CHAPTER – 5

TO MODULATE THE EFFICACY OF METFORMIN WITH BIOACTIVE SWERTIAMARIN

Objective 3: To modulate the efficacy of Metformin with bio active Swertiamarin

Introduction:

In earlier objectives, we had identified the growth inhibition by metformin in different cell lines exhibiting androgen receptor heterogeneity. When starvation was introduced by energy depriving condition in this cancer cell lines, it enhanced autophagy- an inherent property of TME. Hence, it could not inhibit cell proliferation in high energy deprivation rather it started to help the cancer cell to proliferate. Despite of having varied anti-cancer properties, It was found that metformin cannot be considered as an ideal anticancer drug for prostate cancer as AR heterogeneity seems to alter its efficacy. Thus, considering all the observations it was decided to evaluate another compound that can overcome the poor potency of metformin under varied condition.

Previous studies from our lab have identified Swertiamarin- a bitter glycoside from *Enicostemma littorale* as an excellent insulin sensitizer. It has been extensively explored for its anti-diabetic potential and exhibits many properties similar to that of metformin. Our lab have reported that the treatment of swertiamarin in hepatic cells attenuated gluconeogenesis and lipogenesis, ameliorating oleic acid-induced lipid accumulation and oxidative stress. Further, like metformin, it also ameliorates insulin resistance [(T. P. Patel et al., 2016)] and also regulates hepatic and adipose tissue gene expression by targeting PPAR-γ, thus improving insulin sensitivity in experimental NIDDM rat model[(T. P. Patel et al., 2013)]. Moreover, another study have demonstrated the anticancer activity of swertiamarin on human cervical cancer models in which they evaluated the apoptotic activity and showed the suppression of P- MEK and P-ERK expression [(X. Wang & Wang, 2021)]. Chen et.al have shown that swertiamarin ameliorated CS (Chronic cigarette smoke) -induced prostatic collagen deposition and relieved oxidative stress and local inflammation [(J. Chen et al., 2019)]. Swertiamarin was also found to target the expression of proinflammatory cytokines including tumor necrosis alpha (TNF-α) and interleukin (IL)-6 in an acute lung injury animal model by targeting the AKT-pleckstrin homology (AKT-PH) domain which indicated that swertiamarin can regulate inflammatory conditions by

acting as a natural AKT inhibitor [(Muhamad Fadzil et al., 2021)]. All these properties of swertiamarin makes it an ideal candidate for anti-cancer potential. So, taking this further, we evaluated anti-cancer efficacy of swertiamarin on AR-heterogenous cancer cell lines.

Materials:

Chemicals: MTT Reagent (TC191, Hi media), Presto blue (A13261, Thermo Fischer) Paraformaldehyde (TC703, Hi media), crystal violet (TC510, Hi media), Bradford Reagent (B6916, Sigma), Ponceau S treatment (P7170-1L, Sigma), Blotting grade blocker (1706404, Bio rad), Tween20 (P1379, Sigma), Clarity Max Western ECl (1705062, Bio Rad), Fetal bovine serum (10270106, Gibco), Charcoal stripped fetal bovine serum (12-676-029,Gibco), RPMI 1640 (AT171, Himedia), DMEM Ham's F12 (AL127A, Hi media), Trypsin EDTA (TCL179, Hi media), Penstrep(15140122, Gibco), DAPI(Sigma, Cat: D8417), DCFDA(Sigma, Cat: 287810), 17 β-Estradiol(estrogen)(sigma, E2758), Progesterone(sigma,cat:P8783), Swertiamarin (Cayman Chemicals Company, Cat: 27634)

Methodology:

Growth Inhibition Assay:

Growth inhibition of Lncap , PC3 and 22RV-1 AR heterogenic cell lines was performed to evaluate the influence of R1881 on the efficacy of swertiamarin. The growth inhibition of 22RV-1 cells was assessed under androgen induction, as well as under the influence of two additional steroidal hormones(separately), estrogen and progesterone. This evaluation was aimed to determine whether swertiamarin effectively inhibits Pc3 cells under non-specific hormonal induction and evaluated for mutated AR where nonspecific hormones caused proliferation. Growth inhibition assay was performed as per the procedure mention on page no. 59.

Clonogenic Assay: Clonogenic inhibition assays were conducted on 22RV-1, Lncap, and PC3 cells to assess the impact of Swertiamarin on the stopping the

proliferation of AR heterogenous cancer cells. All the procedure of Clonogenic assay was carried out as per page no. 50.

Migration Assay: Cell migration inhibition assays were performed on 22RV-1 cells. These methodologies were employed to gauge the efficacy of the drug in terms of cell survival and metastasis. The migration assay was done according to the protocol described on page no. 50.

Detection of Reactive Oxygen Species and apoptotic markers: Estimating ROS levels resulting from Swertiamarin treatment to assess the impact of Swertiamarin-mediated ROS on cancer cell integrity and death. C-caspase3, and C-PARP1 were subsequently evaluated to elucidate the cell death pathway of swertiamarin. The detection of ROS was done according to the protocol described on page no. 59.

Analysis of Expression of AR protein: The Western blot method was used to investigate the involvement of swertiamarin in expression of AR. All the steps were followed as per page no. 50.

Results:

To understand the inhibitory effect of Swertiamarin, two AR & AR mutated cell line(Lncap and 22RV-1) and one AR negative cell line(PC3) were treated with the Swertiamarin (Figure 1) for which the significant efficacy(91% inhibition) was observed in AR-negative cells at 0.7mM. The inhibitory effects of swertiamarin were observed for Lncap and 22RV1 at 37%and 29% respectively (figure 1). Swertiamarin exhibited greater efficacy in the AR-independent PC-3 cell line, demonstrating significant inhibition up to 0.007 mM. Moreover, in other cell lines such as Lncap and 22RV-1, lower inhibition was observed at a concentration of 0.7 mM. Hence for evaluating the inhibition, we evaluated the R1881 induction effect with higher concentration of 86.6mM.

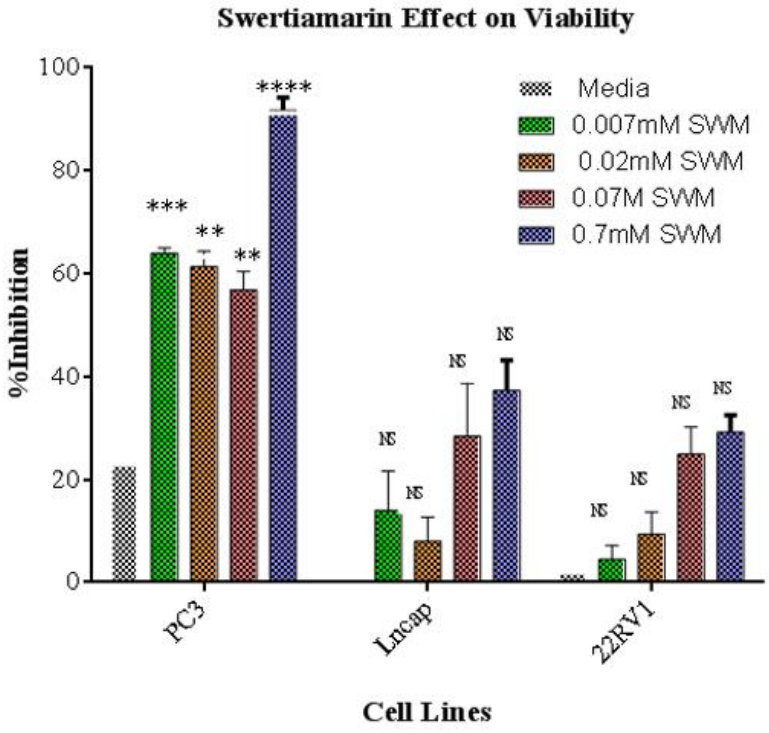
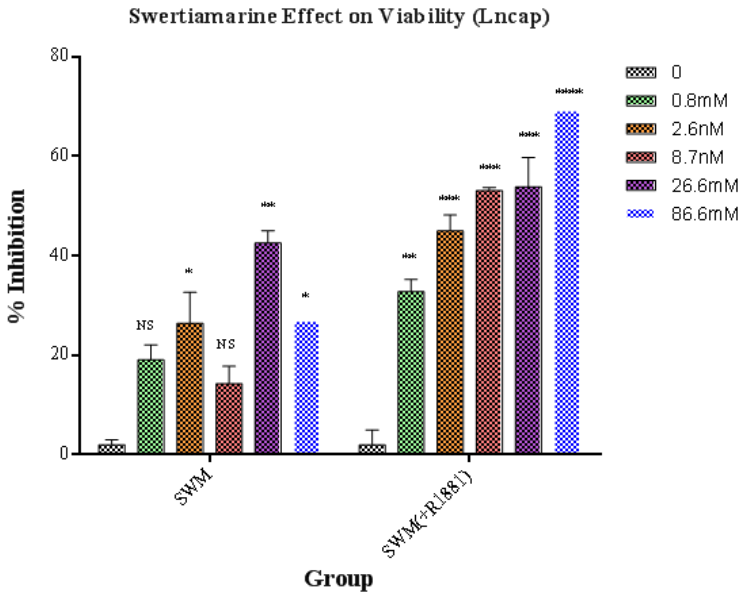


Figure 1 Cell viability accessed after 72 hours following Swertiamarin treatment in 22RV-1, Lncap and in PC3 cells The data was expressed as Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant. Note: P-value calculated compared to Media control

.Now, to evaluate the role of R1881in induction, Swertiamarin treatment was given in the absence and presence of R1881. As per figure 2A, Swertiamarin shows significant inhibition at the dose of 0.87 mM to 86.6 mM both in the absence and presence of 1nMR1881. Surprisingly, another AR +ve cell line(Lncap) swertiamarin demonstrated a maximum of 42% inhibition compared to 70% in 22RV-1(CRPC cell Line) in the absence of R1881 (Figure 2B).). Further, the maximum inhibition of 72% at 26mM od SM for R1881 induction of 22RV1 cells was observed, while estrogen induction achieved maximum inhibition(45%) at 26.01mM whereas, progesterone achieved 68% of significant inhibition at 8.6mM which was lesser than that of estrogen (Figure 3). So, there was no significant inhibitory role of Swertiamarin was observed on the clonogenic ability of all three cell lines (22RV1, Lncap, and PC3) after 6 days of its treatment (Figure 4 and 5). In case of migration inhibition there was no inhibition observed as compared to the media control until Day 6. However, after

day 6 in the event of migration inhibition, similar migration ability as that to the control was observed after Swertiamarin treatment (Figure 6)for 22RV-1 cells. As our focus is to identify drug effect on CRPC cell line, we evaluated migration assay only for 22RV-1 cells

A



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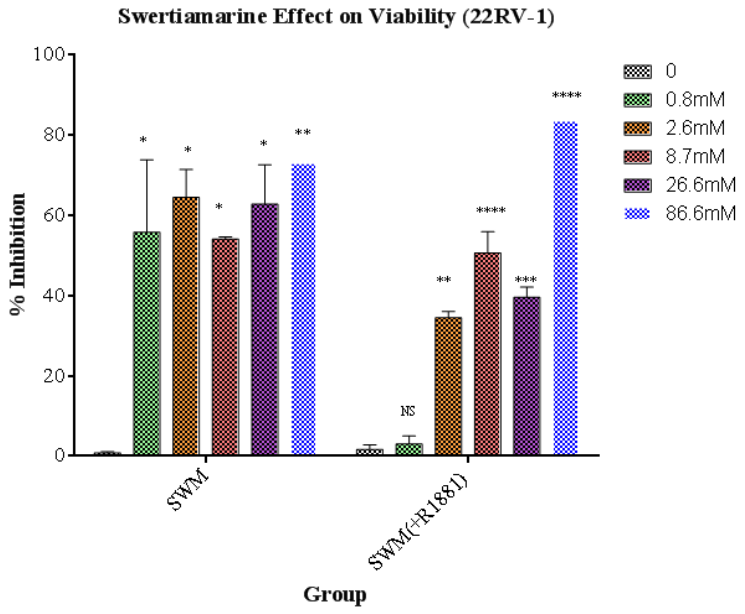


Figure 2 Cell viability accessed after 72 hours following Swertiamarin treatment in (A) 22RV- 1 in presence and absence of 1nM R1881 (B) in Lncap cells with similar condition. the data are expressed as Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant. Note: P-value calculated compared to Media control.

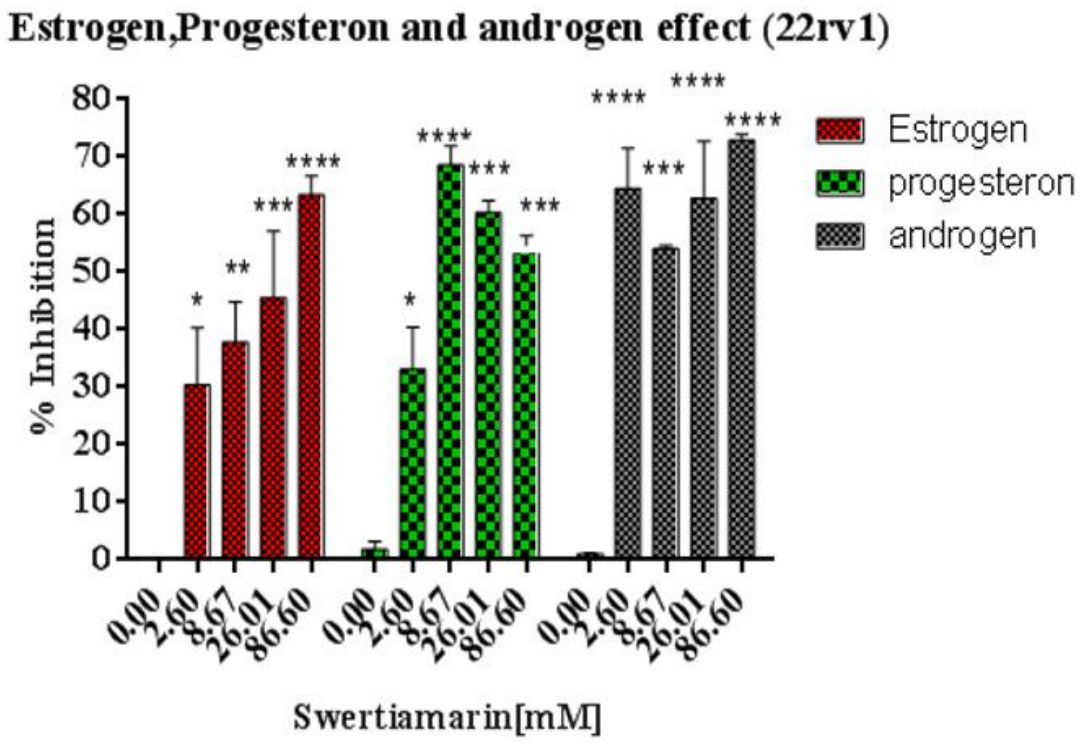
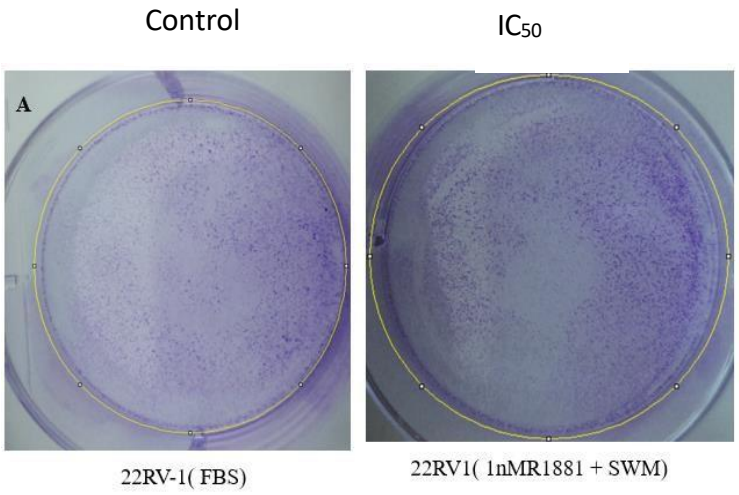


Figure 3 Cell viability accessed after 72 hours following Swertiamarin treatment in (A) 22RV-1 in presence of 1nM R1881,Estrogen and Progesterone. Mean ± SEM (n = 3).*i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant. Note: P-value calculated compared to Media control.**

In Figure 7, Relative expression of AR was evaluated in Lncap cells and it showed a significant decrease in level compared to media control in all three conditions. We excluded the CRPC cell line observations here because the primary antibodies for AR and its splice variants (ARV7) used previously to assess metformin efficacy were found to be malfunctioning. Moreover, the cleaved - Caspase3 and Cleave PARP1 also exhibited a significant increase from media control (Figure 8). Thus, to evaluate inhibition for 1nM estrogen while for 1nM progesterone inhibition was observed at higher concentration86.6nM and 26.01nM**.**



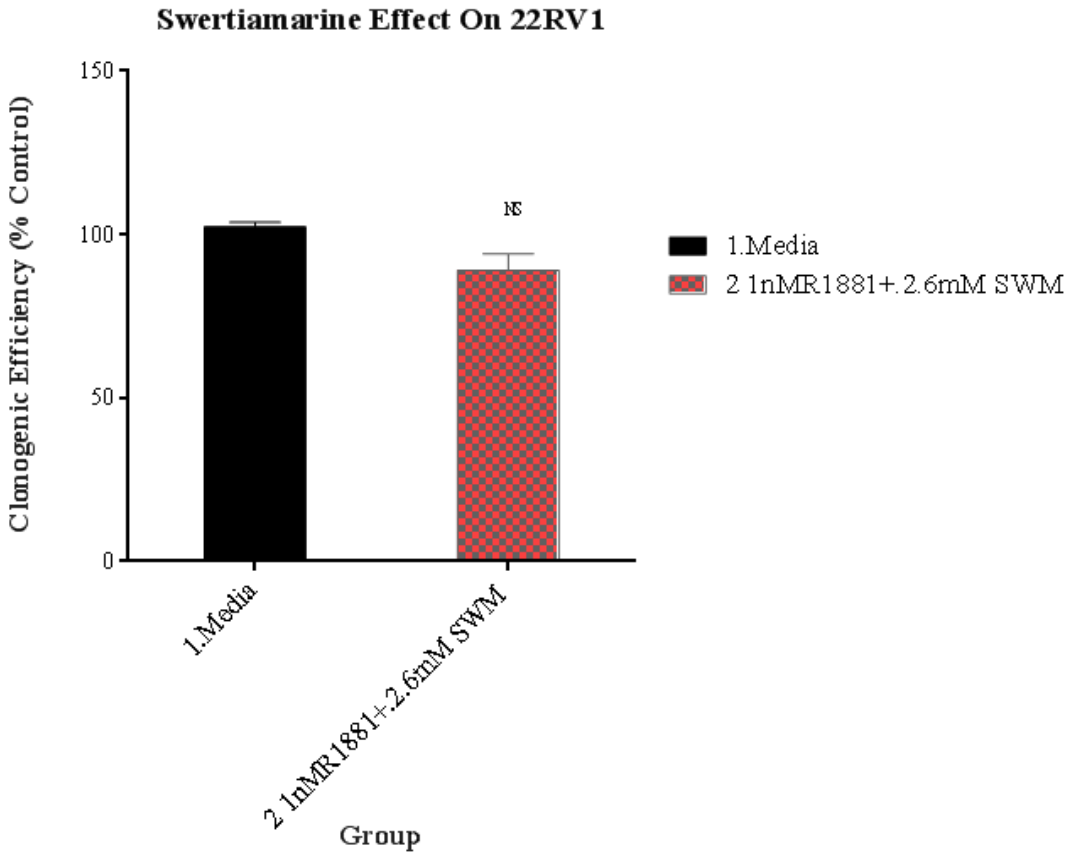
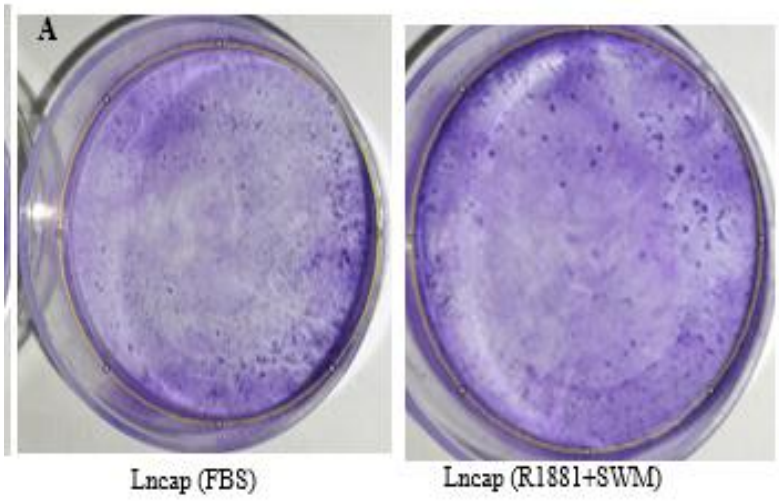
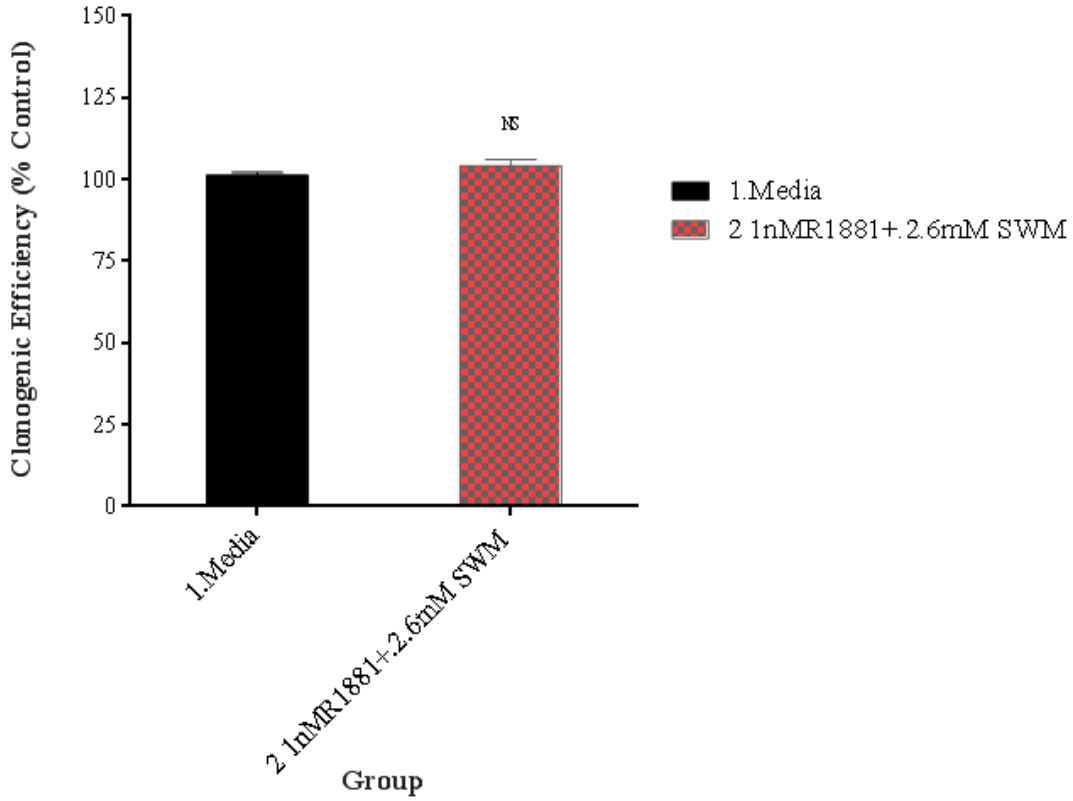


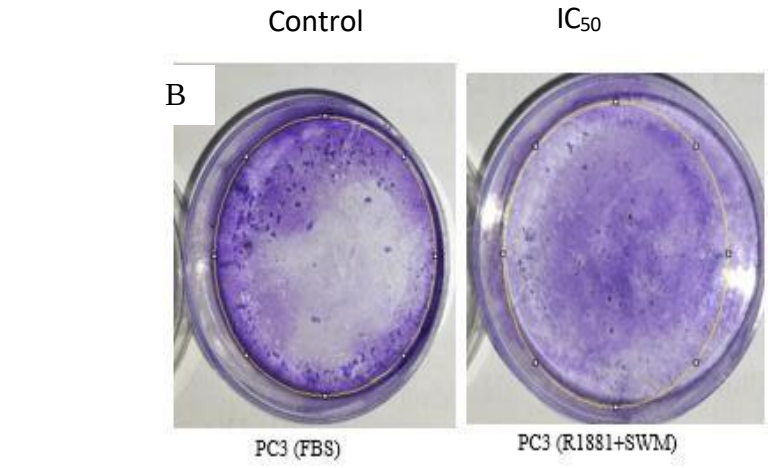
Figure 4 Shows swertiamarin inhibitory role on Clone ability of prostate cancer cells. (A) 22RV1 cells images were taken after 6th day of 2.6mM of swertiamarin treatment and .(B) shows representative data of the images. Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant.

Control IC_{50}



C **Swertiamarine Effect On Lncap**





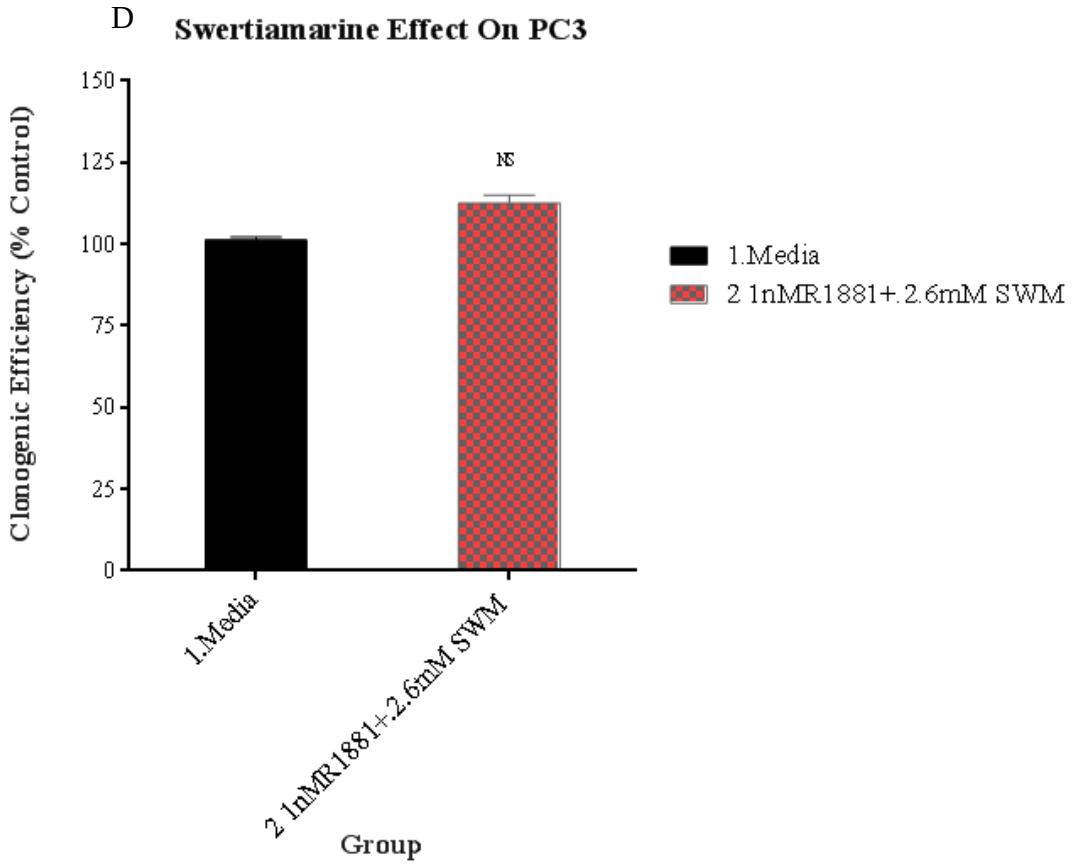


Figure 5 Shows Swertiamarin's inhibitory role on the clone ability of prostate cancer cells. (A) Lncap (B) PC3 cell images were taken after 6th day of 2.6mM of treatment and. (C) and (D) showed representative data of the images. note:1nM R1881 was added in all well. Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant. Note: P-value calculated compared to Media control.

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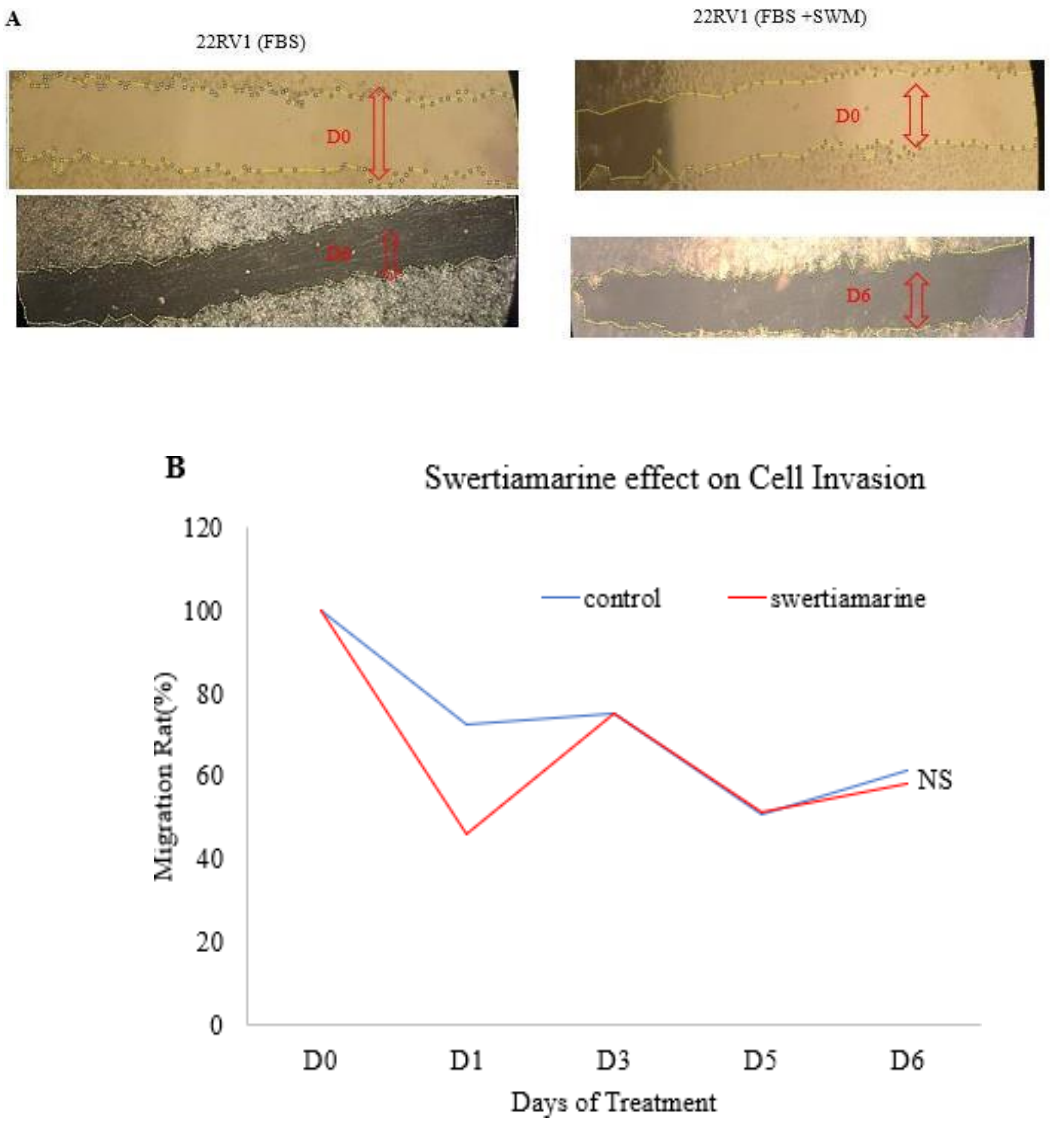

Figure 6 Shows the assessment of cell migration in. (A) 22RV1 Cells images were taken after 6th day of 2.6mM of treatment and .(B) shows representative data of the images. note:1nM R1881 was added in all well Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01,

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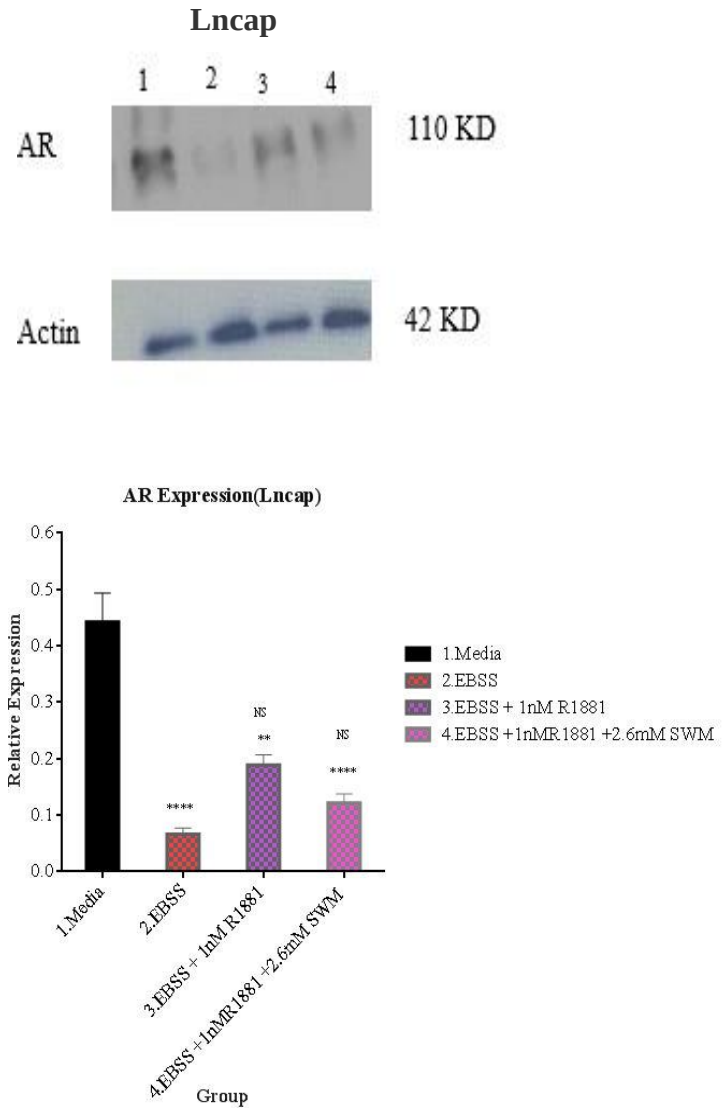
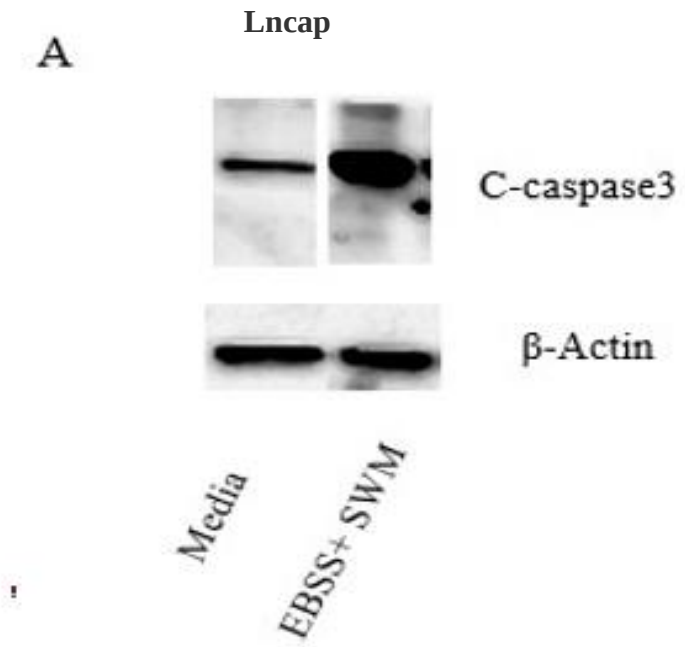
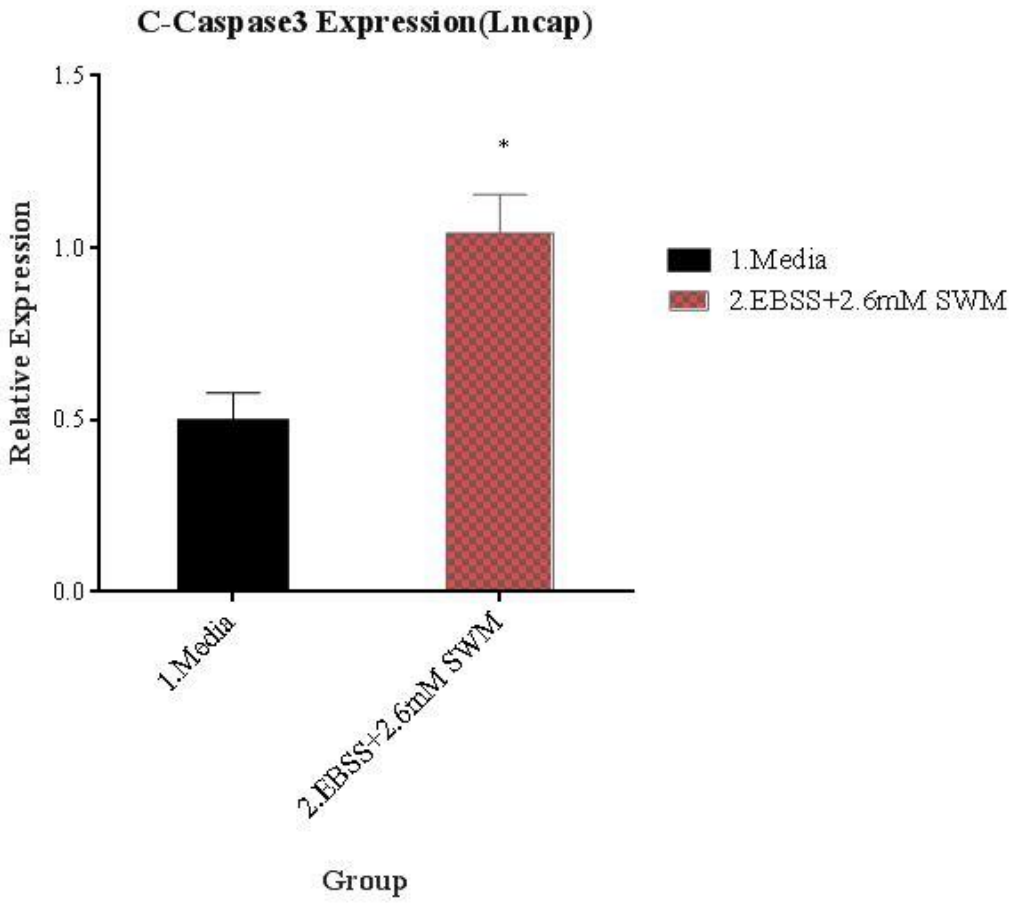


Figure 7 Relative Expression of AR was evaluated in (A) Lncap cells after 24hrs of 2.6mM of Swertiamarin following three different condition(i)Complete Growth Media(ii) only EBSS treatment (iii)EBSS and R1881(1nM). Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant. Note: P-value calculated compared to Media control.


Lncap
A
C-caspase3
β-Actin
Media
EBSS+ SWM


C-Caspase3 Expression(Lncap)
Relative Expression
1.5
1.0
0.5
0.0
*
1.Media
2.EBSS+2.6mM SWM
Group

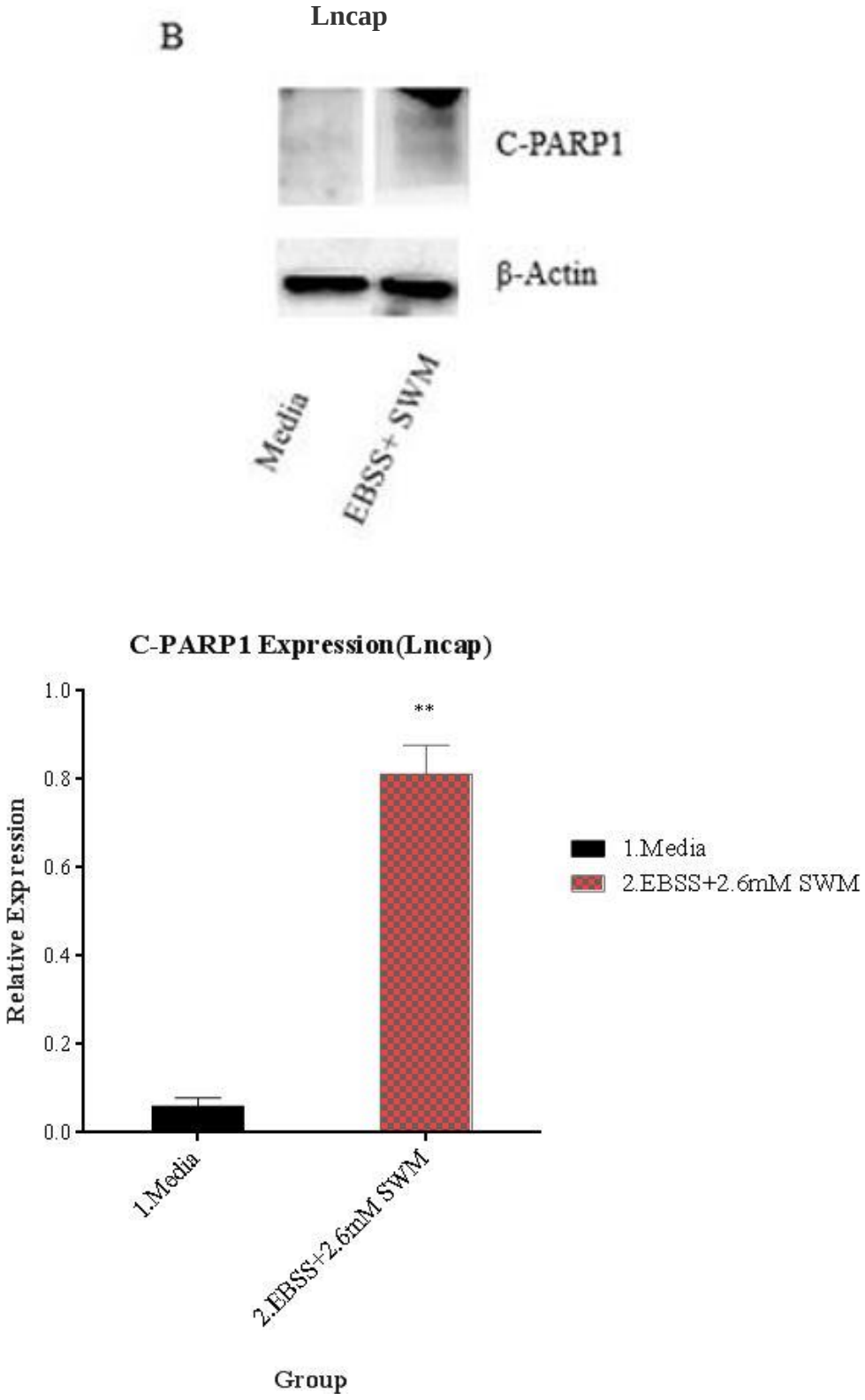


Figure 8 Relative Expression of (A) C-Caspase3 and (B) C-PARP1 was evaluated in Lncap cells after 24hrs of 2.6mM of Swertiamarin following two different condition(i)Complete Growth Media (ii) EBSS and R1881(1nM). Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant. Note: P-value calculated compared to Media control

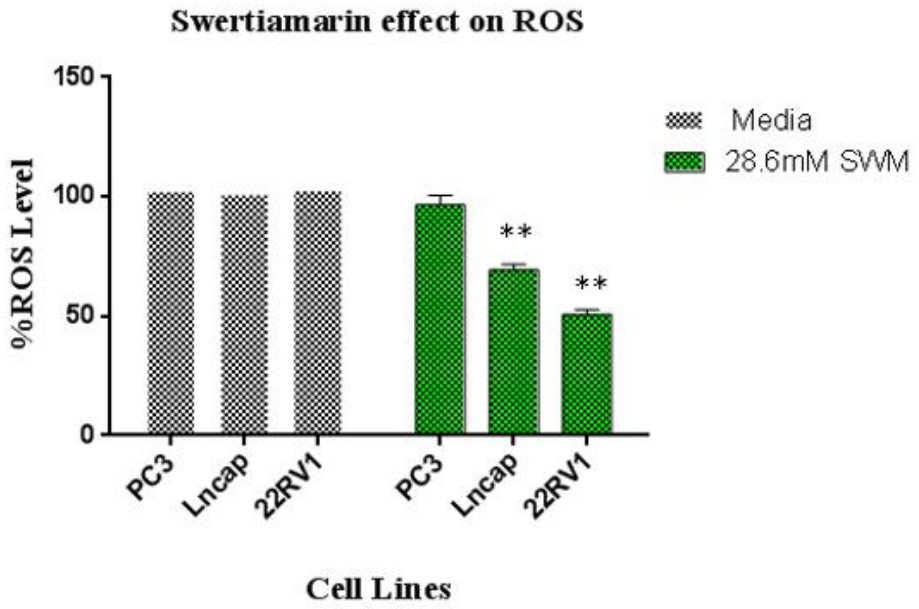


Figure 9 ROS estimation in PC3,Lncap and 22RV1 cells after 24hrs of Swertiamarin treatment followed by 10% charcoal-stripped incubation for 24hrs. Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01,

*** i.e <0.05, NS i.e nonsignificant. Note: P-value calculated compared to Media control.**

Discussion:

After the evaluation of the anti-cancer potential of swertiamarin, in the Pca cell lines, we observed that Metformin and Swertiamarin exhibits anti-cancer activity differently with or without hormonal induction. Metformin has shown highest efficacy in estrogen induction while swertiamarin demonstrated better efficacy in androgen induction in the CRPC cell line. Moreover in case of progesterone induction, swertiamarin exhibited similar inhibition compared to AR but Metformin did not behave similarly which counts an advantage if swertiamarin is considered in combination with progesterone and can target more effectively in CRPC cells (22RV1) cells that were unresponsive to ADT therapy.

Moreover, ROS estimation showed that swertiamarin decreased the ROS level in androgen dependent (LnCap) and CRPC (22RV1) cell line significantly instead of increasing the level, unlike metformin. However, ROS-mediated cell death did not happen in swertiamarin which was observed in figure 8 of o b j e c t i v e . This was supported by the study reported for Hela cells[(X. Wang & Wang, 2021)].

I n the case of metformin, where ROS level was found to decrease when energy deprivation increased respectively and so Metformin started to help 22RV1 cell to grow(Figure 6, chapter 4). If we consider this observation, it can be related to no clonogenic and cell migration inhibition observed with swertiamarin . Secondly, increased levels of caspase 3 and parp-1 may lead to non-autonomous caspase activity where cell death helps adjacent cells to grow[(Mollereau et al., 2013),(Eskandari & Eaves, 2022)].

Thus, for swertiamarin, it has to be explored. Interestingly, swertiamarin targets 22RV-1 more efficiently than metformin, still it is early to state that it is better than metformin because, under energy deprivation and hormonal induction, its efficacy is yet to be evaluated. Further, AR Expression was inhibited significantly as Metformin showed earlier.

Where metformin showed less effect(Figure 5A, Objective 2,) in the

presence/absence of R1881,

Swertiamarin showed 50% inhibition for CRPC cells significantly at 8.6mM which was much less compared to metformin 30mM(under R1881 induction). Thus swertiamarin can be a good bination drug along with Metformin. Further , SM showed better efficacy in androgen-independent PC3 cell line compared to Metformin. However , it is important to evaluate its efficacy under energy deprivation condition and shall be evaluated for ARV7 expression as it's a major prognostic factor in prostate cancer reoccurrence.

Conclusion:

As per the above results, Swertiamarin have the potential to inhibit AR-independent cell line than metformin . In presence of AR induction and energy deprivation (EBSS treatment) its efficacy need to be explored further as metformin anticancer activity gets altered under varied condition. Swertiamarin and metformin combination treatment need to be explored in vitro for combinatorial approach for prostate cancer