CHAPTER 5

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Enhancing Anti-Proliferative and Cytotoxic Activity: Exploring Melatonin Alone and in Combination with *S. virginianum* Leaf Extract

5.1 Introduction

Combinational therapy is a cornerstone in tackling drug-resistant tumours and augmenting overall treatment efficacy (Wang et al.,2015 and Yan et al.,2017). When employing a combination approach, it is imperative to consider factors such as toxicity overlap, neutralized responses, and agonistic mechanisms. Table 5.1.1 exhibits the previously studied combinations for anti-cancer activity evaluations. Till the date, the combinational approach of plant extracts and melatonin were not studied. Therefore, in present study, a very first time, a promising strategy involves integrating natural product-derived substances with anticancer co-activators like melatonin.

Melatonin (Figure 5.1.1), also known as N-acetyl-5-methoxytryptamine, is secreted by the pineal gland in response to darkness in mammals and humans. Beyond the pineal gland, it can be synthesized in various tissues, including the skin, gastrointestinal tract, lymphocytes, retina, and bone marrow (Arendt & Skene, 2005). Recent studies suggest that disruption of melatonin's circadian profile due to exposure to light at night may contribute to the development, promotion, and progression of breast cancer (Kubatka et al.,2018).

The anticancer activity of melatonin primarily involves two membrane-associated G-protein coupled receptors (GPCRs), namely the MT1 receptor (previously Mel1a) encoded by the MTNR1A gene and the MT2 receptor (formerly Mel1B) encoded by the MTNR1B gene, both present in humans and mammals. Melatonin has been found to have anti-cancer effects via MT1/MT2 receptor-dependent mechanisms through G-protein coupled receptor activity. It

also demonstrates non-receptor-dependent mechanisms such as immune modulation, tumour surveillance, and telomerase activity (Menéndez-Menéndez, & Martínez-Campa, 2018).

In previous chapter it was found that Sv leaf extracts were showing promising anti-cancer activities against MCF-7 and MDA-MB-231 cell lines. In continuation, we hypothesize that Sv leaf extracts, in combination with melatonin, will synergistically lower down breast cancer proliferation rate and survival, serving as a potent molecule for anticancer therapy.

Questions regarding synergistic interactions between Sv leaf extracts and melatonin and the pathways involved in mediating this inhibitory effect merit exploration. Understanding these mechanisms could provide valuable insights into novel therapeutic targets for breast cancer treatment.

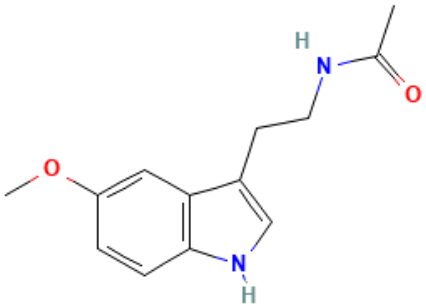

Figure 5.1.1: Structure of Melatonin. Image curtsy: PubChem

https://pubchem.ncbi.nlm.nih.gov/

This chapter delves into the investigation of melatonin's role as a potential combinational treatment alongside *Sv* leaf extract, aiming to address the broader research question of whether such a combined approach could offer enhanced efficacy compared to a single leaf extract. This inquiry is particularly pertinent in cancer treatment, where tumours often harbour multiple pathway mutations. Additionally, evaluating the potential of leaf extract as an adjuvant in combination with established single-molecule treatments is crucial for clinical

applications. Melatonin, renowned for its potent antioxidant properties and well-documented oncostatic effects, presents a unique candidate for such combinations due to its ability to target cancer cells at multiple levels.

Table 5.1.1: Combinational studies shows anti-cancer activity				
Combinational compounds		**Type of study**	**Effect**	**References**
Arctigenin (isolated from *Arctium lappa* L. seeds)	Doxorubicin	*In-vitro*	Increase Doxorubicin cell uptake and suppress multidrug resistance-associated protein 1 (MRP1) gene expression in MDA-MB-231 cells	Lee et al.,2020
Jatamanvaltrate P is a novel iridoid ester isolated from *Valeriana jatamansi* Jones	PI3K inhibitor : 3-MA	*In-vitro*	Cell destructor pathway induction through autophagy	Yang et al.,2017
Crocin (Carotenoid derived from saffron)	Crocetin (Carotenoid derived from saffron)	*In-vitro* *In-vivo*	Antimetastatic effects by disturbing the Wnt/β-catenin target genes	Arzi et al.,2020
Curcumin (*Curcuma longa* derived)	Paclitaxel	*In-vitro*	Induction of apoptosis	Calaf et al.,2018
Curcumin	Malphalan	*In-vitro*	Cell cycle arrest and Apoptosis	Passos et al.,2023
Quercetin	Doxorubicin	*In-vitro*	Increase Doxorubicin cell uptake and suppress multidrug resistance-associated protein 1 (MRP1) gene expression in MDA-MB-231 cells	Li et al.,2018

5.2 Materials and method

5.2.1 Cell viability analysis for combinational group: MTT assay

The MCF-7 and MDA-MB-231 cell lines were cultured following the protocol outlined in section 2.2.1 (Chapter 2), and the cell viability was determining for melatonin with concentration range 6.25 µg/mL-200 µg/mL. The combination experimental groups were considered on the basis of the IC_{50} obtained for melatonin and *Sv* leaf extracts on MCF-7 and MDA-MB-231 (Schmidt et al.,2020). The study aimed to evaluate the impact of the melatonin/extract combination on cell viability. For the combinational treatment of extract and melatonin, fold ratios of IC_{50} (1.25, 1.0, 0.5, and 0.25) for extract/melatonin combinations were considered, ensuring non-toxic concentrations for MCF-7 and MDA-MB-231 cells.

Table 5.2.1: Extract and melatonin combinational groups for MCF-7

Combinational groups	***Sv* Leaf extract (Aqueous) concentration (**µg/mL)	**Melatonin Concentration (**µg/mL)	**Combination Fold ratio of extract/melatonin IC_{50}**
Control	0	0	0
Combination 1	2.5	6	0.25
Combination 2	5	12	0.5
Combination 3	10	24	1.0
Combination 4	12.5	30	1.25

Table 5.2.2 Extract and melatonin combinational groups for MDA-MB-231			
Combinational groups	***Sv* Leaf extract (Methanolic) Concentration (**µg/mL)	**Melatonin Concentration (**µg/mL)	**Fold ratio of extract/melatonin IC_{50}**
Control	0	0	0
Combination 1	3	12.5	0.25
Combination 2	6	25	0.5
Combination 3	12	50	1.0
Combination 4	15	62.5	1.25

5.2.2 Statistical Analysis

All experiments were conducted in triplicates, and the data are presented as the mean ± standard error mean (SEM) of three independent measurements per extract were subjected to One Way ANOVA. Addtionaly, post hoc dunnets multiple comparison test was performed and significance were noted at $p<0.01$; $p<0.001$ through Graphpad Prism 8.0.

5.3 Result

The IC_{50} values for *Sv* leaf aqueous extract for MCF-7 and *Sv* leaf methanolic extract for MDA-MB-231 were previously determined to be 10 µg/mL and 12 µg/mL, respectively (Chapter 3). Figure 5.3.1 illustrates the cell viability (%) for melatonin treatment on MCF-7 and MDA-MB-231 cell lines (24 hr). For the study, a melatonin dose range of 6.25–200 µg/mL was administered to cells previously seeded with 90–95% confluency in a 96-well plate. With increasing concentrations, melatonin exhibited lower viability in both cell lines (MCF-7, MDA-MB-231) in a dose-dependent manner. The MTT assay determined the IC_{50} of melatonin for MCF-7 and MDA-MB-231 as 24 µg/mL and 50 µg/mL, respectively.

Figure 5.3.2 (A-D) present the results for combinational groups of extract/melatonin on MCF-7 and MDA-MB-231 cell lines for 24 hr and 48 hr. These graphs compare five combinational groups, including a control group, and demonstrate the significance of combination treatments in MCF-7 and MDA-MB-231 cells.

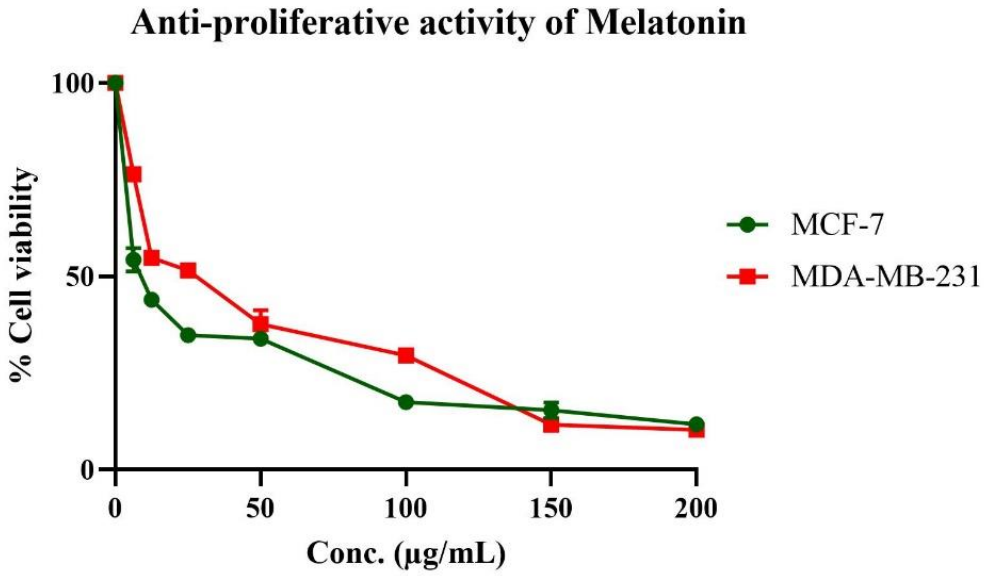

Figure 5.3.1: Anti proliferative effect of melatonin has shown dose dependent reduction in % cell viability in MCF-7 and MDA-MB-231 cells.

The MTT assay evaluated the effect of extract/melatonin combinations on cell viability in MCF-7 and MDA-MB-231 cell lines after 24 hr and 48 hr exposures. Cell viability (%) was calculated for each combinational group, and the values were plotted in Figure 5.2(A-D). Results showed that combinational group 1, comprising *Sv* leaf aqueous extract at 2.5µg/mL and melatonin at 6µg/mL concentration, had a non-significant lower inhibitory effect on MCF-7 cells with a 3.22% reduction in cell viability. Combinational group 2, consisting of *Sv* leaf aqueous extract at 5µg/mL and melatonin at 12µg/mL concentration, exhibited significant inhibition for MCF-7 cells with a reduction in cell viability of 24.66%. The most significant reduction in cell viability (33.77%) for MCF-7 cells was observed in combinational group 3, which included *Sv* leaf aqueous extract at 10µg/mL and melatonin at 24µg/mL (Figure 5.2 A&B).

All combinational groups of *Sv* leaf methanolic extract and melatonin showed significant inhibitory effects on MDA-MB-231 cells in a dose-dependent manner. Additionally, for both MCF-7 and MDA-MB-231 cell lines, 48-hour exposure to combinational groups significantly reduced viable cell percentages (Figure 5.2 C&D).

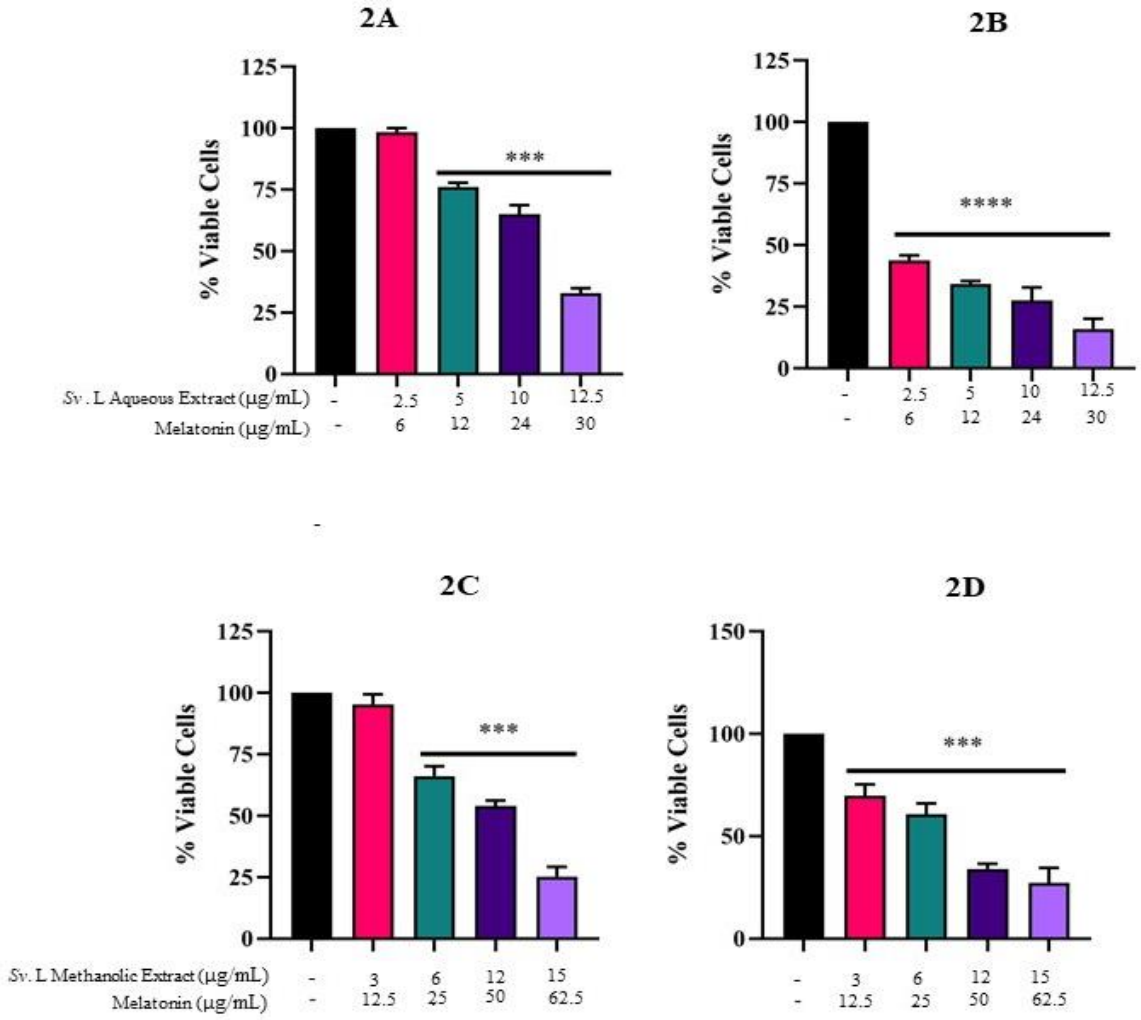

Figure 5.3.2 (A-D): Anti-proliferative activity of melatonin in combination with *Sv* leaf extracts at IC_{50} (µg/mL) combinational group. Figure A and B show the anti- proliferative activity for MCF-7 cell line at 24 hr and 48 hr incubation respectively. Figure C and D shows the anti- proliferative activity for MDA-MB-231cell line at 24 hr and 48 hr incubation respectively. All treated combinational groups were compared to control group (untreated cells) by Student t-test and the significance * represents p<0.005 and **** represents as <0.001.**

5.4 Discussion

In recent years, interest has increased in exploring natural compounds for their potential in cancer treatment. Combining melatonin and plant extracts as a novel approach that holds promise for developing innovative and improved cancer treatment strategies. While there have been reports on phyto-molecular combinations as anticancer therapeutics, previous studies have primarily focused on combinations involving established anticancer drugs and bioactive compounds (Pezzani et al.,2019). However, the combinational approach using melatonin and plant extract presents a fresh and potentially effective strategy for combating cancer. This study opens new possibilities for developing innovative and improved cancer treatment strategies.

Melatonin, known for its free radical scavenging and antioxidant properties, has been widely studied against the toxic effects of doxorubicin in rats (Öz & İlhan 2006). The findings revealed that rats treated with doxorubicin exhibited increased levels of oxidative stress markers and decreased levels of antioxidant enzymes, indicating the damaging effects of the drug. However, co-administration of melatonin alongside doxorubicin significantly decreased oxidative stress markers and restored antioxidant enzyme levels to normal. This suggests that melatonin can protect against the toxic effects of doxorubicin by reducing oxidative stress and enhancing antioxidant defence mechanisms (Öz & İlhan 2006).

Previous studies have examined the combination of melatonin and doxorubicin, demonstrating improved survival rates in hepatoma cancer and mitigation of cardiotoxicity (Valentina et al., 2001 and Hanna et al.,2022). Additionally, a study on sensitive pleiotropic cells treated with melatonin and doxorubicin showed cell growth, cytotoxicity inhibition, and increased survival time in resistant P388 mouse leukaemia cells (Granzotto et al.,2001). Moreover, melatonin was found to increase doxorubicin intracellular concentrations,

potentially inhibiting P-glycoprotein-mediated efflux, thus preserving doxorubicin's efficacy in cancer treatment (Mohammad et al., 2020; Guo-Ping et al., 2010;).

Furthermore, a study found that melatonin synergizes with doxorubicin to induce apoptosis in breast cancer cells by decreasing AMPK α1 expression. This synergistic effect was also observed in other cancer cell lines, suggesting that reducing AMPKα1 could increase cancer cell sensitivity to doxorubicin treatment (Quynh et al., 2021).

Combining drugs in therapy offers several significant benefits, such as decreased dosages and treatment duration, lower costs, and reduced development of drug resistance. The present study aimed to explore the potential synergistic effects of combining plant extract and melatonin to overcome these challenges. The combinational therapeutic approach of plant extract and melatonin was studied for the first time to combat barriers encountered in cancer therapy.

In the present study, observations indicated that the sub lethal doses ($IC_{50/2}$) combination and IC_{50} combination of Sv leaf extract and melatonin showed promising reductions in cell viability at 24 hr of exposure. These combinations (extract/melatonin) and exposure duration (24 hr) were considered for further assays (Chapter 6 and 7).

Overall, findings of this chapter highlight the presence of active molecule in the extracts and the combination of it with melatonin shows a novel and effective strategy in cancer treatment. Thus, to unleashed the active principal components present in the plant extracts, next chapter deals with understanding the diversity of it through analytical methods.