrix. This degradation enables cancer cells to infiltrate surrounding tissues and gain access to the bloodstream or lymphatic system. Elevated levels of specific MMPs, notably MMP-2 and MMP-9, have been linked to unfavorable prognosis in breast cancer patients. Consequently, targeting MMPs has garnered attention as a potential therapeutic approach to impede tumor progression and metastasis.

Interleukins in breast cancer

Interleukin-4 (IL-4) and interleukin-10 (IL-10) are immunoregulatory cytokines with intricate roles in breast cancer. IL-4 is often associated with promoting Th2-type immune responses and allergic inflammation, while IL-10 is known for its immunosuppressive and anti-inflammatory properties. Their roles in breast cancer are diverse and context-dependent, influencing tumour progression, immune responses, and treatment outcomes.

IL-4 has been implicated in breast cancer advancement by influencing tumour cell growth, invasion, and metastasis. It promotes the polarization of macrophages towards an M2-like phenotype, which is associated with tumour-promoting actions such as angiogenesis and tissue remodeling. However, some studies have suggested potential anti-tumor effects of

IL-4, including its ability to induce programmed cell death in breast cancer cells and enhance immune effector cell cytotoxicity.

On the other hand, IL-10 is an anti-inflammatory cytokine with immunosuppressive properties. In breast cancer, IL-10 has been shown to inhibit the production of pro-inflammatory cytokines and reduce chronic inflammation in the tumor microenvironment. This may limit tumor growth and metastasis.

Targeting the signaling pathways of IL-4 and IL-10 holds promise for breast cancer treatment, either alone or in combination with other therapeutic approaches.

Breast cancer therapeutics and *In silico* studies:

In silico techniques, such as molecular docking, molecular dynamics simulations, and pathway analysis, have become effective tools in the field of drug discovery and development (Roy et al.,2022). These computational methods facilitate the prediction of interactions between ligands and receptors, the assessment of binding strengths, and the understanding of atomic-level drug-target interactions (Feng et al.,2020). Through the use of structural biology and bioinformatics methods, *in silico* investigations accelerate the process of identifying lead compounds, improving the pharmacokinetic qualities, and designing new therapies for breast cancer (Fend et al.,2020). *In silico* docking research allows for the exploration of connections between plant based substances and cellular receptors, offering important insights into how ligands bind, their strength of attachment, and their specificity (Kadioglu et al.,2021). Phytochemicals have the potential to impact signaling pathways associated with cellular growth, viability, and dissemination by specifically targeting these receptors, potentially leading to anticancer properties (Kadioglu et al.,2021).

In silico pathway analysis combines omics data with network pharmacology methods to understand the molecular mechanisms behind the anticancer properties of plant compounds. Through mapping drug-target networks and pathway interactions, computational

research offers valuable insights into the complex nature of breast cancer and identifies possible combinations of drugs that work together synergistically for combination therapy (Daina et al.,2019).

HYPOTHESIS

AND

OBJECTIVES

HYPOTHESIS AND OBJECTIVES

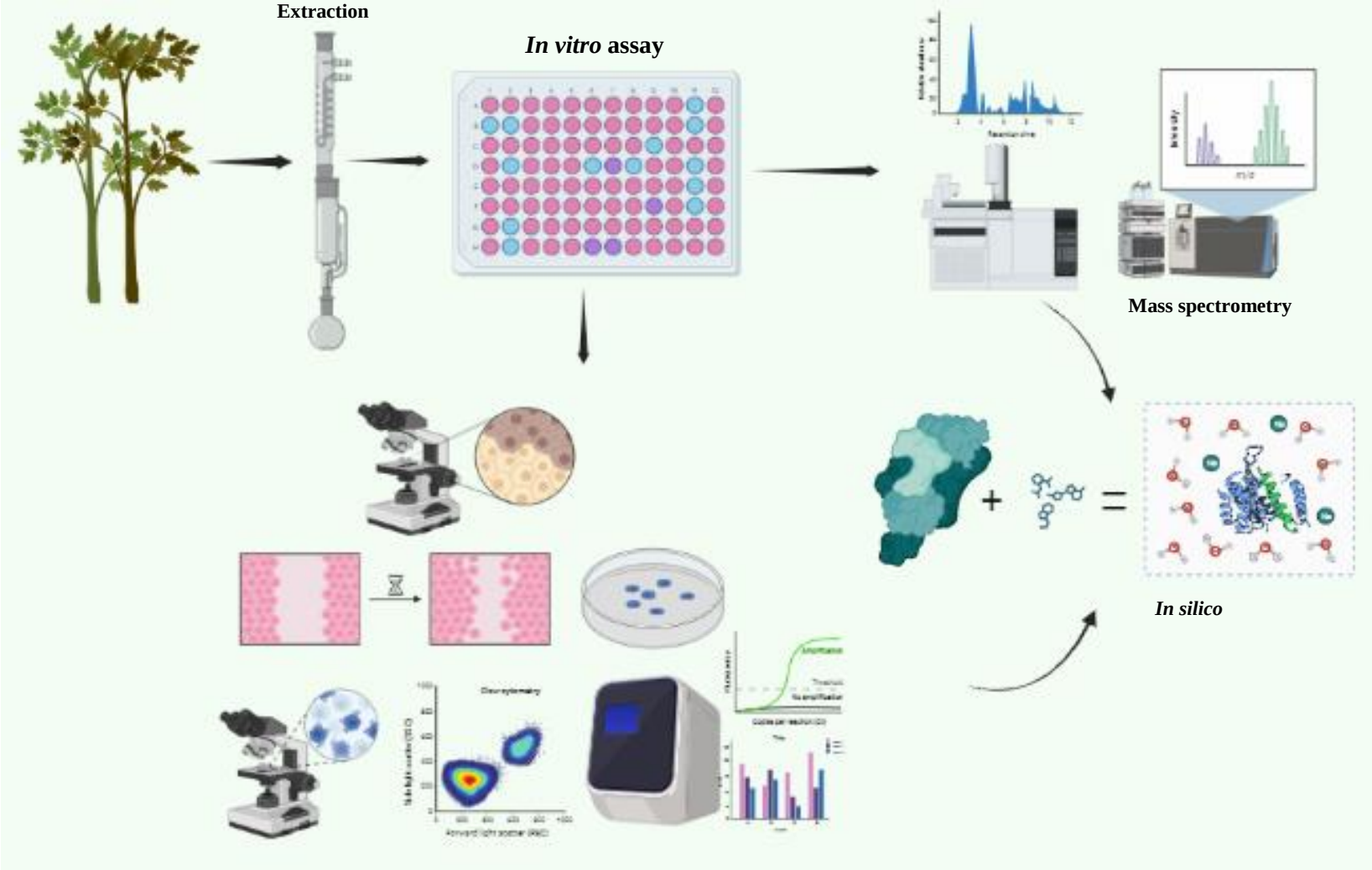
Phytochemicals of the identified plants will exhibit anti-breast cancer properties that can be further potentiated by Melatonin in combination.

Objective 1: To screen selected four medicinal plants for their anti-proliferative activity on breast cancer

Objective 2: To identify phytochemicals in the selected medicinal plant extract (*S. virginianum* L.).

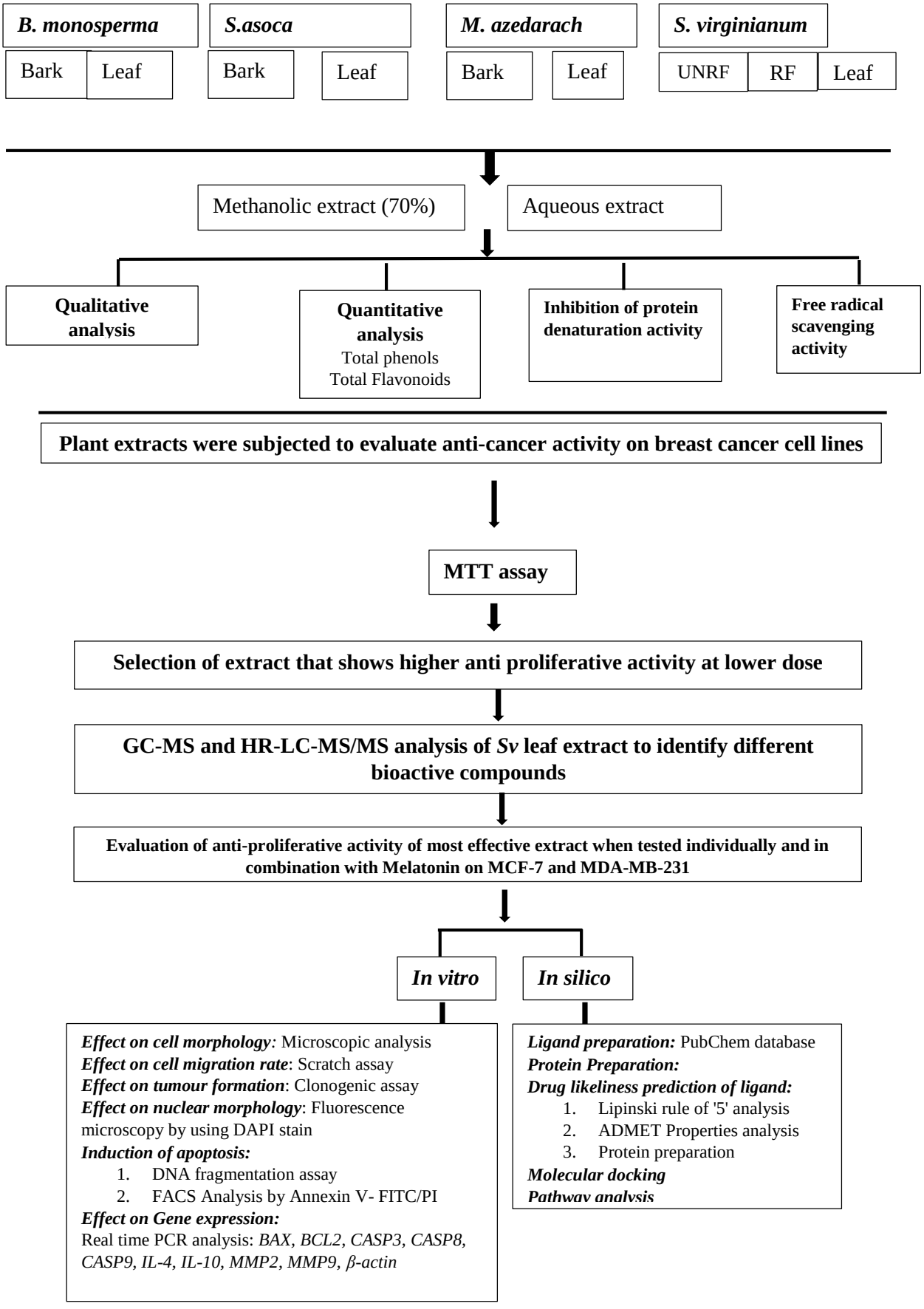
Objective 3: To identify potential bioactive compounds through *in silico* studies.

Objective 4: To evaluate the combinatorial action of melatonin in presence or absence of selected plant extract (*S. virginianum* L.) for its anti-proliferative action on breast cancer cells.



Flow chart explaining the work done

Overall experimental design of the work



Scope of the proposed work

Notably, there is been limited exploration into the combined effects of natural substances and their role in inhibiting metastasis. Phytochemicals, in particular, have shown significant potential in preventing the spread of cancer cells from the primary tumour to other parts of the body. This research has the potential to lay the groundwork for developing phytochemicals as potent anti-metastatic agents, alongside melatonin, for treating breast cancer.

The current study is centered on exploring phytotherapeutics for breast cancer and additionally aims to target metastatic breast cancer cells using a combination approach with melatonin. This study encompasses phytochemical analysis, as well as *in vitro*, *in silico*, and molecular investigations of human breast cancer using breast cancer cell lines.