CHAPTER - 4

TO STUDY THE MECHANISM OF ACTION OF METFORMIN ON CELL DEATH

Objective 2: To Study the mechanism of action of metformin on cell death

Introduction:

In the previous objective, we used all three cell lines and observed that the AR heterogeneity alters the metformin efficacy and thus anticancer activity. Metformin can play an anticancer role in many ways, as it exhibits various anticancer characteristics such as anti-inflammatory effects, regulation of the mTOR pathway, apoptotic effects, and regulation of ROS levels, all of which influence tumor progression and drug resistance [(Skuli et al., 2022),(X. Zhou et al., 2023)] thus systematically assessing ROS levels, apoptosis induction, and autophagy activation following metformin treatment, researchers can gain insights into its mechanisms of action and efficacy as an anticancer agent. As discussed Metformin behavior in prostate cancer is ambiguous [(Skuli et al., 2022)]. The reason behind the ambiguity is not well-researched for In-vitro models of prostate cancer considering tumor microenvironment(TME). Whether Metformin can be affected by TME or it can reprogram the TME is interesting to analyze. Energy deprivation which has a vital impact on TME plays a major role in developing hypoxia and consequently creates ROS and angiogenesis to overcome the energy and cell repair mechanism [(Dewhirst et al., 2008)]. Increased ROS levels contribute to cell survival and proliferation of cancer cells by various pathways like PI3/AKT, and MAPK/ERK1/2.However, achieving toxic levels of ROS can also be responsible for cell death by apoptosis causing a mitochondrial membrane disruption [(England & Cotter, 2005)]. ROS also activates p53 in cancer cells and can be interlinked with apoptotic and autophagic pathways, which can alter the effects of DNA-targeting chemotherapeutic drugs sensitivity. Thus, energy deprivation & ROS level determination may give a better picture of metformin ambiguity in prostate cancer PTEN loss in cancer also increases the chromosomal instability [(Rieckhoff et al., 2020)] which is elevated in response to ROS level. All above circumstances can be affected by amino acid starvation So, in vitro development of amino acid starvation using EBSS treatment can provide a better understanding of metformin ambiguity between clinical and in vitro efficacy. So, in this objective, we evaluated the metformin effect on cell death pathways in

heterogenic condition like amino acid starvation (using EBSS) and androgenic stimulation(using R1881).

As per the conclusion of the previous objective introduction of R1881 alters the efficacy of metformin for prostate cancer's different models having an AR heterogeneity as AR signaling pathway is involved in cell proliferation via PI3K/Akt and MAPK pathway. Not only that, AR activation affects to CSC and is associated with stemness and self-renewal. Androgen can be produced and secreted by cancer cells itself called autocrine or either produced by the testis & adrenal gland which released and proliferate prostate cells via endocrine or autocrine signaling [(Quintero et al., 2023)]. Schroeder et al. demonstrated that The activity of AR can influence additional signaling pathways that support cell survival like STAT3 expression increased which maintains pluripotency of CSC when AR is inhibited[(Schroeder et al., 2014)]. To consider such a scenario R1881 treatment was given to the in vitro model before metformin treatment along with amino acid starvation

Material and Methods:

Materials:

Cell Lines: PC-3(CRL-1435, ATCC), Lncap (CRL-1740, ATCC), 22RV-1(CRL-2505, ATCC).

Culture Media: PC-3 was cultured in DMEM F12(AL127A, Himedia) + 10%FBS + 1% Pen Strep (15140122, Gibco) media. Lncap and 22RV-1 cells were cultured in RPMI-1640(AT171, Himedia) + 10%FBS+1% Pen Strep media

Chemicals: MTT Reagent (TC191, Himedia), Presto blue (A13261, Thermo Fischer) Paraformaldehyde (TC703, Himdeia), crystal violet (TC510, Himedia), Bradford Reagent (B6916, Sigma), Ponceau S treatment (P7170-1L, Sigma), Blotting grade blocker (1706404, Biorad), Tween20 (P1379, Sigma), Clarity Max Western ECl (1705062, BioRad), Trizol (9109, Takara), Metformin (317240-5GM, sigma), Fetal bovine serum (10270106, Gibco), charcoal-stripped fetal bovine serum (12-676-029, Gibco), Trypsin EDTA (TCL179, Himedia) Antibodies: actin (4967s, Cell signaling), PARP (9542s, Cell signaling), caspase 3 (9662S, Cell signaling), GAPDH

(5174, Cell signaling), BCL2 (AB32124, Abcam), LC3B (2775,Cell signaling), DAPI (Sigma,Cat:D8417), DCFDA (Sigma, Cat:287810), 17β-Estradiol(estrogen)(sigma, E2758), Progesterone (sigma,cat:P8783)

Methods:

Detection of LC3-Autophagic marker:

Cells were plated at a density of 0.5×10^6 Cells/Well in the six-well plate. After 24 h cells were starved for 24hrs using 10% charcoal-stripped media Except Control well. After 24 hrs starvation, media were replaced by EBSS in all wells except Control Metformin treatment for 4hr was given along with the final concentration of 1nM R1881 in respective wells and 20mM NH_4CL treatment will be given to all the wells. Bafilomycin was added as a positive control. Cells were harvested in Laemmli Sample Buffer (after an ice-cold PBS wash. The protein concentration of samples was determined by Bradford assay. The equal protein was loaded and resolved on 12% SDS-PAGE. The electro-blotting of protein onto an activated PVDF membrane (methanol charged) was conducted at a voltage of 100 V for 90 minutes, with the incorporation of an ice pack within the chamber. Transfer of protein was checked by Ponceau S treatment for 3-5min. After PBS wash the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS), 0.1% Tween-20 in TBS-0.02 M Tris-Cl, 0.15 M NaCl) for 1 h at room temperature. The membrane was incubated overnight with a specific(LC3, β-actin) primary antibody (1:1000). After incubation, the membrane was washed three times with TBS-T (TBS containing 0.1%Tween-20) for 10 min and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and the signal was visualized by UVTEC gel documentation system using Clarity Max Western ECl. Signal was normalized by the intensity of the housekeeping gene.

Detection of Caspase 3 and PARP1- Apoptotic Marker:

Cells were plated at a density of 0.5×10^6 Cells/Well in the six-well plate. After 24 h cells were starved for 24hrs using 10% charcoal-stripped media. After 24 hrs starvation, Metformin treatment for 24hr was given along with R1881. Cells were harvested in Laemmli Sample Buffer (after an ice-cold PBS wash. Protein The

protein concentration of samples was determined by Bradford assay. The equal protein was loaded and resolved on 10% SDS-PAGE. For assessing Caspase-3 and PARP-1 using western blot method as mentioned earlier(Page No: 68)

Estimation Reactive Oxygen species:

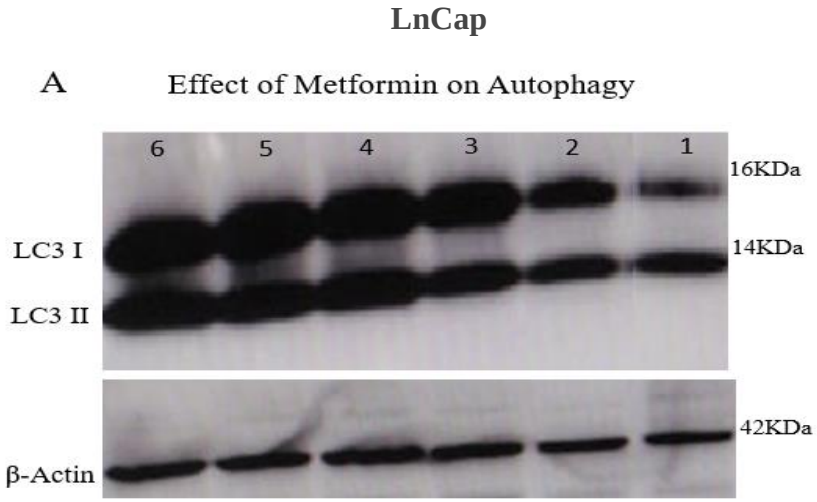
Cells were plated at a density of 0.5×10^6 Cells/Well in the six-well plate. After 24 h cells were starved for 24hrs using 10% charcoal-stripped media. After 24 hours of starvation, Metformin treatment for 24hr was given along with R1881 in respective wells. 100µM NAC was added in respective well as a positive control. Next day media was replaced by 500µl PBS.After PBS wash DCFDA (10µg/ml) was added per well, then incubated for 20 min in the dark. After incubation, PBS wash was applied, 10µg/ml DAPI solutions was added in each well for 10 min in the dark. The plates were read at Ex 485 and Em 535nm for DCFDA fluorescence detection and . Ex330/Em 470nm for DAP.ROS readout was normalized by DAPI to calculate the ROS production per cell.

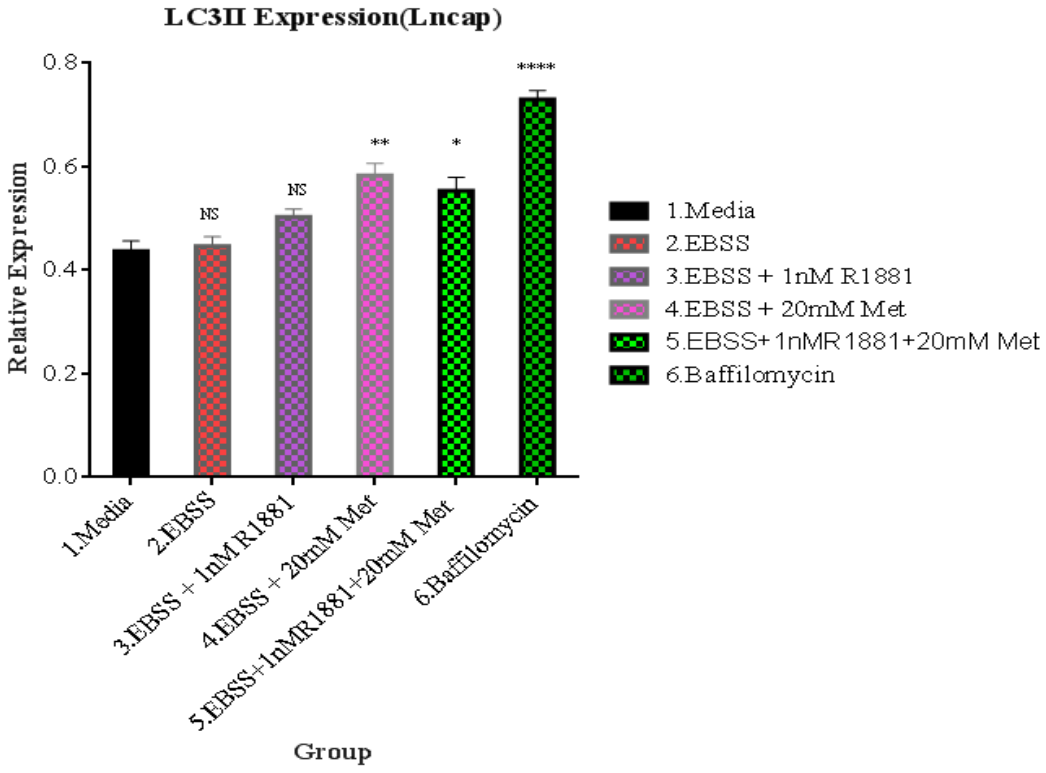
Growth Inhibition assay under Energy Deprivation :

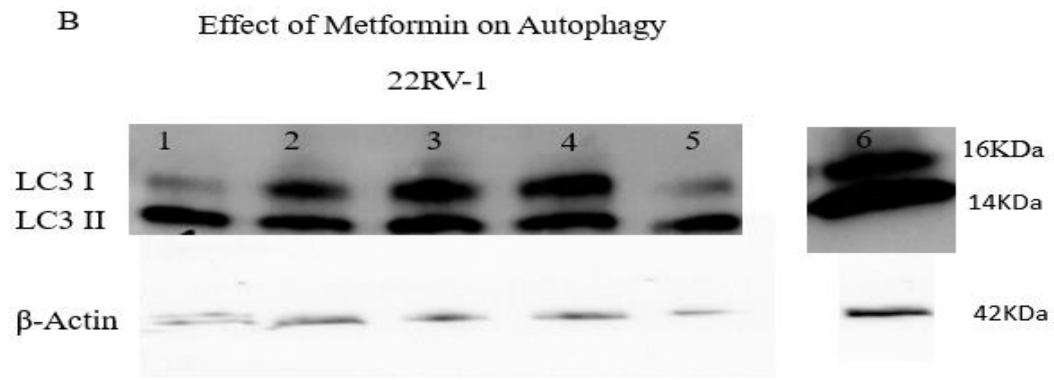
All cell lines were seeded at a density of 5000 cells/well in 96-well plates and starved for 24 hours. The experiments were conducted in the presence or absence of 1nM R1881 and the presence of different concentrations of EBSS(%V/V). On the same day, Metformin treatment was given to the respective well. After 48 hrs of Metformin incubation, prestoblue viability reagents were added in all the wells and after 4 hrs of viability reagents, fluorescence readings were taken at 560nm Ext and 590nm Em. % survival and % inhibition were calculated in comparison to media control and EBSS control.

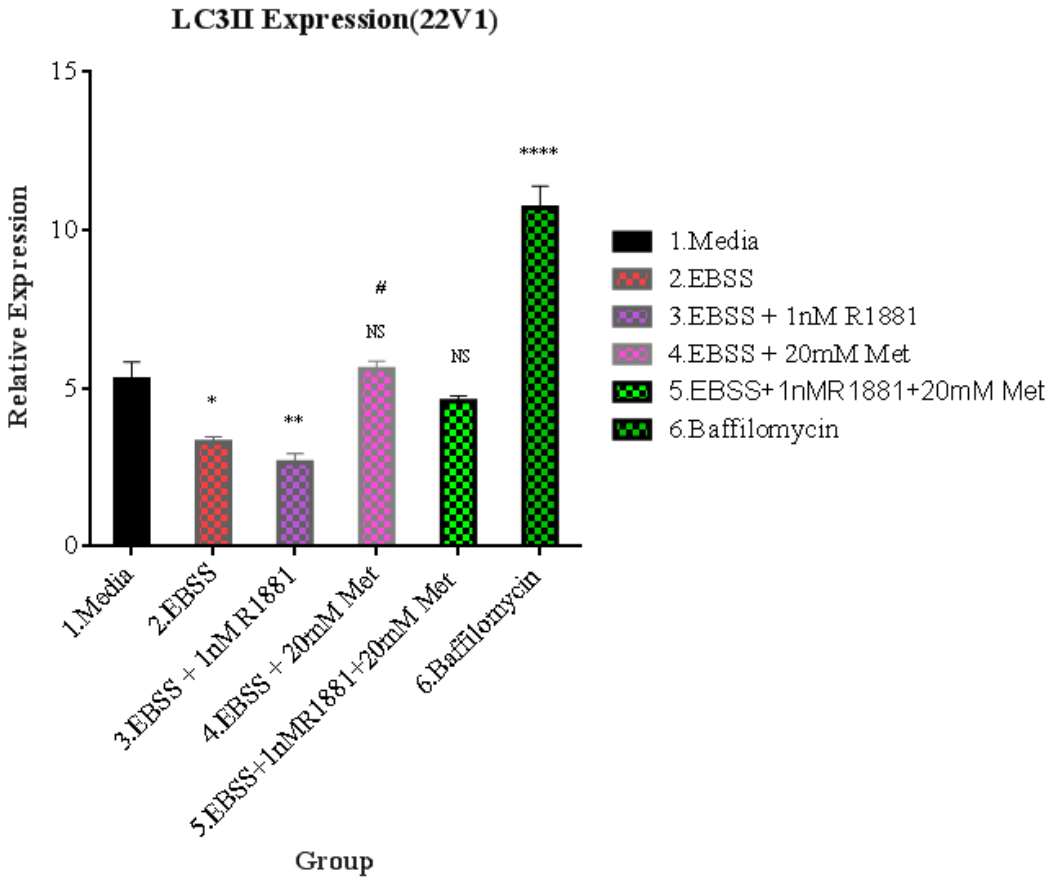
Results:

Autophagic and androgenic condition alters Metformin effect on LC3 an Autophagic Marker:






B
Effect of Metformin on Autophagy
22RV-1
1
2
3
4
5
6
LC3 I
LC3 II
β-Actin
16KDa
14KDa
42KDa


LC3II Expression(22V1)
Relative Expression
15
10
5
0
*
**
#
NS
NS
****
1.Media
2.EBSS
3.EBSS + 1nM R1881
4.EBSS + 20mM Met
5.EBSS+1nMR1881+20mM Met
6.Baffilomycin
Group

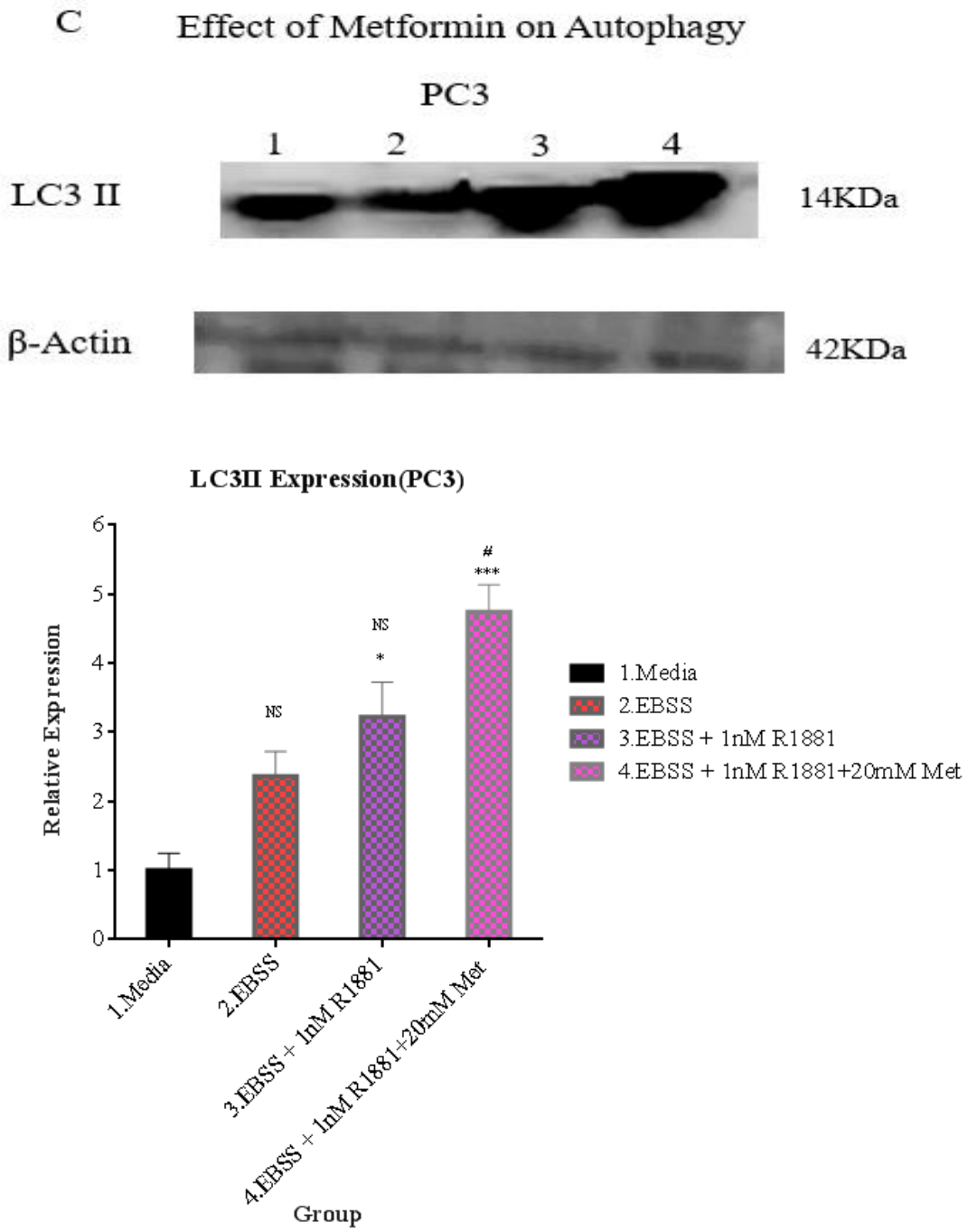
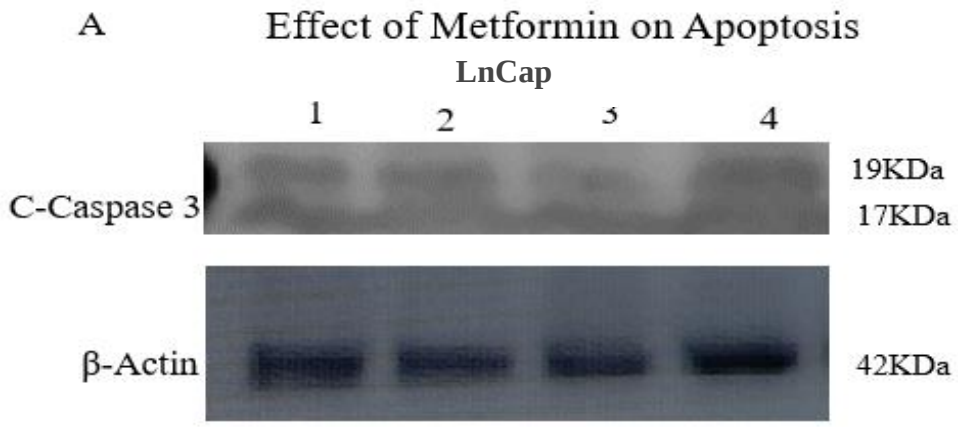
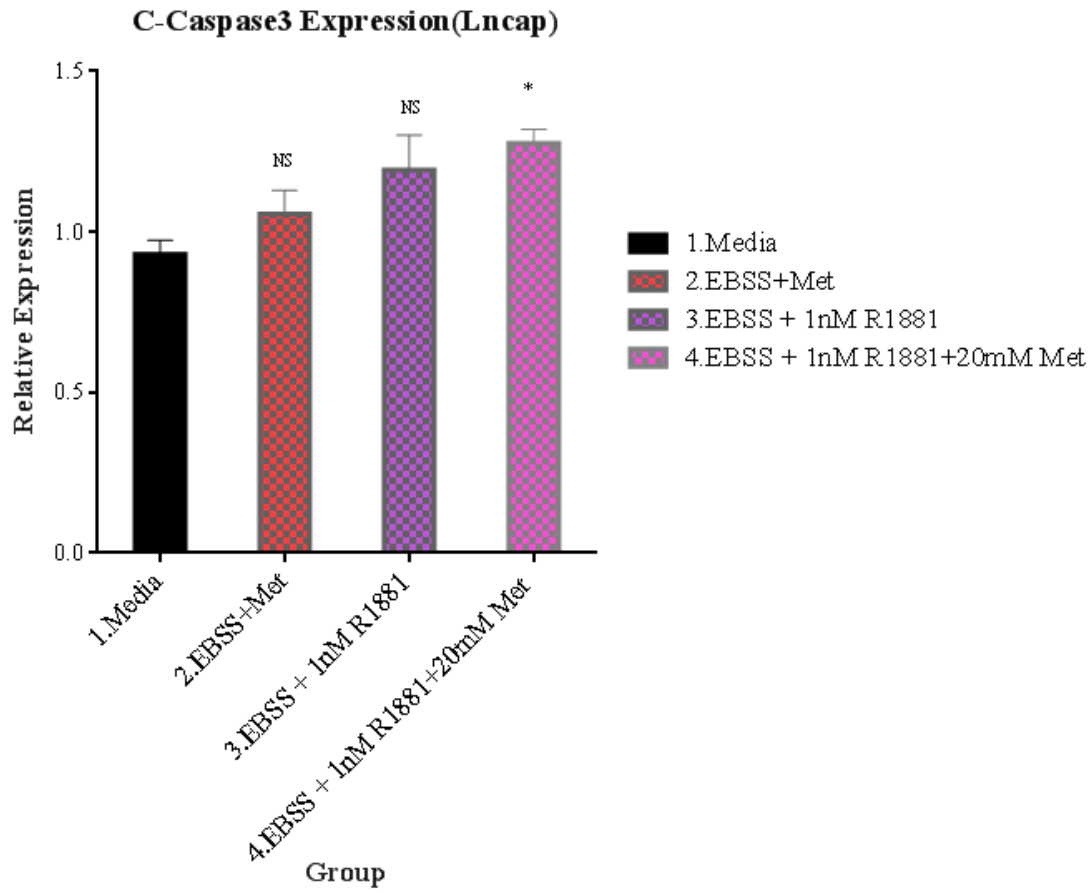
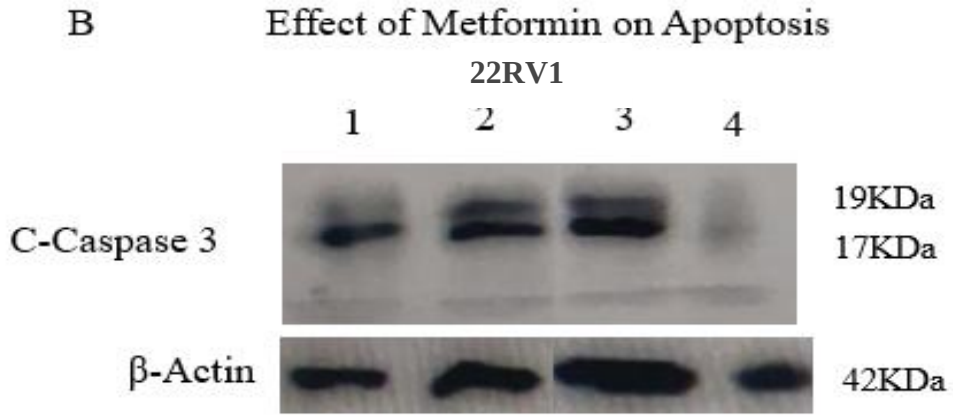


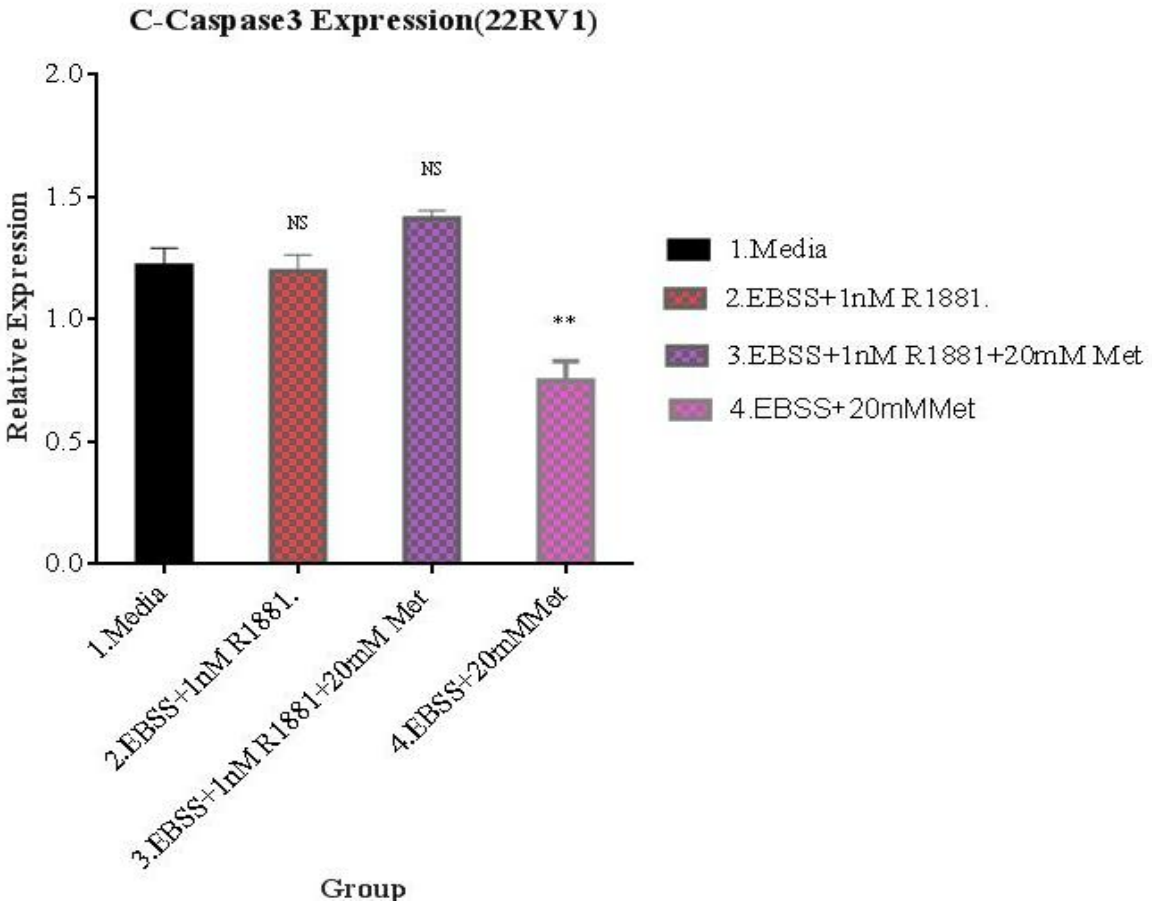
Figure .1 Relative expression of LC3 II was examined for Lncap (A), 22RV-1 (B) and PC3cells (C) following treatment with Metformin (20mM) with five different conditions (i) Complete Growth Media(ii) only EBSS treatment (iii)EBSS and R1881(1nM) (iv) EBSS and Metformin(20mM) and (v) EBSS, R1881(1nM) and Metformin(20mM) treatment for an equivalent duration. Bafilomycin was taken as a positive control. Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i.e nonsignificant Note: P-value determined against media control(*), EBSS control (#), EBSS+R1881($).

Following amino acid deprivation and androgen deprivation, three distinct in vitro models of prostate cancer exhibited varying increases in LC3II expression upon treatment with 20 mM Metformin. Figure 1 shows an expression of LC3II for three different cell lines Lncap cells(Figure 1A) 22RV1Cells (Figure 1B) and PC3 cells (Figure1 C) under five different conditions. The treatment with 20 mM Metformin exhibited varying increments of expression. However, only energy deprivation (EBSS) and androgenic Condition (1nM R1881) could not similarly increase the LC3II expression in all three cell lines, particularly in 22RV-1. In LNCaP cells, LC3II expression appears to be nonsignificant under only EBSS (without R1881) treatment, but after Metformin treatment, it increased significantly. A similar effect was also observed after adding Metformin under the EBSS + 1nM R1881 condition. In the case of 22RV1 LC3II was shown to decrease significantly under EBSS and EBSS+R1881 which was increased significantly after metformin treatment compared to EBSS condition. In PC3 cells R1881 and R1881+Met increased LC3II expression significantly under EBSS condition.

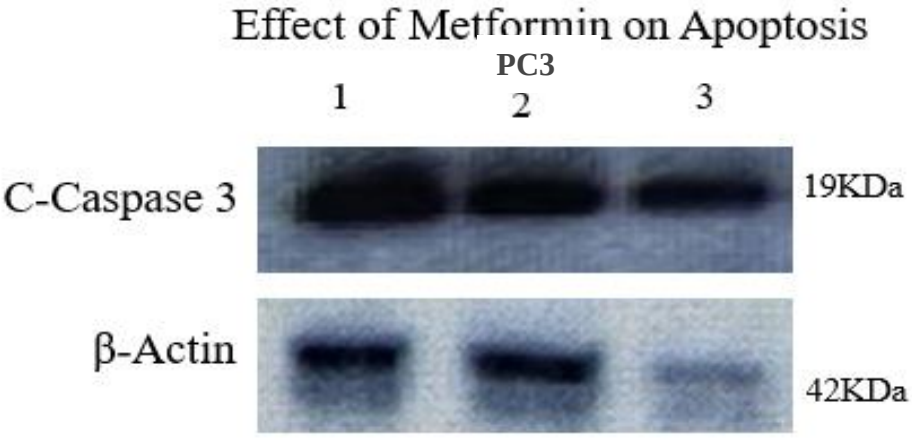



C-Caspase3 Expression(Lncap)
Relative Expression
1.5
1.0
0.5
0.0
NS
NS
*
1.Media
2.EBSS+Met
3.EBSS + 1nM R1881
4.EBSS + 1nM R1881+20mM Met
Group


B
Effect of Metformin on Apoptosis
22RV1
1
2
3
4
C-Caspase 3
19KDa
17KDa
β-Actin
42KDa


C-Caspase3 Expression(22RV1)
Relative Expression
2.0
1.5
1.0
0.5
0.0
NS
NS
**
1.Media
2.EBSS+1nM R1881.
3.EBSS+1nM R1881+20mM Met
4.EBSS+20mMMet
Group

C


Effect of Metformin on Apoptosis
PC3
1
2
3
C-Caspase 3
19KDa
β-Actin
42KDa

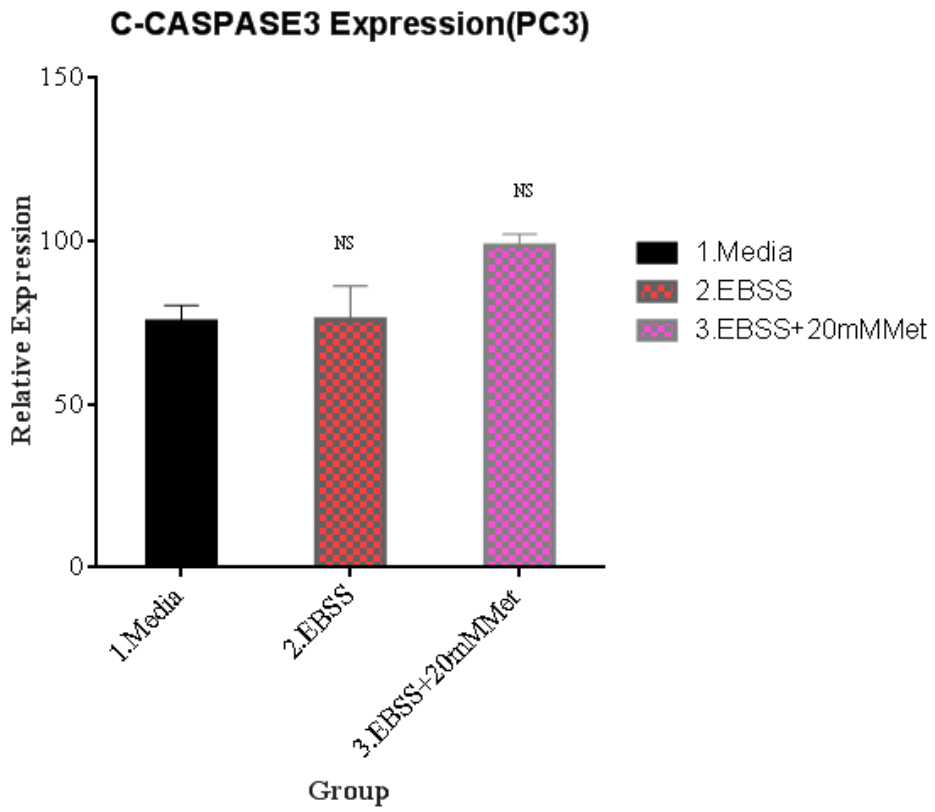
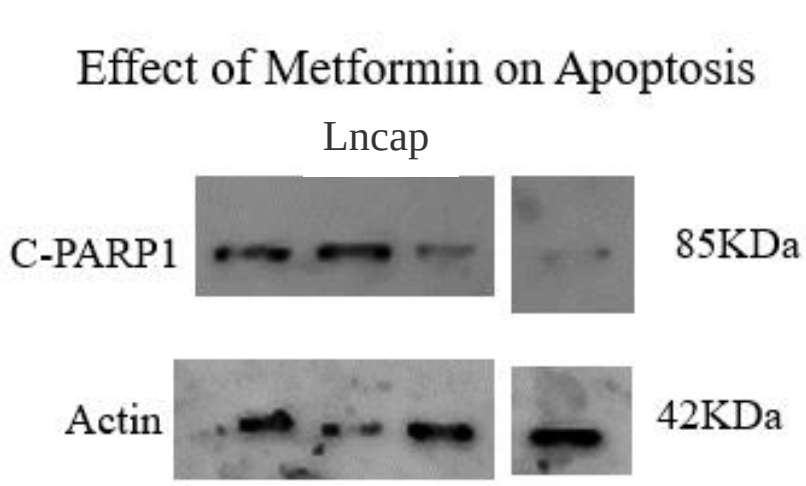
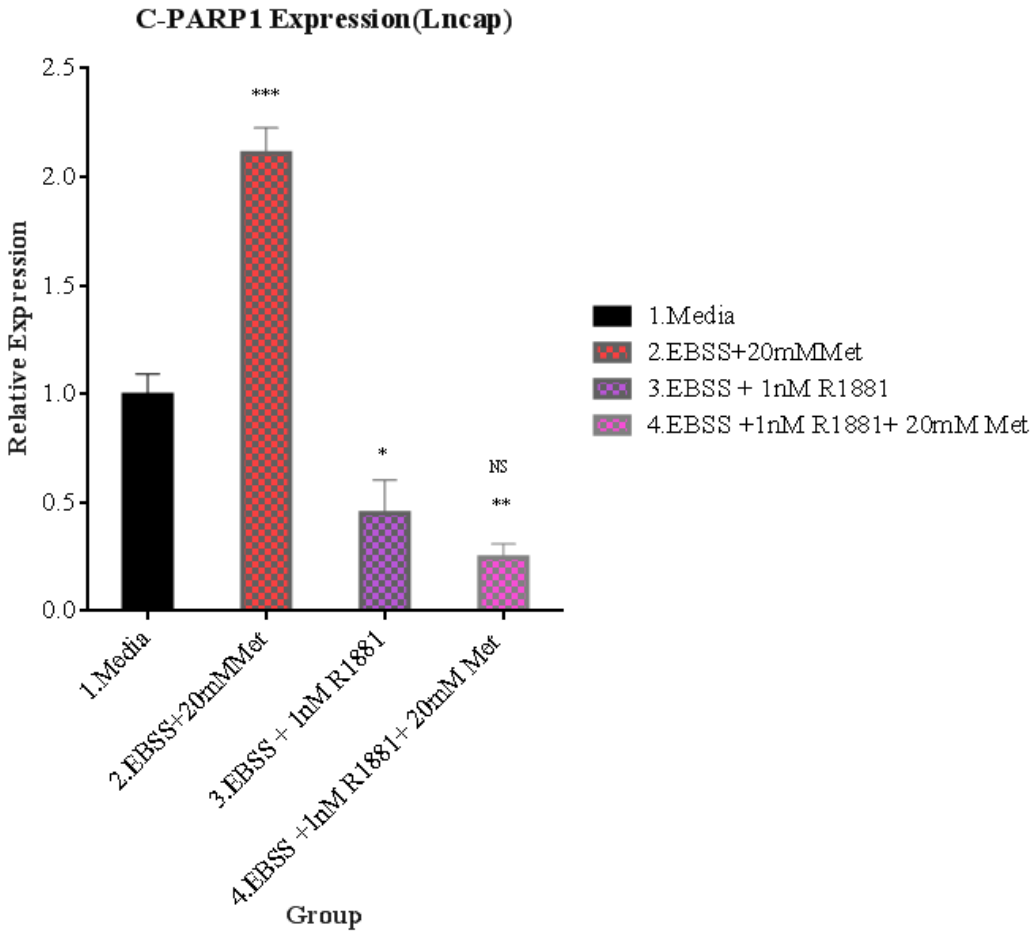


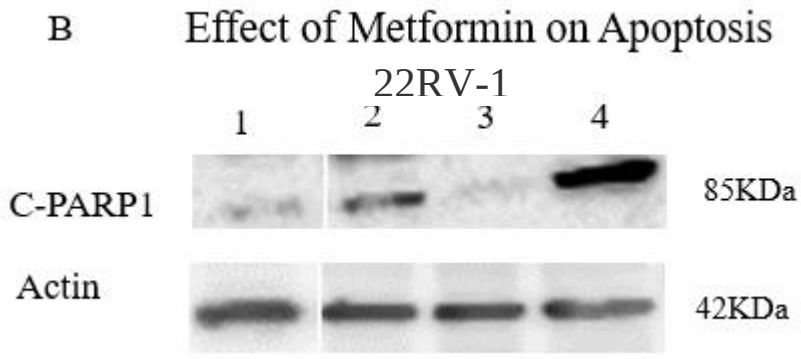
Figure.2 The relative expression of C-Caspase3 was examined for Lncap (A), 22RV-1 (B) and PC3 cells (C) following treatment with Metformin (20mM) with four different conditions (i)Complete Growth Media (ii) EBSS and Metformin (iii) EBSS and R1881(1nM) and (iv) EBSS, R1881(1nM) and Metformin(20mM) treatment for an equivalent duration. Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant.

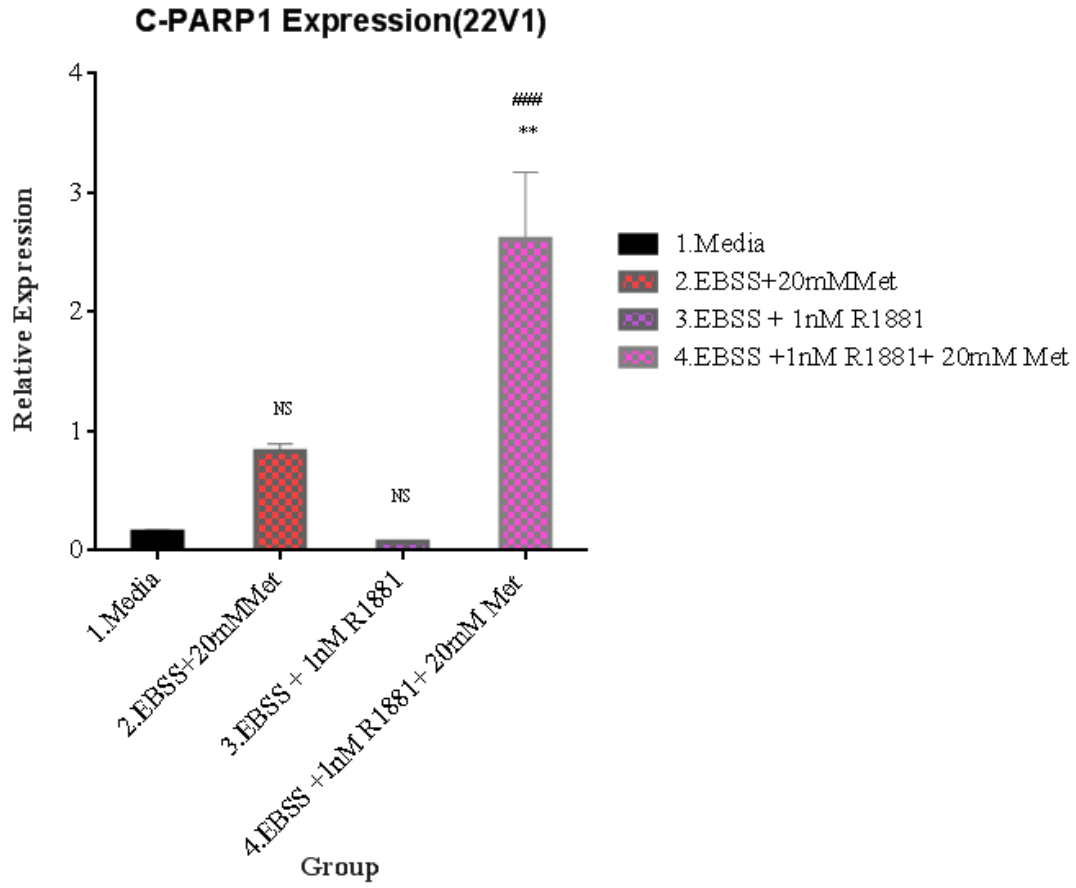
According to Figure 2(A-C) after Metformin treatment, C-Caspase3 (Cleaved caspase 3) was significantly increased in Lncap cells; however, no other cell line showed a significant increase. In 22RV1 C-caspase3 showed a significant decrease after Metformin treatment under solely EBSS condition. Other conditions did not increase significantly. Similarly in PC3 Metformin treatment did not show an increase in C-caspase 3 level.

A


Effect of Metformin on Apoptosis
Lncap
C-PARP1
85KDa
Actin
42KDa


C-PARP1 Expression(Lncap)
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
***
*
NS
**
1.Media
2.EBSS+20mMMet
3.EBSS + 1nM R1881
4.EBSS +1nM R1881+ 20mM Met
Group


B
Effect of Metformin on Apoptosis
22RV-1
1
2
3
4
C-PARP1
85KDa
Actin
42KDa


C-PARP1 Expression(22V1)
Relative Expression
0
1
2
3
4
NS
NS
####
**
1.Media
2.EBSS+20mMMet
3.EBSS + 1nM R1881
4.EBSS +1nM R1881+ 20mM Met
Group

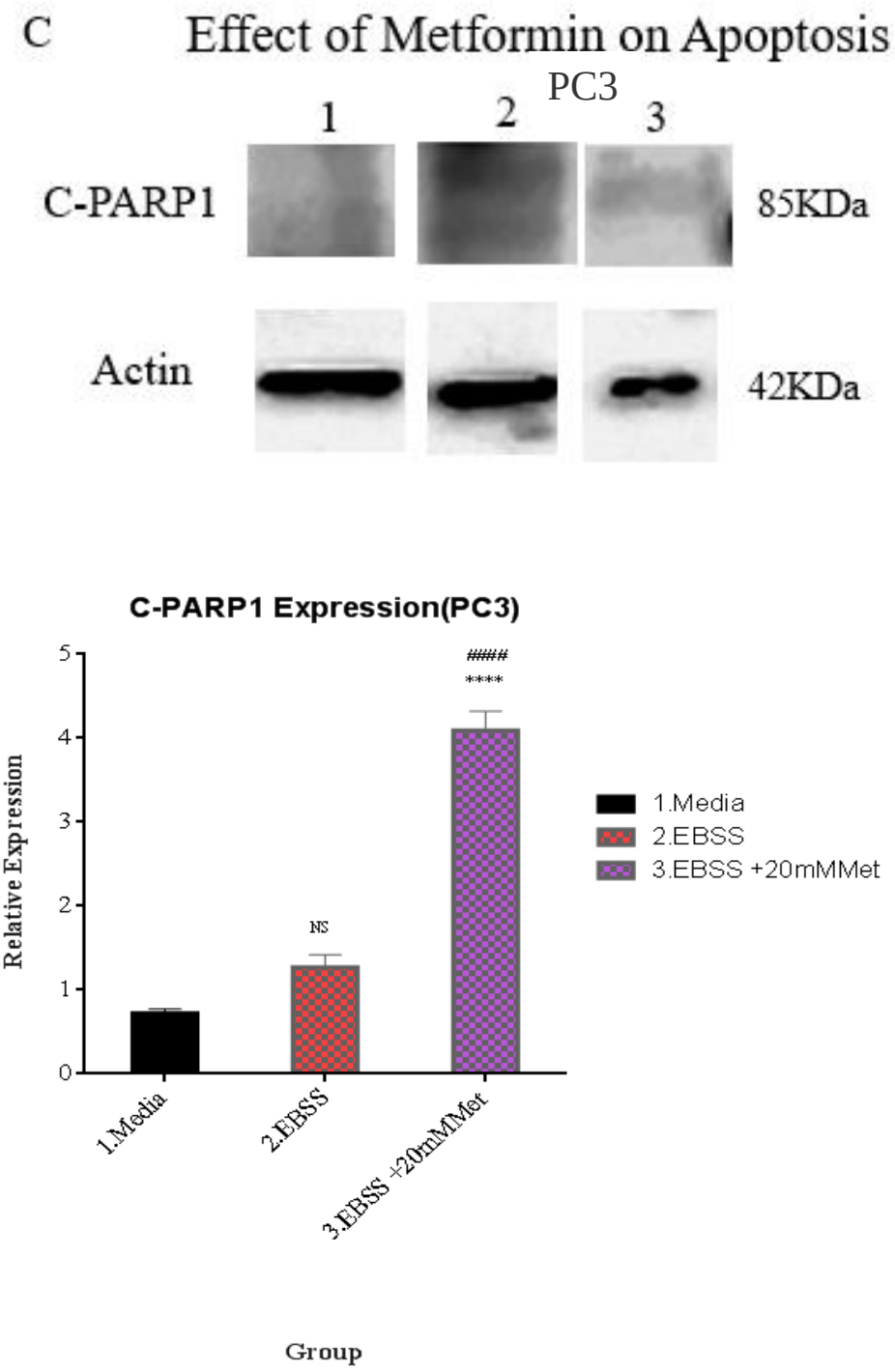
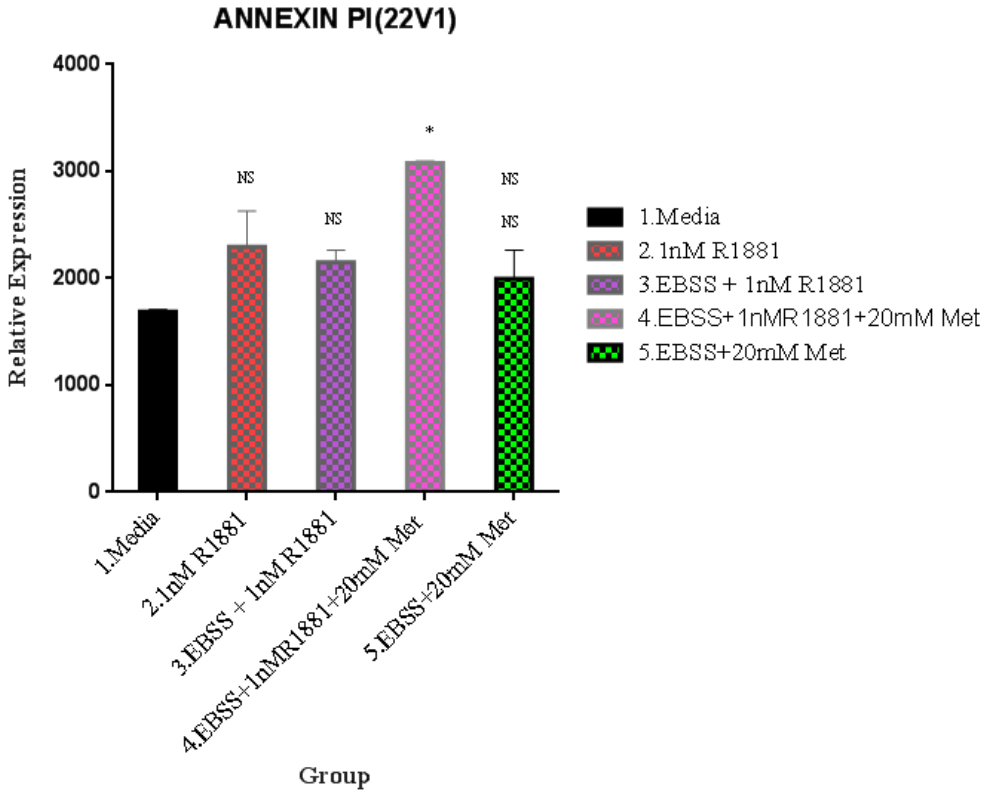


Figure.3 The relative expression of C-PARP1 was examined for Lncap (A), 22RV-1 (B) and PC3 cells (C) following treatment with Metformin (20mM) with four different conditions (i)Complete Growth Media (ii) EBSS and Metformin (iii) EBSS and R1881(1nM) and (iv) EBSS, R1881(1nM) and Metformin(20mM) treatment for an equivalent duration. Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant, Note:P-value determined against media control and EBSS+1nM R1881

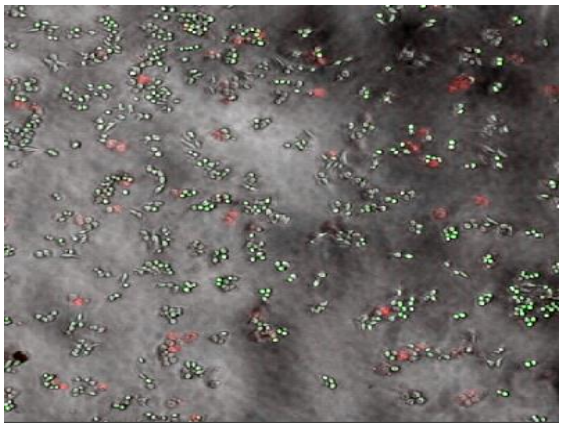
PARP1, another marker of apoptosis, was evaluated for Lncap, PC3, and 22RV-1 (Figure 3A-C), and its change is very unconventional after treatment. For Lncap Metformin reported a highly significant increase in expression when introduced under EBSS, and after R1881 addition, a significant decrease was observed (EBSS+R1881). Under the EBSS condition, Metformin treatment did not show a significant effect on 22RV-1 cells (Figure 3B). C-PARP1 expression significantly increased after metformin treatment in EBSS+1nMR1881 conditions for 22RV1 cells. Similarly, a highly significant trend was observed in PC3 cells, showing significant results compared to the EBSS condition.

Similar results were observed as shown in Figure 4 where Annexin/PI ratio was examined for 22RV1 cells showed a significant increase after Metformin treatment. As autophagy and apoptosis both are interlinked and can be inhibited by amino acid starvations

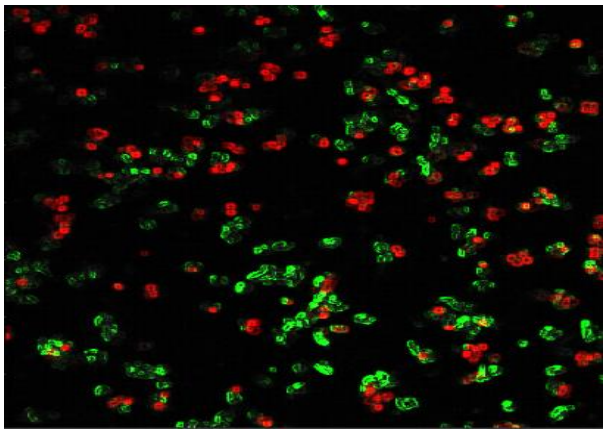
A



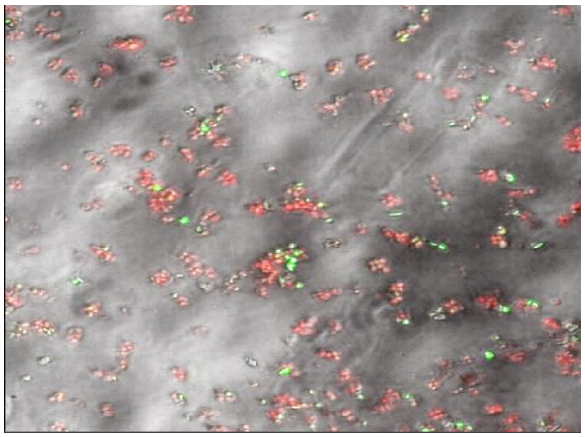
B



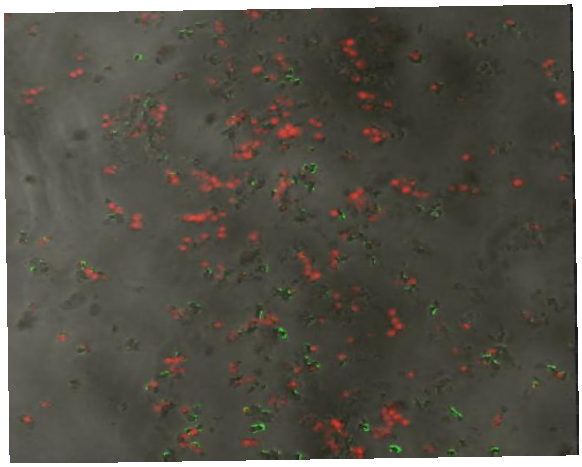
(MEDIA)



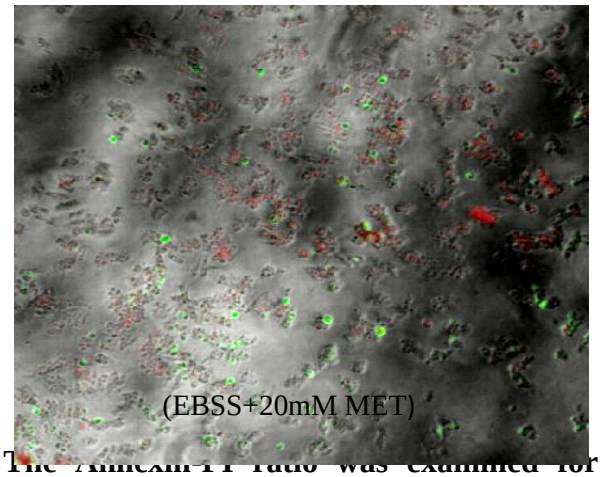
(1nMR1881)



(EBSS+1nMR1881)



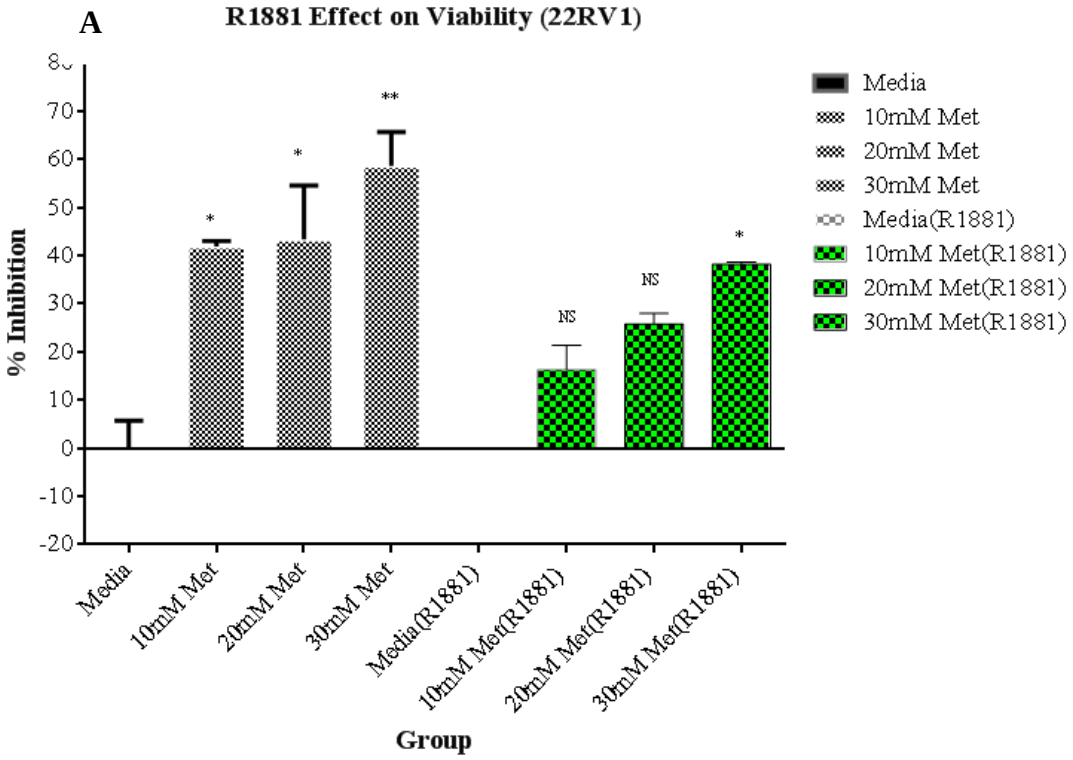
(EBSS+1nM R1881+20mM MET)



(EBSS+20mM MET)

Figure.4 (A) The Annexin-PI ratio was examined for the 22RV1 Cell line following treatment with Metformin (20mM) along with five different conditions (i)Complete Growth Media (ii) 1nM R1881(iii) EBSS and R1881(1nM) (iv) EBSS, R1881(1nM) and Metformin(20mM) and (v) EBSS and Metformin treatment for an equivalent duration.(B) Representative images Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant Note:P-value determined against media control and EBSS+1nM R1881.

Viability was examined under different starvation pressures and different Metformin doses in the absence /presence of R1881.In Figure 5A Significant inhibition in viability was observed with all Metformin concentrations (10-30mM) without R1881 induction. When R1881 was introduced less inhibition was observed. Only at 30mM, Metformin achieve significant observation. Similarly, in PC3 cells only 30mM dose of Metformin achieved significant observation.As per Figure 6A In 22RV1 cells different Starvation can also affect the viability unconventionally.10-90% of EBSS show 40% inhibition to 20% proliferation.where 90% EBSS treatment achieved significant proliferation upto 20%.R1881 introduction to system having different EBSS pressure affect to metfomin's efficacy at 10%. But higher EBSS(40%-90%EBSS) pressure restricted to lower efficacy of metformin instead of converting into the proliferation(As observed in absence of R1881).



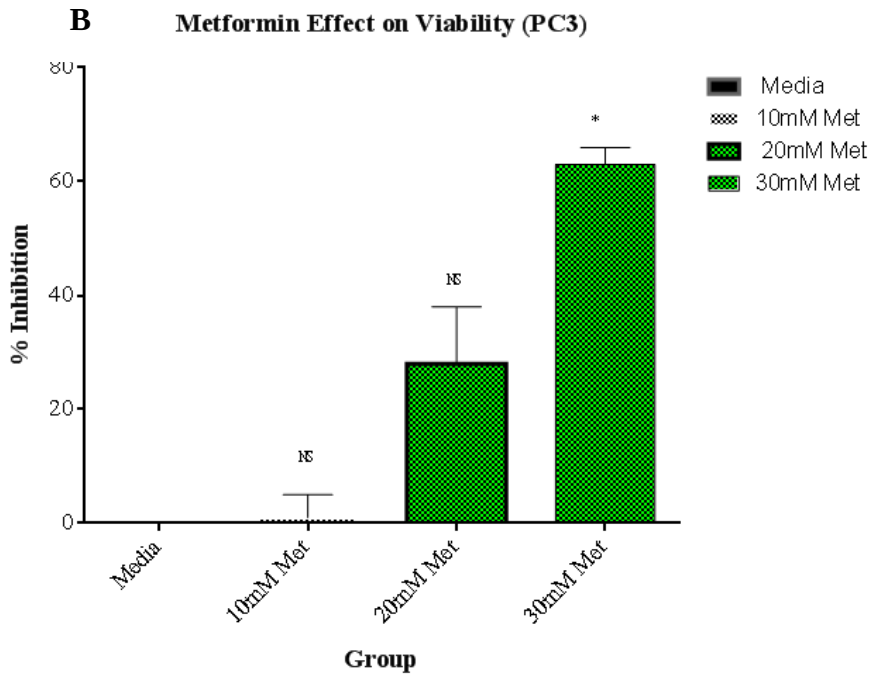
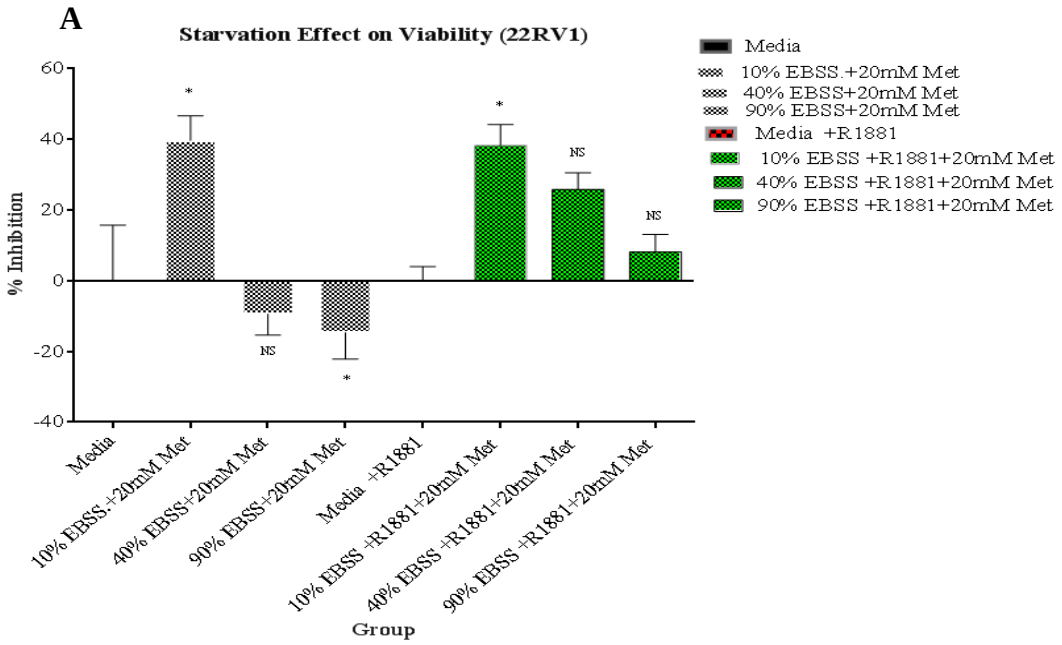


Figure 5 Metformin Inhibition shows (A) 22RV1 cells and (B) PC3 cells were exposed to varying concentrations of metformin (10, 20, 30mM) with or without 1nM R1881, using a 10% CSS media, for a 72-hour incubation period. Data represents were Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant. Note: P-value determined against media control.



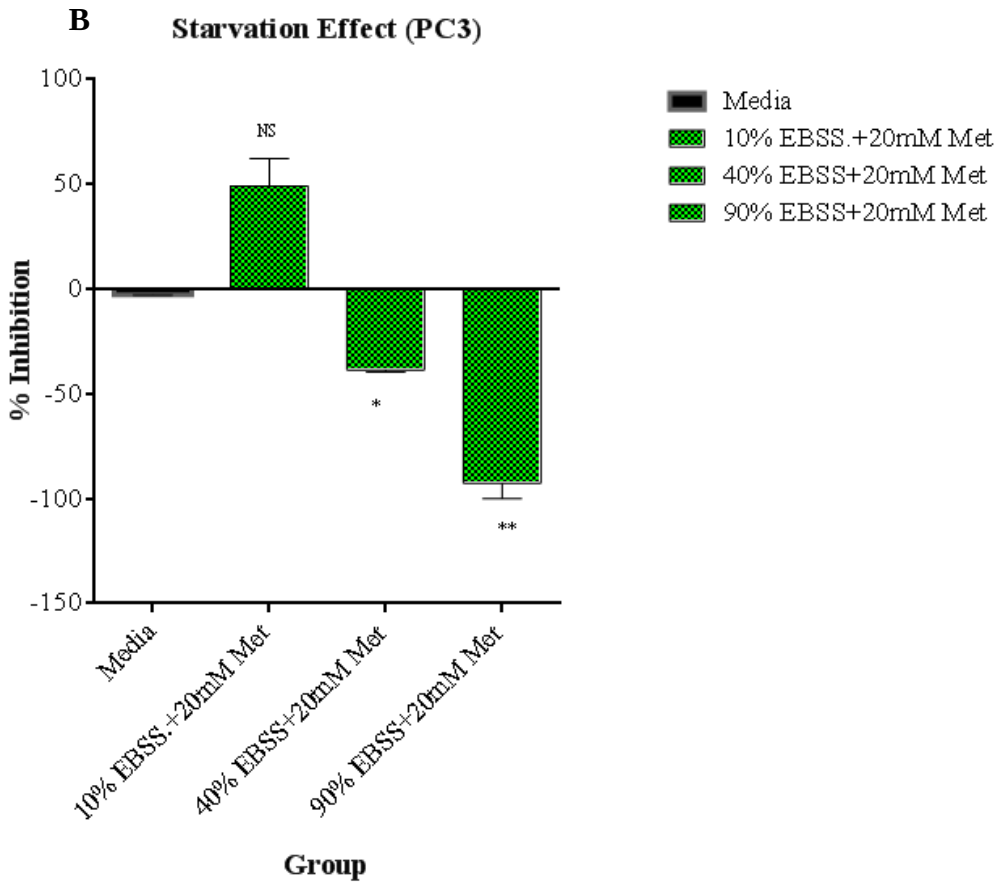


Figure 6 Starvation effect on % Inhibition in viability was evaluated in (A) 22RV-1 and in (B) PC3 cells with varying percentages of EBSS addition in to10% CSS media for 48-hour incubation. Data represented were Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant. Note: P-value determined against media control

As per Figure 7, non-specific ligand estrogen showed significant inhibition after metformin treatment for all concentrations. Maximum inhibition was observed at 86.6mM and 50% inhibition was achieved at 2.6mM. Progesterone also showed significant inhibition up to 26.01mM where maximum inhibition was 50% at 8.67mM. Metformin showed significant inhibition at 26.01mM and an 86.6mM dose.

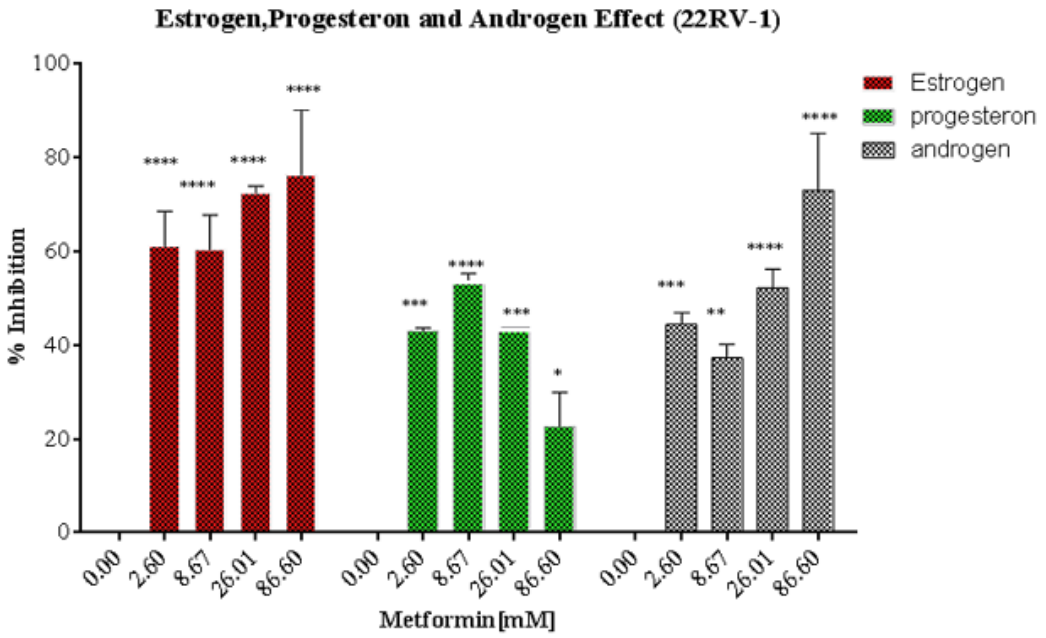


Figure 7 Steroid Hormone effect on %viability of 22RV1 cells was evaluated in presence of (A)1nM R1881, 1nM Estrogen and 1nM progesteron with varying concentration of swertiamarin in to10% CSS media for 72-hour incubation. Data represented were Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant. Note:P-value determined against media control

As ROS is an influential factor in the cellular metabolism of cancer cells, ROS levels were also examined in two cell lines with increasing % of EBSS in 10% charcoal-stripped serum-containing growth media and also with increasing a Metformin dose in 10% charcoal-stripped serum growth media. Lncap cells were excluded for ROS evaluation for two reasons first is its androgen-dependent cell line which was not in our focus and second, its adherent property is poor.

In Figure 8. A ROS level showed a decrease in percentage (Compared to media control) when androgenic induction was not given (absence of R1881) while in the presence of R1881 metformin could not alter the level of ROS. However, ROS produced from media control in the presence of R1881 shows a significant decrease compared to media without R188.1 Similarly, in the case of AR-negative PC3 cells (Figure 8B) under EBSS increment Metformin showed very less effective (non-significant) except at 10%EBSS treatment.

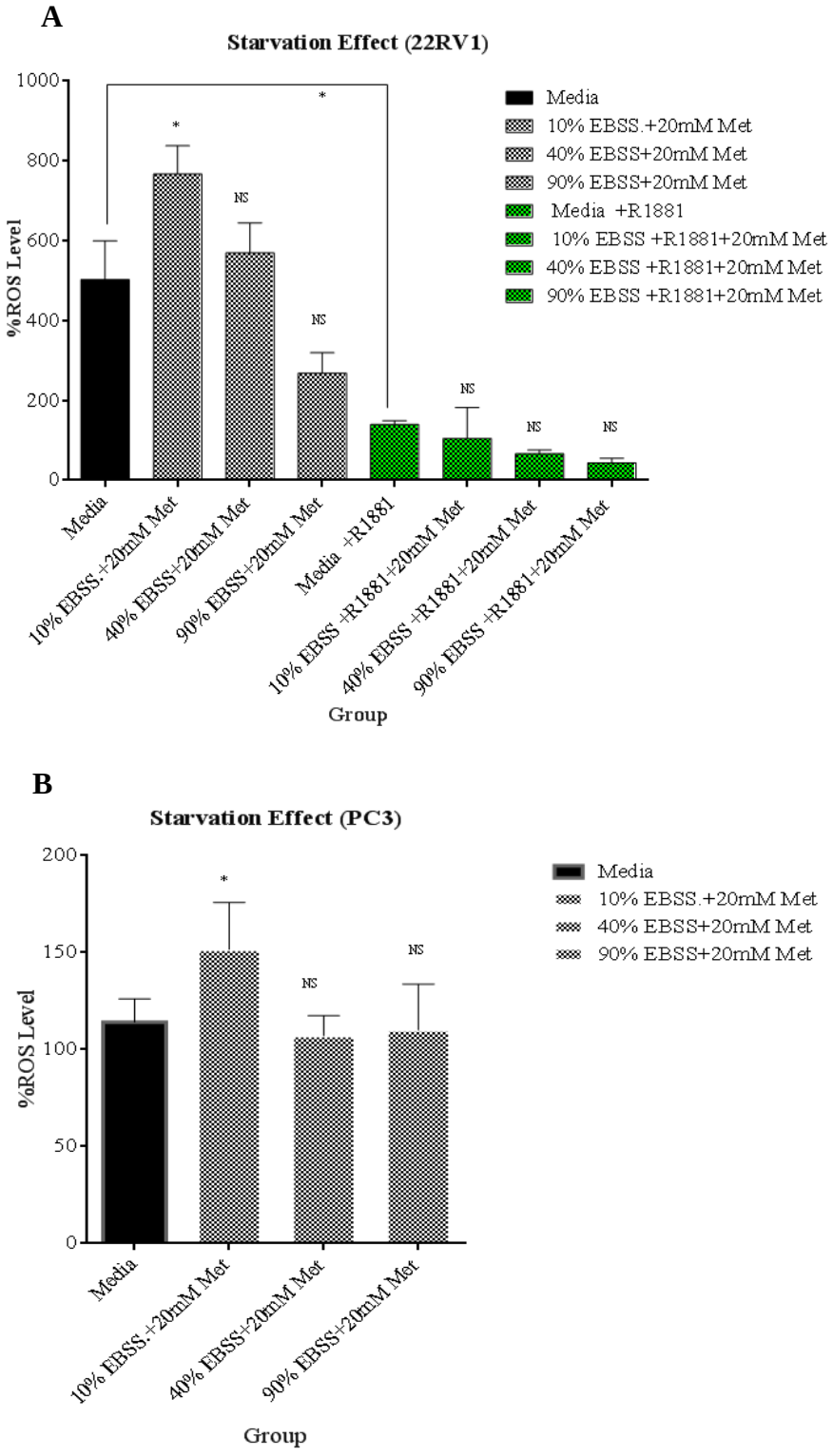


Figure 8 Starvation effect on %ROS level was evaluated in (A) 22RV-1 and in (B) PC3 cells with varying percentages of EBSS addition in to10% CSS media for 48-hour incubation. Data represented were Mean ± SEM (n = 3). ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant. Note: P-value determined against media control

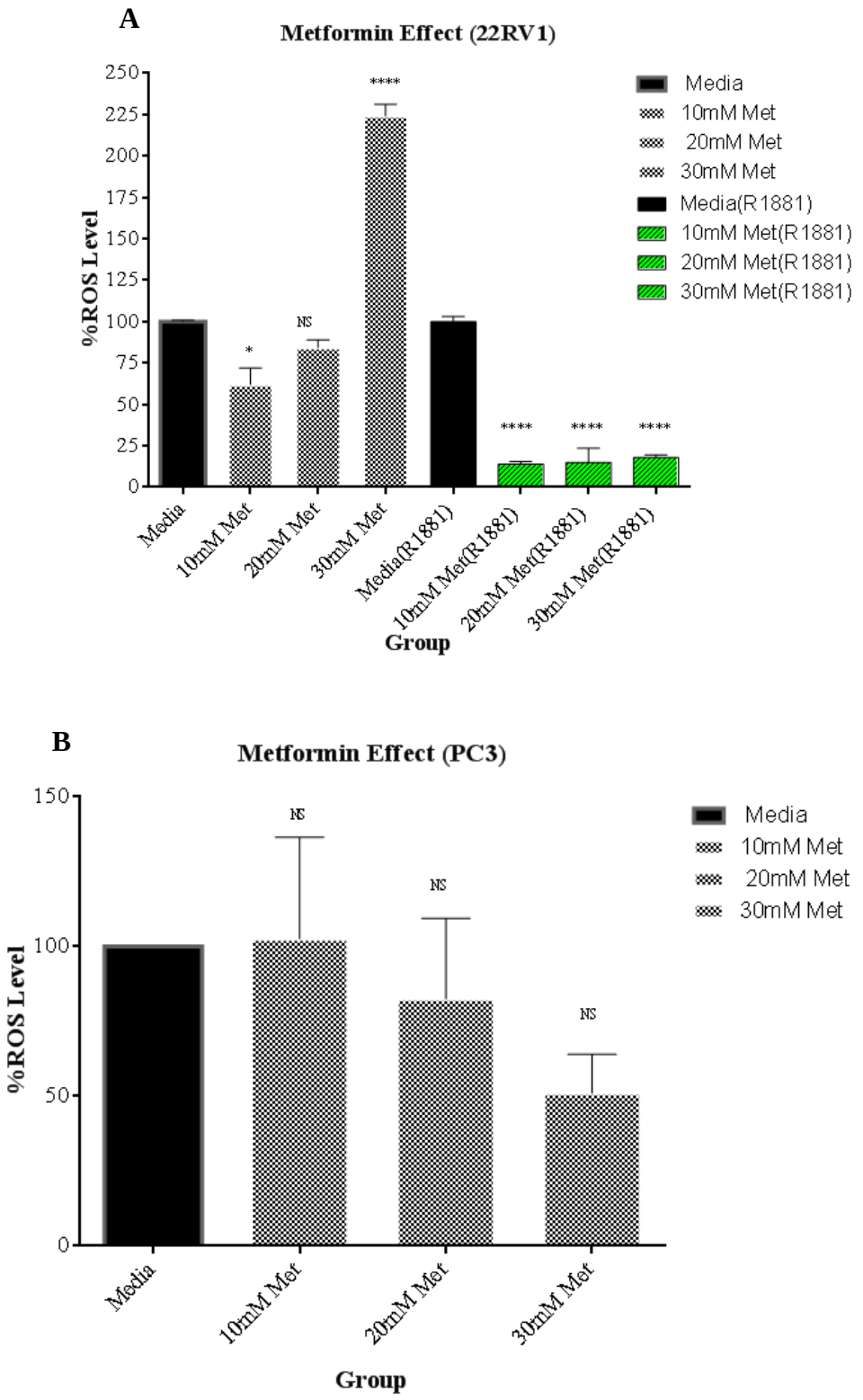


Figure 9 Metformin effect on ROS was evaluated in (A) 22RV-1 and in (B) PC3 cells varying concentrations of metformin(10mM,20mM,30mM) to10% CSS media for 72-hour incubation and viability inhibition was evaluated.Data reported were N:3 ***i.e. < 0.001, ** i.e.<0.01, * i.e <0.05, NS i,e nonsignificant Note:P-value determined against media control

When Metformin concentration increased ROS levels were significantly decreased in 22RV-1 cells in the absence of R1881 (Figure 9. A) and in the presence of R1881 from 10mM to 30mM Metformin showed a significant increase. In PC3 cells ROS levels are not significantly affected (Figure 9B). Here due to AR-negative cells, the metformin ROS effect in the presence of R1881 was not examined in PC3. Figure 8 shows different % in EBSS treatment alters the efficacy of 20mM Metformin. In Figure 9A, R1881 induction significantly inhibits %ROS level while without R1881 from10 to 30mM reverse the trend significantly. while in the absence of androgenic induction around 250% of the significant level was observed at 30mM which gradually decreased as the Metformin dose decreased means Metformin helps to produce ROS in the absence of R1881.In PC3 ROS levels were not affected by Metformin in different doses of Metformin

Discussion:

In the first objective we saw an AR role in altering the metformin anti-cancer activity. Metformin degraded AR and AV7 translationally. Moreover, AR-negative PC3 cells are also inhibited with higher concentrations of metformin. It has been reported that metformin can induce AR degradation by disrupting the MID1 complex suggesting the inhibitory role of metformin in downregulating AR and ARV7 expression in prostate cancer. [(Xie et al., 2021)]

Hence, in this objective, we had introduced AR induction and amino acid starvation condition along with the treatment of metformin on prostate cancer cells causing an alteration in autophagy or apoptotic state in order to evaluate its anti-cancer efficacy. Earlier studies have mentioned metformin's main anti-cancer effect done by two major cell death pathway, autophagy (which activated by AMPK) and apoptosis (by activation of caspase-dependent and caspase-independent Pathway)

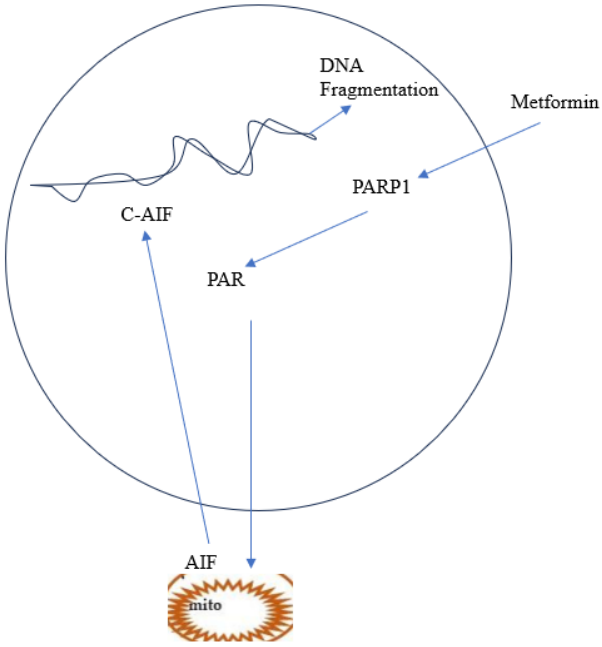
Hence, we assessed the efficacy of Metformin with both the condition simultaneously using 22RVI and PC3 Cell lines which represent CRPC and androgen-independent cells respectively while Lncap is considered as androgen-sensitive cell line. The anti-cancer behavior of metformin under the given stressful condition was evaluated by analyzing the status of autophagy, apoptosis and ROS level as the three most crucial parameters that influence the cell death/inhibition/

proliferation of cancerous cells.

Further, the induction of autophagy was observed by the increase in LC3 II marker in LnCap and PC3 cells post metformin treatment. While in 22RV1 cells, it was found that there were no significant changes in expression of LC3 II after the metformin treatment. . The applied stress conditions also depicted the significant decrease in LC3 II expression in 22RV1, suggesting the role of different cell death pathway in the inhibition of in 22RV1 cells. Moreover, the cleavage of caspase 3 was observed only in LnCap cells after the treatment which signifies that caspase dependent activity is enhanced by metformin but in 22 RV1 EBSS & Metformin treatment demonstrated significant decrease in caspase 3 activity.

Caspase 3 cleavage was observed only in Lncap after treatment means caspase is dependent activity enhanced by metformin but in 22RV-1 EBSS and Metformin treatment show a significant decrease in C-caspase 3 activity similar results observed earlier also where lncap show c-caspase activity but not in V-cap cells line (AR and ARV7) [(Xie et al., 2021)]. This can be validated by the fact that decrease in BCL2 leads to the release of cytochrome c in the cytoplasm. This decrease in BCL2 and increase in BAX followed by cytochrome c triggers the apoptotic process which can be seen by the increase in LC3 II signal post metformin treatment. Hence, in LnCap cells, Metformin led the cell death via autophagy but the same cannot be said for CRPC cell line- 22RV1 because there the LC3II marker did not depict significant increase under the starvation and was rather found to decrease. Also, another reason behind why metformin led AR sensitive LnCap cells and not CRPC-22RV1 cells is the difference in level of C-PARP1. C-PARP1 tends to decrease significantly in LnCap cells while it increased in CRPC-22RV1 cells. Similar results were also observed by Xie. Et al for ARAT. which concluded that PARP1 cleavage usually signals apoptotic programmed cell death. This was proved here as well. In another way, we can say that autophagy diverts the apoptotic cell death pathway by getting activated when apoptosis is suppressed [(Ouyang et al., 2012)]. This occurs due to the disruption of Beclin-1-VPS34 complex due to BCL2 phosphorylation under stress conditions.[(Siddiqui et al., 2015)]. In CRPC cell line(22RV-1), metformin (under starvation + AR induction) might also follow the non-apoptotic PCD where increased PARP-1 made AIF nuclear localization (Via production of PAR) and thus caused

DNA fragmentation which led to cell death [(Xie et al., 2021)]. In case of PC-3 cells, metformin seems to be causing autophagic death as LC3II marker significantly increased while C-Caspase3 did not exhibit significance.



Further, the addition of EBSS in 10% CSS media starvation effect not only causes amino acid starvation but also alters the glucose content in systems hence, creating the energy deprivation state in cells. Under different starvation condition and different concentrations of Metformin, we elucidated that cellular viability and ROS levels tend to be affected by metformin. Moreover ROS status also influences the different cell death pathways. When energy deprivation increases it activates AMPK(AMP/ATP ration) and MAPK(Stress Stimuli) pathways which can control either cellular component degradation or ROS production. Energy deprivation affects the cell viability of CRPC differently in absence and presence of R1881. 10% EBSS without R1881 showed significant inhibition in 22RV1 and similar inhibition observed in the presence of R1881. But as EBSS content increases difference in inhibition changes drastically. At 40% and 90% EBSS, cells tend to proliferate instead of inhibition after treatment of 20mM Metformin but when R1881 is introduced, AR ligand is restricted to the function of cell proliferation. This Means

not only AR /ARV7 presence but also the energy deprivation can cause either cell proliferation or cell inhibition depending on the Autophagic/ energy deprivation magnitude. Aljofan et.al. demonstrated that whether poor outcome of metformin in renal cancer cells may be influenced by the nutrient condition.[(Aljofan & Riethmacher, 2019)]. They observed that treatment of cells with metformin under normal conditions led to a notable inhibition of cell growth. However, altering the cellular environment from normal to glucose-deprived conditions reversed the growth suppression induced by metformin, suggesting that metformin may facilitate cell growth under such circumstances. Whereas ROS production in CRPC cells(22RV1) seemed to be increased under lower energy deprivation after metformin treatment but at 90% EBSS, ROS levels decreased significantly. This depicts that the energy deprivation diminishes the ROS generating capability of metformin which causes DNA fragmentation via AIF production (as mentioned earlier) and starts to help cancer cells in survival [(Aljofan & Riethmacher, 2019), (Xie et al., 2021)]. ROS can also activates ASK1 downstream of the MAPK-JNKC pathway activated by stress and inhibits phosphorylation of BCL2 but in deprivation conditions, metformin treatment inhibits the ROS production and thus inhibit the ASK may lead to the activation of BCL2 and thus helps in proliferation[(Ouyang et al., 2012), (Aljofan & Riethmacher, 2019)]. In PC3 Cells, the starvation effect could not alter the efficiency of metformin mediated ROS production due to AR absence. As reported earlier, AR ablation increases the ROS production in cells, however the exact mechanism is not well explored.PC3 is more resilient to ward metformin effect and not showing inhibition at 40% and 90% EBSS conditions instead significant proliferation observed at 40% EBSS Metformin efficacy decreased when R1881 treatment given to 22RV-1 cells indicates that AR push back the Metformin's ability to inhibit signaling pathway but when EBSS stress introduced its help to metformin for cell proliferation might be due to the activation of AR nongenomic pathway which influences the cell viability either positively. Like IGF-1 enabling AR transactivation even in the absence of androgen. HER2 is mostly found in highly expressed in androgen-independent pathway and causing cell proliferation of cell without AR genomic pathway[(Craft et al., 1999)]

The mtDNA encodes for 12 subunits of the OXPHOS system which is source of intracellular ROS so reduced levels or defective mtDNA can cause imbalances in the

structure of the OXPHOS complexes and result in defective mitochondrial respiration. Preventing apoptosis and triggering PCa progression is a result of mtDNA depletion mainly due to activation of PI3K/Akt pathway. Loredana Moro et.al demonstrate that mtDNA depletion in less invasive and androgen-dependent Lncap cells became aggressive and androgen-independent like PC3 cells that had disturbed membrane potential due to depleted mtDNA. Depleted mtDNA causes less production of PARP-1 which reflected when we introduced metformin to the prostate cancer cell line. Due to these factors, we noticed that PC3 exhibited greater resilience in terms of ROS production (induced by metformin) following starvation(EBSS) treatment but CRPC did not follow the same because AR and ARV7 may attributed to higher mtDNA in 22RV1 cells.

Apart from starvation and AR induction, other steroidal hormone can also affect the metformin anticancer efficacy in case of mutation in AR (specifically in ligand binding sites). Hence, to validate this the effect of metformin was evaluated with estrogen, progesterone and androgen induction for CRPC cells 22RV-1 which bear mutated AR. The introduction of Estrogen and progesterone alters the metformin efficacy positively.. Estrogen introduction demonstrated significant inhibition in dose dependent manner for all metformin concentrations and achieved 80% inhibition which was the highest. In case of progesterone significant inhibition was achieved at lower concentrations (8.6 and 2.6mM) while androgen induction(R1881) showed significant inhibition above 8.6mM concentration. Thus Progesterone treatment with metformin therapy might helpful in case of CRPC. From above results, estrogen treatment seems to be better combination rather than progesterone but it should not be advisable due to it may create an oncogenic effect as it is a ligand of ER-α which plays an oncogenic role[(Furic et al., 2015)]. more over its decrease the T/E ratio which is also oobserved in old age male. ,where testosterone converted to estrogen and low Testosterone and high estrogen level promote cell survival. Progesterone which was found to help metformin in inhibiting 22RV1 cells significantly.and males also normally produce progesterone. Unfortunately, progesterone levels in males drop with aging Hence, Progesteron treatement with metformin therapy might helpful in case of CRPC . This might be due to the fact that progesterone can stimulate the activity of a protective gene called “p53(Petrow et al., 2011) [Lee, J. “prostate disease and hormones.” The John R. Lee, M.D. Medical Letter Feb. 2002]. V. Petrow et al

reported results that tumors can be inhibited by 6-methylene progesterone as it inhibited the •5-alpha-reductase[(Petrow et al., 2011)].

Conclusion: Hence, the above study demonstrated that LC3II expression altered unconventionally by EBSS and R1881 treatment in AR positive cell line. Also, LC3II expression increased after metformin treatment. Moreover, Energy deprivation by EBSS act as dual edge sword and can alter metformin's pro-death effect and pro-survival effect in prostate cancer cells. Caspase 3 cleavage induced after metformin treatment under androgenic induction and energy starvation but only energy deprivation cannot increase C-caspase 3 for 22RV-1 which is also reflected in viability of 22RV-1 cells after different starvation condition. Thus, Progesterone combination therapy can be vital option as progesterone addition gives a boost to cell death of CRPC cells(22RV1) and might be made by inhibition of 5-alpha-reductase.