Chapter 6

Chapter 6

To analyse the effect of active extract/fraction /isolated phytocomponent/s of *Bauhinia variegata* L. on cell death parameters in breast cancer cell lines.

6.1 Introduction:

The aqueous extract of *Bauhinia variegata* leaves inhibits cell proliferation in both hormone responsive and triple negative breast cancer cell lines. Alkaloids and flavonoids were detected by qualitative tests of aqueous extract. HRLC-MS of aqueous extract of *Bauhinia variegata* leaves showed presence of three anti-cancer phytocomponents, two alkaloids: Berbamine and Papaverine; and a stilbene glycoside, Rhapontin. These compounds were present in the fractions of aqueous extract of *Bauhinia variegata* leaves when run along with the standards on the TLC plates. The aqueous extract of *Bauhinia variegata* leaves also exhibited TNF-α modulating property as it inhibits cell proliferation and migration in presence of TNF-α in breast cancer cell lines. The aqueous extract of *Bauhinia variegata* leaves restricts cell invasion in MCF-7 spheroids when exposed to TNF-α and estradiol both individually or in combination. The phytocomponents in the aqueous extract of *Bauhinia variegata* leaves are anti-proliferative, anti-migratory and anti-invasive in nature but whether the extract as a whole is effective, or individual compounds (Berbamine, Papaverine and Rhapontin) or combination of these compounds are more effective needs to be elucidated. The molecular mechanisms by which the aqueous extract of *Bauhinia variegata* leaves induces cell death in the breast cancer cell lines also needs to be explored. Previous reports suggest the role of TNF-α as a mediator of cell death, apart from enhancing the cell proliferation ability in breast carcinoma (Burow et al., 1998).

6.2 TNF-α-regulates mitochondria and apoptosis in cancer.

TNF- α has a dual role in regulating the innate immune system and sustaining cellular homeostasis. It regulates a complex signalling network in a way that is essential for a maintaining a balance between cell death and cell survival and hence is the one of the most investigated cytokines (L. Wang et al., 2008);(Balkwill, 2009). In the tumor microenvironment, TNF-α, as a major proinflammatory cytokine promotes the neutrophils and monocytes activation and recruitment while impeding T cells antitumor effector functions and cytotoxic activity of macrophages (Alberto Mantovani & Dejana, 1989). A continuous production of TNF- α by host and tumor cells at low levels is an important factor involved in tumor progression starting from initiation, to the metastasis of primary breast tumors. TNF-α has also been strongly linked to cancer cachexia, a symptom associated with an advanced-stage cancer with poor survival outcome (Han et al., 2012). An increased level of TNF-α has been consistently observed in different tumor types (Bodo E. Lippitz, 2013). In a murine cancer model, localised high concentrations of TNF-α demonstrated an antitumoral response, whereas systemically injected TNF-α was linked to catastrophic organ failure. Similar to this, a randomised trial for advanced pancreatic and oesophageal cancer demonstrated that local delivery of a TNF-α expressing vehicle coupled with chemotherapy was more beneficial and safer (Glauben Landskron, Marjorie De la Fuente, Peti Thuwajit, Chanitra Thuwajit, & Marcela A. Hermoso, 2014). On the other hand, persistent TNF-α production paradoxically inhibits the growth of tumours.

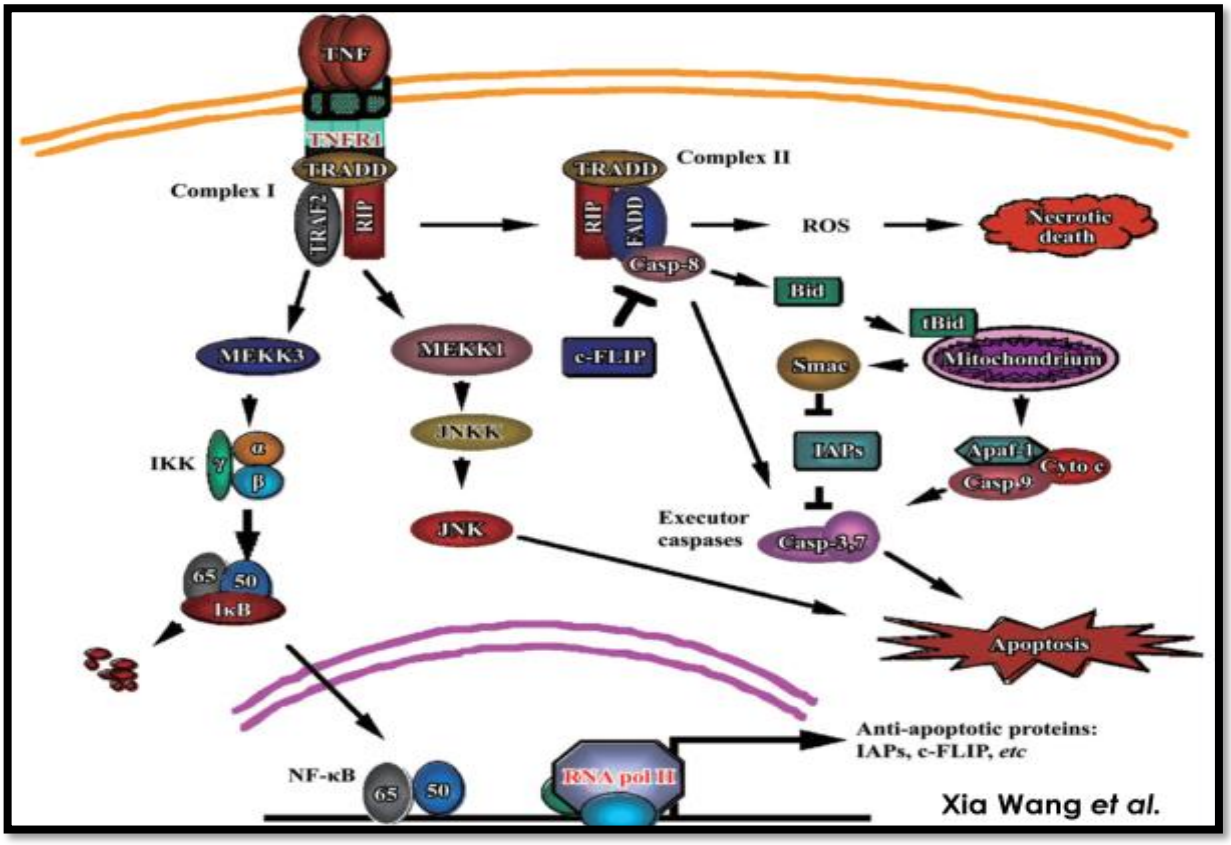


Figure 6.1: TNF- α regulates a complex signalling network for a maintaining a balance between cell death and cell survival. (Adapted from Wang et al., 2008)

TNF-α, in either form, soluble (sTNF-α) or membrane bound (mTNF-α), is recognised by two receptors: TNFR1 and TNFR2 (H. Blaser et al., 2016). TNF-binding to TNFR1 activates the prosurvival pathway by forming proximal plasma membrane bound complex I, which includes TNF receptor-associated protein with death domain (TRADD), TNF receptor associated factor 2 (TRAF2), and receptor-interacting protein kinase 1 (RIP1) (Figure 6.1) (L. Wang et al., 2008). Complex I formation causes rapid transcriptional activation of RIP1-mediated NF-κB responsive genes as well as an antiapoptotic response by cIAP-1 and TRAF2 via an intermediate kinase cascade. Alternatively, in an unknown process, complex I undergoes ubiquitin-dependent modifications, proteolysis, and internalisation to form complex II, a sub-cytosolic pro-death multiprotein complex. The association of complex II with Fas-associated death domain (FADD) and procaspase-8 initiates a non-reversible proteolytic cascade by activating caspase-8 which leads to apoptosis (Micheau & Tschopp, 2003).

Interestingly, TNFα is also known to play important role in regulation of mitochondrial bioenergetics and function during tumorigenesis. On the other hand, evidences show the emerging role of mitochondria in regulation of inflammatory pathways (Missiroli et al., 2020). In the presence of TNF-α, NLRX1 (a mitochondrial NOD-like receptor protein) and Caspase-8 subunits are localized to mitochondria regulating the mitochondrial ROS generation and maintaining ATP levels in the presence of TNF-α, sensitizing breast cancer cells to TNF-α induced cell death (K. Singh et al., 2015). A recent study has shown that TNF-α was able to differentially modulate mitochondrial proteome, mitochondrial activity and mitochondrial super complex assembly, ATP and ROS levels in two different breast cancer cell lines MCF-7 and MDA-MB-231 (Shinde et al., 2021).

6.3 Mitochondria and ROS: Important regulators of tumorigenesis

Mitochondria is one of the major sources of ROS release during TNF-α induced cell death. The active pool of caspase-8 has been reported to localize to mitochondria (Navarro-González & Mora-Fernández, 2008). Emerging evidences suggest an important role of caspases in mediating the proteolytic degradation of key subunits of OxPhos complex to regulate ROS generation during different stress conditions (Ricci, Gottlieb, & Green, 2003). Under malignant condition, there is cell reprogramming that perturbs mitochondrial functions resulting in stress. The mitochondrial stress response triggers the acquisition of malignant traits, thereby promoting cancer progression and dissemination (Porporato, Filigheddu, Pedro, Kroemer, & Galluzzi, 2018). The cellular stress causes abrupt increase in the opening of permeability transition pores (PTP) enhancing the permeability of the inner mitochondrial membrane followed by matrix swelling and rupturing of outer membrane (Javadov & Karmazyn, 2007). The opening of the pore may be an essential factor for provoking the cell death either by necrosis or apoptosis (Crompton, 1999).

In normal conditions, the mitochondrial metabolism and homeostasis, ROS is accumulated as one of the by-products which needs to be neutralized. This is maintained by regulation of the mitochondrial permeability transition pores (mPTP). In response to the redox stress, their activation with subsequent redox changes in intra-mitochondrial and inter-mitochondrial environment leads to the release of potentially

detrimental levels of ROS from mitochondria. This sequence of mitochondrial ROS generation and release is termed as ROS-induced ROS release (RIRR). The opening of the mitochondrial permeability transition pores (mPTP) is a reversible process but increased ROS levels induces longer mPTP openings and release a ROS burst. As a consequence, there is destruction of mitochondria, and if sustained for a longer period of time, causes destruction of the cell itself (Zorov, Juhaszova, & Sollott, 2014). Hence targeting the mitochondrial stress response shall prove as an effective anti-cancer therapeutic strategy (Ghosh, Vidal, Dey, & Zhang, 2020); (Y. G. Lee, Park, & Chae, 2022).

6.4 TNF-α-induced ROS generation in cancer

TNF-α enhances the process of tumorigenesis by regulating the generation of genotoxic molecules such as nitric oxide (NO•) and superoxide ($O2^{-}$) radicals with subsequent loss of genomic stability and DNA integrity (Heiko Blaser, Catherine Dostert, Tak W. Mak, & Dirk Brenner, 2016). During TNFR1 signaling, the high levels of ROS have a crucial role in regulating NF-κB responsive genes such as enhanced transcription of cFLIP and Bcl-XL antiapoptotic genes (Brenner, Blaser, & Mak, 2015). TNF-α is known as an important regulator for the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). TNF-α dependent cell death pathway include ROS derived from mitochondrial sources or non-mitochondrial sources. (Heiko Blaser et al., 2016) has demonstrated that both TNF-α signaling and ROS generation influence each other in a positive feedback loop. The high levels of TNF-α in TME along with chemotherapeutic agents induces extensive metabolic changes in tumor and increases cellular ROS levels resulting into cell death and tumor regression (Habtetsion et al., 2018). Dysregulated ROS intensifies tumor formation process and its progression via DNA mutations, immune escape, metastasis, angiogenesis and telomere extension through the activation of various oncogenic signaling pathways (Kirtonia, Sethi, & Garg, 2020). On the other hand, the therapeutic effect gets antagonized by the neutralization of oxidative stress via preventing TNF-α signaling in tumor cells.

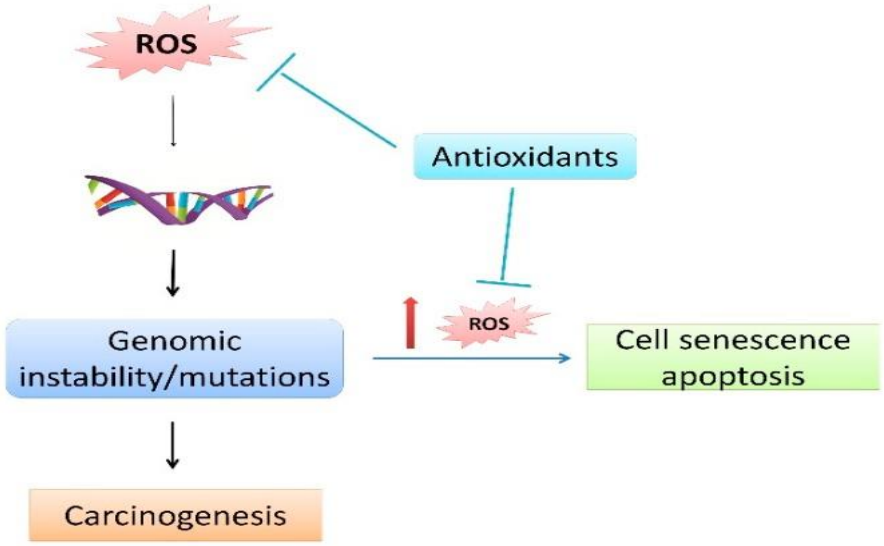


Figure 6.2: Dual role of ROS in tumorigenesis. (Adapted from (Milkovic, Cipak Gasparovic, Cindric, Mouthuy, & Zarkovic, 2019).

At early neoplastic stage, anti-oxidant activity is on a decline with the rise in tumor initiation process promoted by ROS. Contradictorily, in the advanced stages of cancer, the tumor cells escape apoptosis via production of high levels of intracellular antioxidants like NADPH and GSH, to curb extensive production of ROS. Interestingly, massive accumulation of ROS inhibits tumor growth in two ways: (1) by blocking cancer cell proliferation by suppressing the proliferation signaling pathway, cell cycle, and the biosynthesis of nucleotides and ATP and (2) by inducing cancer cell death via activating endoplasmic reticulum stress pathway, mitochondrial pathway, and p53 apoptotic pathways and the ferroptosis pathway. Unfortunately, cancer cells can adapt to ROS via a self-adaption system (R. Huang et al., 2021). Hence, ROS is a double-edged sword, at low levels they facilitate cancer cell survival while high levels of ROS can supress tumor growth (R. Huang et al., 2021) and can cause cancer cell apoptosis (Aggarwal et al., 2019). Therefore, it is important to understand the modulation of TNF-α signaling along with ROS signaling for any new anti-cancer therapy proposed against breast cancer.

For the regulation of the tumor cell survival, progression and advancement, understanding the interplay between TNF-α signalling and mitochondrial ROS generation in the tumor microenvironment is crucial. A thorough understanding of

communication between TNF-α signalling, ROS-redox changes and mitochondrial metabolism will help in the development of better therapeutic targets for solid tumors.

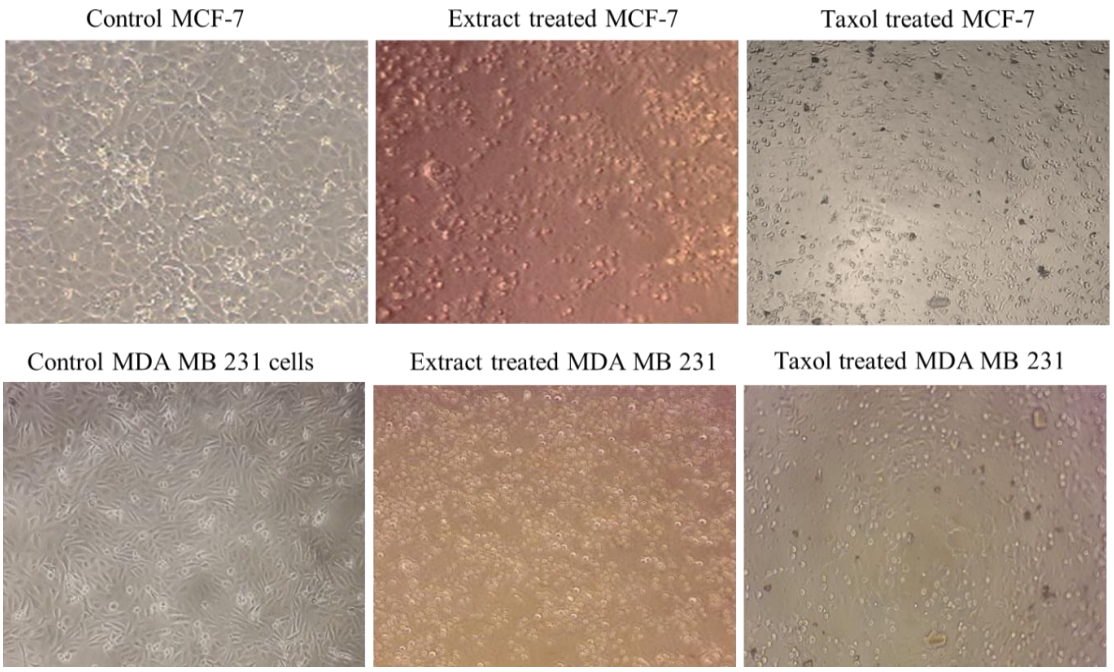
In view of the above discussion, the ability of the aqueous extract of *Bauhinia variegata* L. to modulate ROS leading to cell death of the breast cancer cells was studied.

Results:

6.5 Morphological changes in the MCF-7 and MDA-MB-231 cells when exposed to different compounds present in the aqueous extract of *Bauhinia variegata* L.

The experimental protocol followed is as given in materials and methods. The breast cancer cell lines were observed for any morphological changes compared to the untreated group in presence of Taxol, aqueous extract, and the different standards (Berbamine, Rhapontin and Papaverine) which are shown to be present in the aqueous extract of *Bauhinia variegata* L. by HRLC-MS analysis in the present study.

a)



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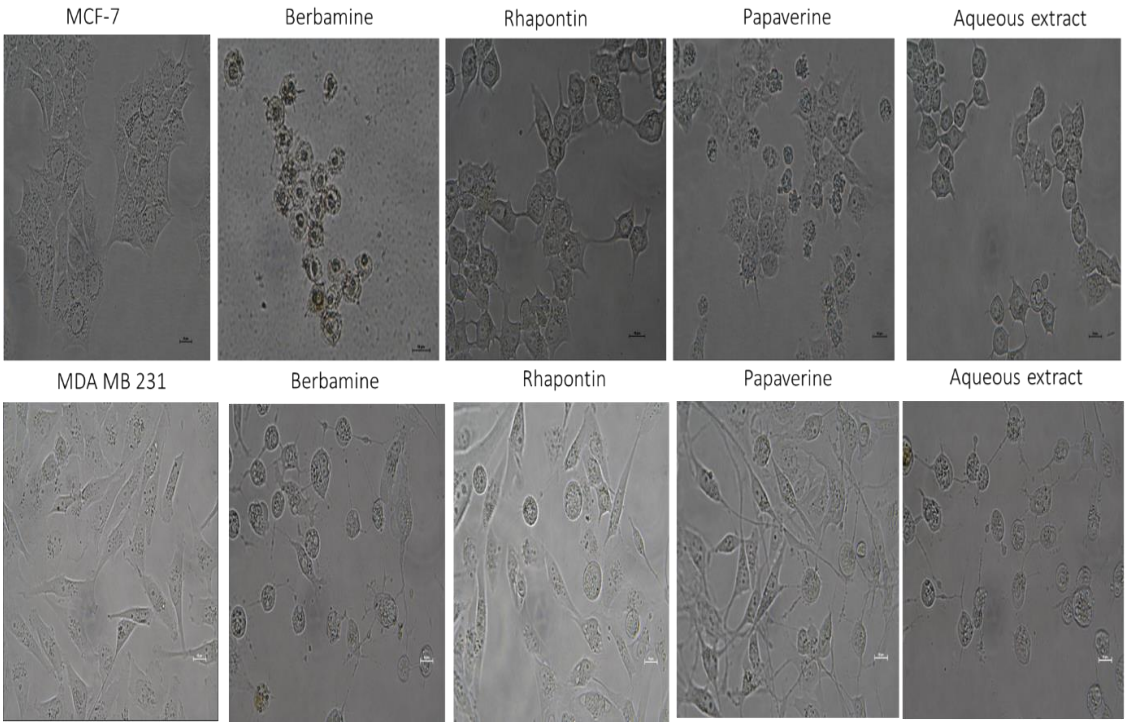


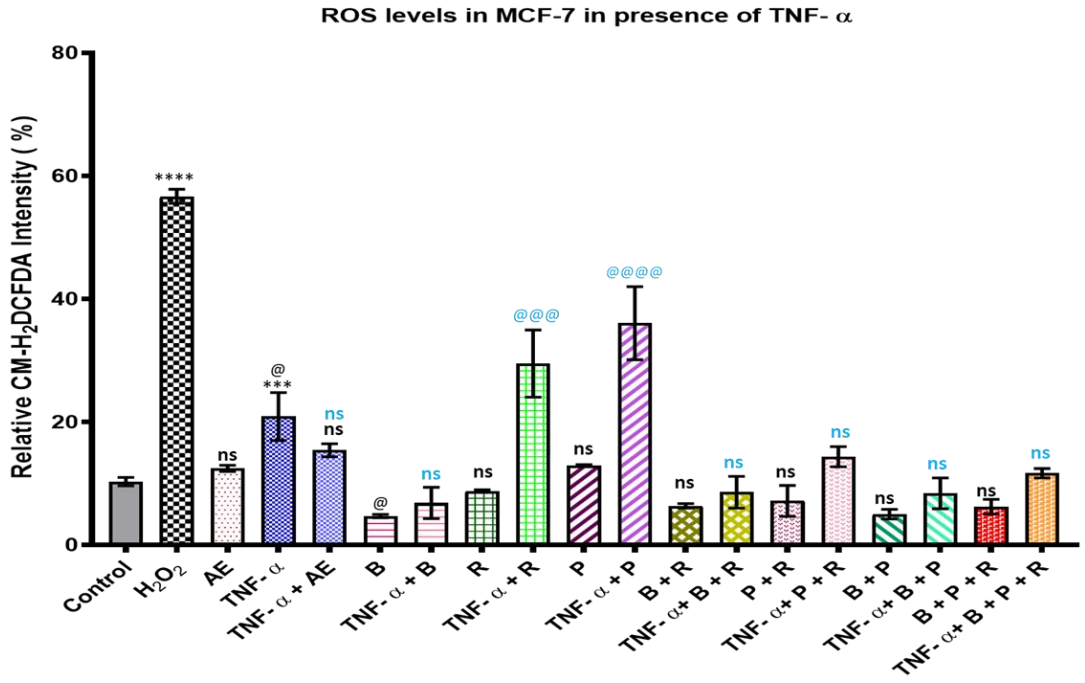
Figure 6.3: Morphological changes in breast cancer cell lines (MCF-7 and MDA-MB-231). a) after treatment with extract b) After treatment with different compounds. Images were taken at 20x.

Figure 6.3 a) shows that the untreated cancer cells (MCF-7 and MDA-MB-231) have intact cell structure. While the morphological examination of the cells after aqueous extract and Taxol treatment showed cell shrinkage and rounding of the cells which are typical features of cell death. Breast cancer cells MCF-7 were compared in presence of the phytocomponent standards, it was found that the untreated cancer cells (MCF-7 and MDA-MB-231) have intact cell structure in the control group (figure 6.3). The prominent morphological changes like rounding of the cells, shrunken cells with rounded morphology, enlarged rounded morphology, cell membrane blebbing and lamellipodia like protrusions were observed in the cells treated with different standards Berbamine and Papaverine whereas minute changes were observed in presence of Rhapontin. The changes observed in the aqueous extract seems to be similar to that observed in Berbamine and papaverine though the combinatorial effect of all the three compounds together or with presence of some other phytocomponent cannot be neglected. When the breast cancer cells MDA-MB-231 were compared in presence of the phytocomponent standards, it was found that the control MDA-MB-231 cells showed the normal feature of a healthy cell line whereas prominent changes were observed in the cells treated with Berbamine. Papaverine and Rhapontin treated MDA-MB-231 showed no significant morphological changes in their presence. MDA-MB-231 cells also showed similar morphological changes to Berbamine when treated with aqueous extract. Thus, Berbamine seems to be prominent phytocomponent affecting both the cell lines that is notable via morphological changes. Nevertheless, the additive effects of papaverine and Rhapontin, along with presence of some more bioactives cannot be ruled out. To find out the involvement of these phytocomponents, analysis of various cell death parameters was done which have been described further in this chapter.

6.6 To study the effect of aqueous extract on ROS levels in breast cancer cell lines

The effect of the aqueous extract was checked on the ER/PR positive cell line; MCF-7 and triple negative cell line MDA-MB-231 in presence and absence of inflammatory cytokine, TNF-α to check any effect on ROS levels. Further along with the aqueous extract, the standards Berbamine, Rhapontin and Papaverine were also analysed to check individual or synergistic effect of these phytocomponent, compared to the aqueous extract (Figure 6.4).

a)



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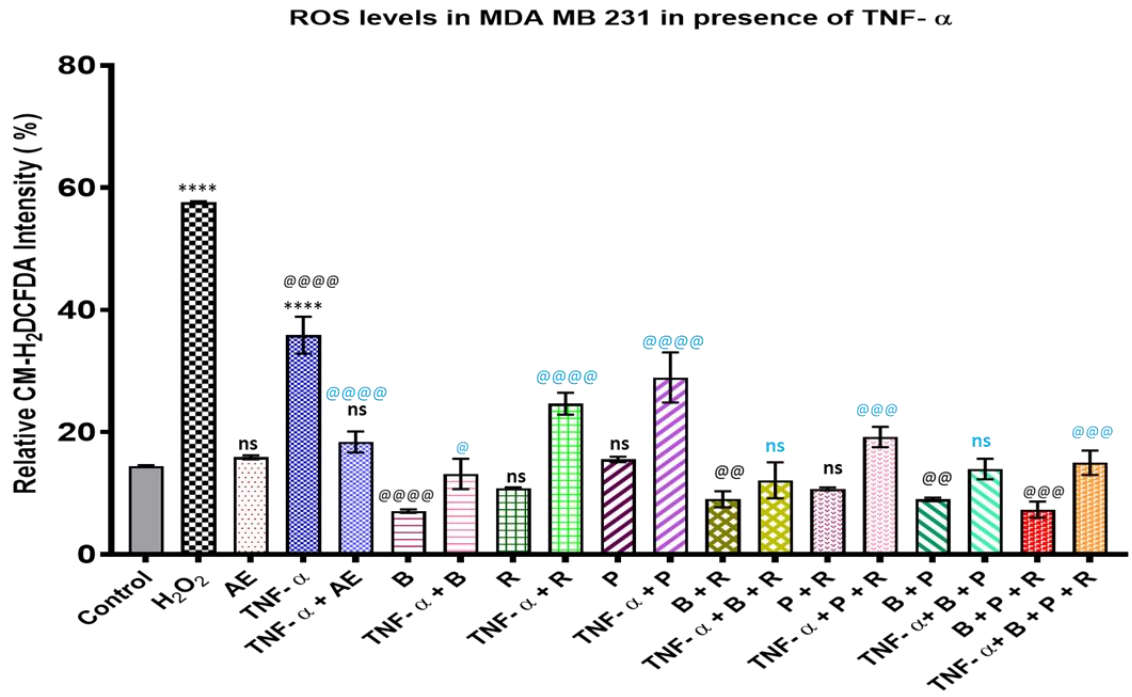


Figure 6.4: Effect of aqueous extract and compounds on ROS level in MFC-7 and MDA-MB-231; Alterations in ROS levels at 24 hours was measured in MCF-7 and MDA-MB-231 cell lines stained with CMH2DCFDA, (a) MCF-7 cells exposed to TNF-α. (b) MDA-MB-231 cells exposed to TNF-α. H_2O_2 was used as positive control. The experiment was repeated thrice in triplicates. B indicates Berbamine,

P indicates Papaverine and R indicates Rhapontin. p value indicates p > 0.05 (ns), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.005 (***), p ≤ 0.001 (****)

Comparison was made with respect to control indicated by * and ns (black color), with respect to aqueous extract indicated by @ and ns (black color) and between TNF-α treated and TNF-α untreated groups indicated by @ & ns (blue color). combination group of standard refers to B+R, P+R, B+P, B+P+R

The breast cancer cells (MCF-7 and MDA MB231) were treated with aqueous extract (AE) and with different standards individually or in combination (B+R, P+R, B+P, B+P+R) to assess alterations in ROS production.

In MCF-7 cell lines, cells exposed to H_2O_2, showed significant increase in ROS levels compared to the control cells. Also, significant increase was also observed in the cells exposed to TNF-α compared to the control and aqueous extract (AE) group. There was no significant difference between the AE group and TNF-α + AE group in MCF-7. In MCF-7 cells, treated with individual or combination groups of standards showed no significant difference with respect to AE, except with Berbamine where significant decrease was seen. In MCF-7 cells no significant difference was observed in all the groups (TNF -α absent) when compared with the presence of TNF-α, with exception of R and P group, where significant increase was seen compared to R + TNF-α and P + TNF-α, respectively. There was no significant difference between AE and P+B+R groups in MCF-7.

In MDA MB231 cell lines, cells exposed to H_2O_2, showed significant increase in ROS levels compared to the control cells. There was significant increase in the cells exposed to TNF-α compared to the control and aqueous extract (AE) group. In MDA-MB-231 cells, significant increase was observed between the AE group and TNF-α + AE group. In MDA-MB-231 cells, between individual and combination groups there was significant decrease with respect to AE, except Rhapontin, Papaverine, and P+R group. In contrast to MCF-7, significant increase was observed in all the groups when compared to the respective groups with TNF-α in MDA-MB-231. In B + R and B + P group, where there was no significant difference with respect to TNF-α + B + R and TNF-α + B + P whereas with AE group, significant decrease was seen compared to

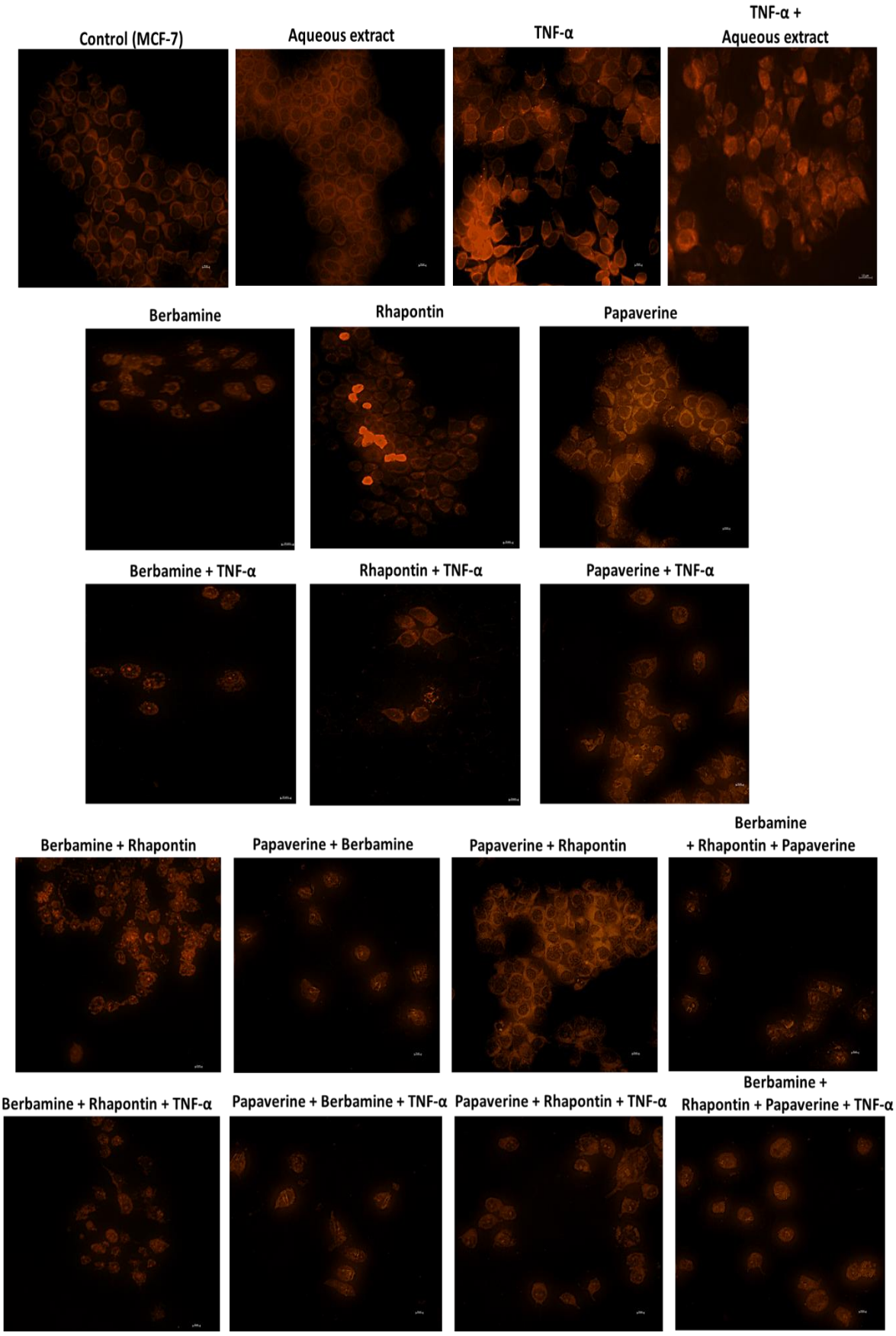
TNF-α + AE group in MDA-MB-231 cells. A significant difference was observed between the AE and P+B+R in MDA-MB-231.

These results suggest enhanced ROS levels in MDA-MB-231 cells as compared to MCF-7 cells. This results also points out that the aqueous extract has the ability to alter the ROS levels.

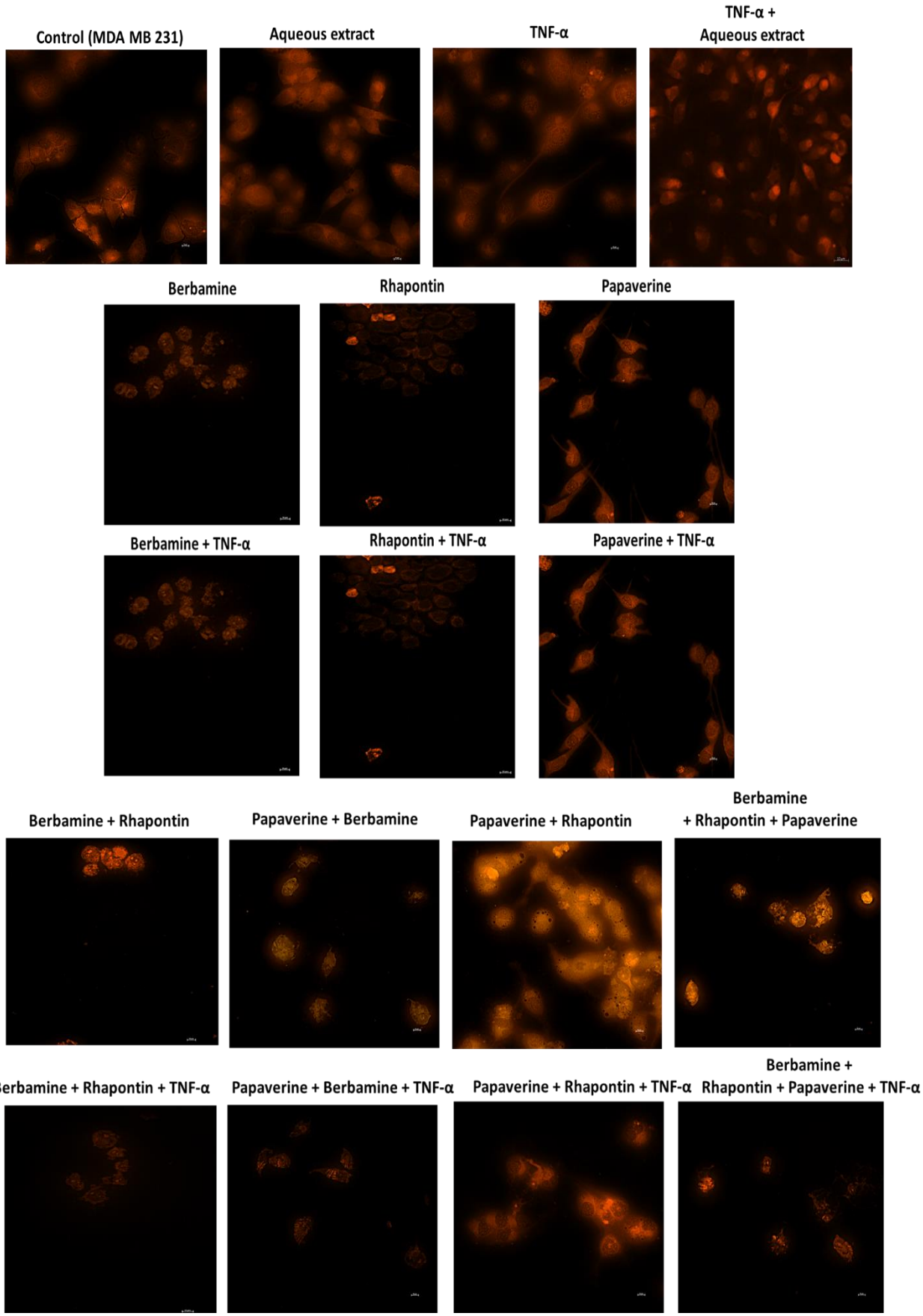
6.7 Analysis of mitochondrial transmembrane potential in breast cancer cell lines in response to *Bauhinia variegata L.* aqueous extract.

As, mitochondrial membrane potential is direct indicator of mitochondrial functional status, TMRM (tetramethylrhodamine, methyl ester) staining was used to evaluate the effect of aqueous extract on both the breast cancer cell lines in presence and absence of the inflammatory cytokine, TNF-α. The aqueous extract showed decreased staining hence loss of transmembrane potential in MCF7 and MDA-MB-231 cells. Mitochondrial transmembrane potential by TMRM Assay (Figure 6.5).

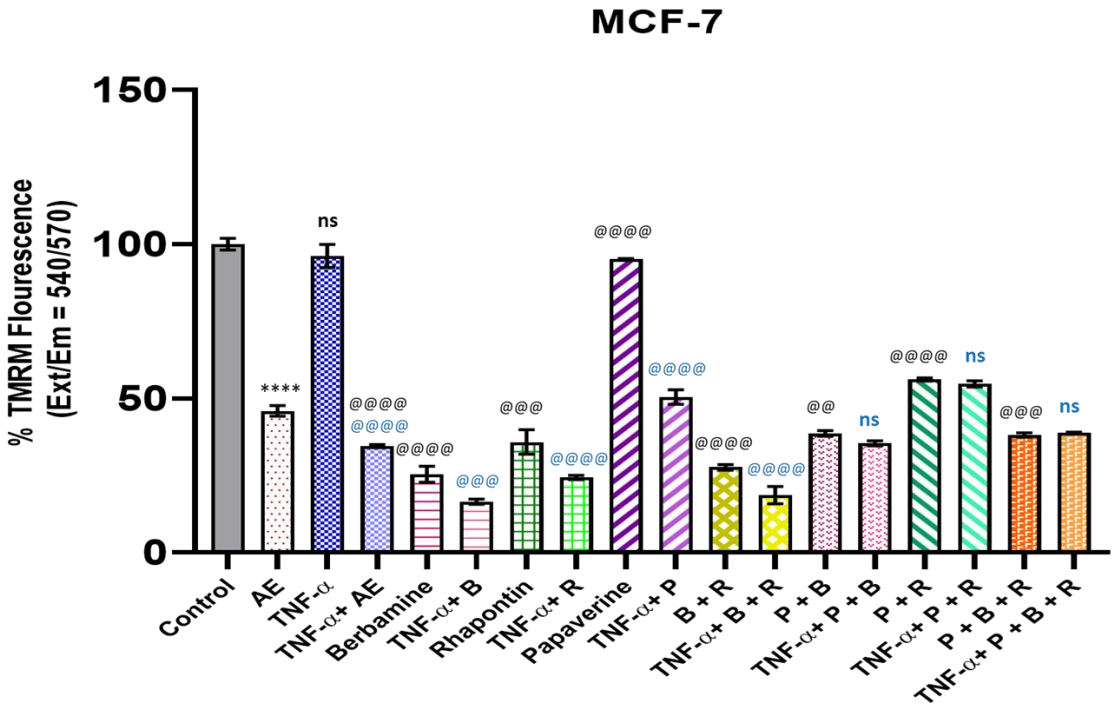
a)


Control (MCF-7)
Aqueous extract
TNF-α
TNF-α +
Aqueous extract
Berbamine
Rhapontin
Papaverine
Berbamine + TNF-α
Rhapontin + TNF-α
Papaverine + TNF-α
Berbamine + Rhapontin
Papaverine + Berbamine
Papaverine + Rhapontin
Berbamine
+ Rhapontin + Papaverine
Berbamine + Rhapontin + TNF-α
Papaverine + Berbamine + TNF-α
Papaverine + Rhapontin + TNF-α
Berbamine +
Rhapontin + Papaverine + TNF-α

b)


Control (MDA MB 231)
Aqueous extract
TNF-α
TNF-α +
Aqueous extract
Berbamine
Rhapontin
Papaverine
Berbamine + TNF-α
Rhapontin + TNF-α
Papaverine + TNF-α
Berbamine + Rhapontin
Papaverine + Berbamine
Papaverine + Rhapontin
Berbamine
+ Rhapontin + Papaverine
Berbamine + Rhapontin + TNF-α
Papaverine + Berbamine + TNF-α
Papaverine + Rhapontin + TNF-α
Berbamine +
Rhapontin + Papaverine + TNF-α

c)



d)

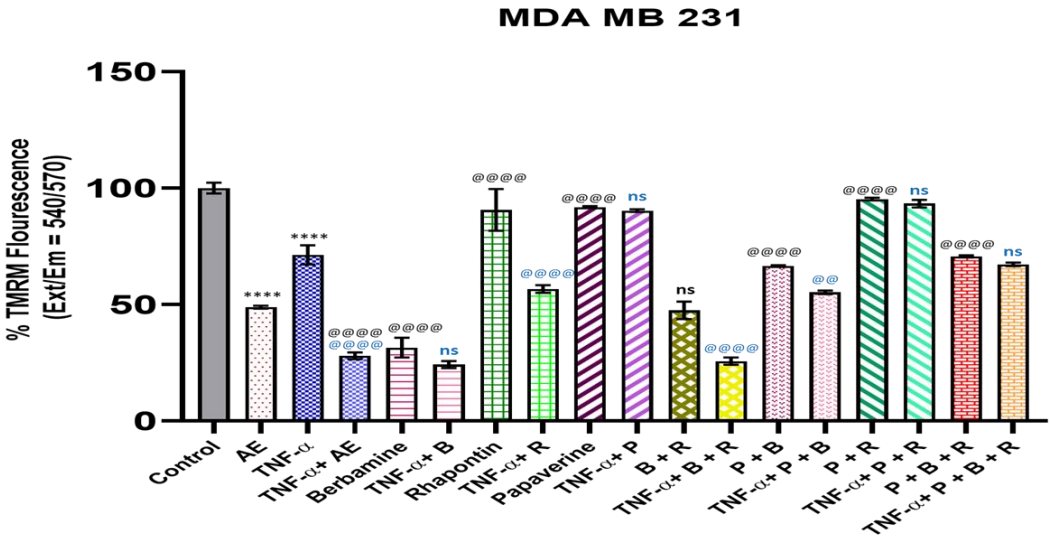


Figure 6.5: Mitochondrial transmembrane potential by TMRM Assay in MCF-7 and MDA-MB-231cells in presence and absence of TNF- α. Images a) and b), breast cancer cell lines MCF-7 and MDA-MB-231 respectively, treated with different compounds individually and in combination (Images were taken at 20x in fluorescent microscope). Image c) and d) TMRM results MCF-7 and MDA-MB-231, respectively. The experiment was repeated thrice in triplicates. p value indicates p > 0.05 (ns), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.005 (***), p ≤ 0.001 (****)

Comparison made with respect to control is indicated by * and ns (black color), comparsion done with respect to aqueous extract (AE) indicated by @ and ns (black color) and third comparison made between TNF-α treated and TNF-α untreated groups indicated by @ & ns (blue color). combination group of standard refers to B+R, P+R, B+P, B+P+R

In MCF-7 cells, no significant difference was evident between control and TNF-α group. A significant reduction in the intensity was observed in the cells treated with aqueous extract (AE) compared to untreated control group in the breast cancer cell-line, MCF-7.

In the absence of TNF-α, in MCF-7 cells, significant decline in the fluorescence intensity was observe in all the treatment groups (individually or in combination) except Papaverine (significant increase was seen) compared to AE. Notably, a significant reduction was observed in aqueous extract plus TNF-α group compared to AE only.

When the individual and combination groups, with TNFα and without TNF-α were compared, significant decline was observed in the groups exposed to TNF-α, except P+B, P+R, and P+B+R groups which showed non-significance when compared to TNF-α + P+B, TNF-α + P+R , TNF-α + P+R respectively.

In MDA-MB-231 cells, significant reduction was evident between the control and TNF-α group. A significant reduction in the intensity was observed in the cells treated with aqueous extract (AE) compared to untreated control group in the breast cancer cell-line, MDA-MB-231. In case of MDA-MB-231, in the absence of TNF-α, significant increase in the fluorescence intensity was observed in all the treatment groups (individually or in combination) compared to AE. Berbamine group showed decrease in fluorescence intensity than B+R group which was non-significant. Similar to MCF-7, a significant reduction was observed in aqueous extract plus TNF-α group compared to AE only.

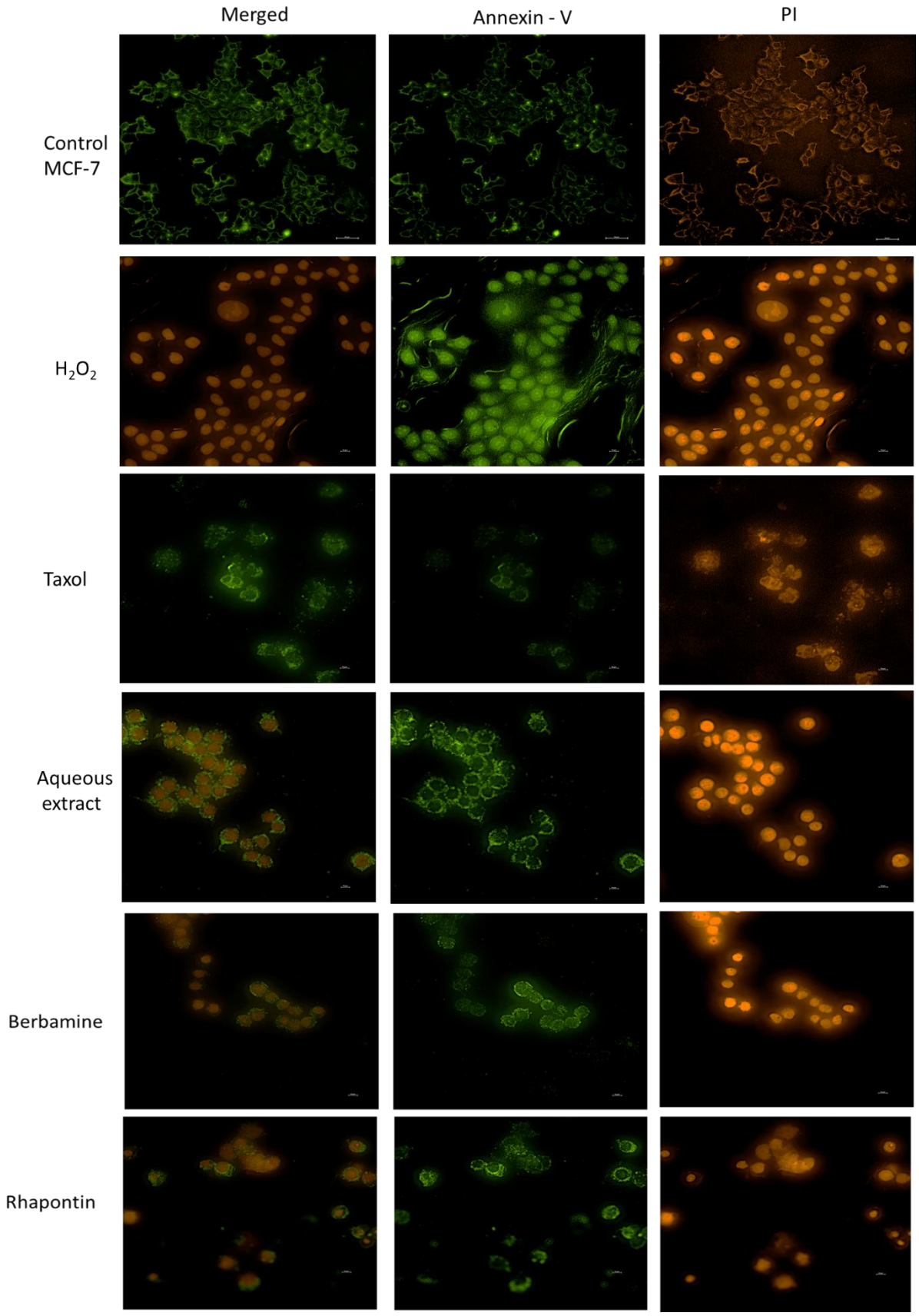
The comparison of individual and combination groups, with TNFα showed no significant difference (B, P, P+R and P+B+R) when exposed to TNF-α compared to without TNF-α. Significant decrease was observed in the TNF-α + AE, TNF-α +R, TNF-α +B+R, TNF-α + P+B groups with respect to their unexposed TNF-α group.

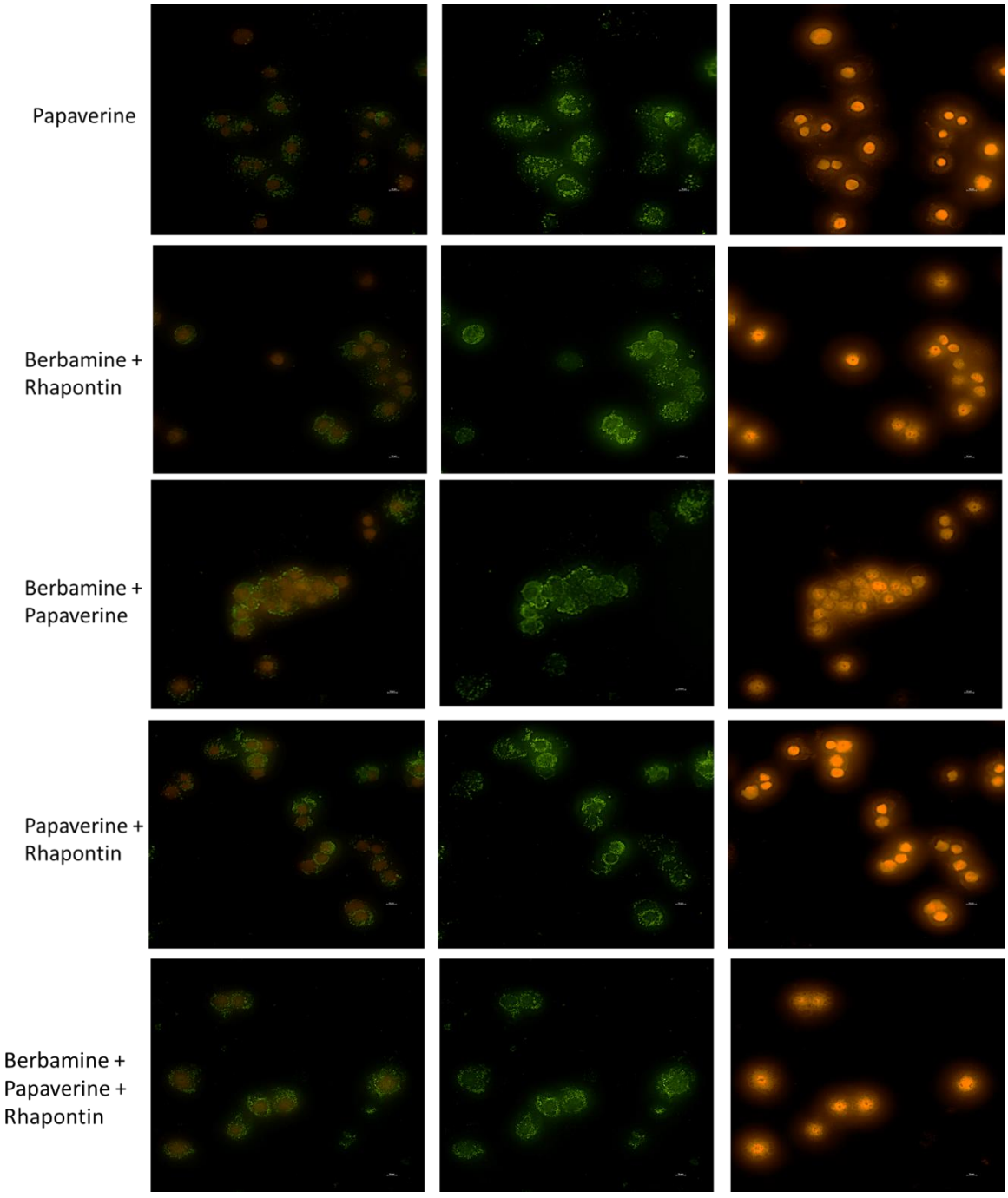
Overall observation shows that the loss in mitochondrial membrane potential is more in MCF-7 cells as compared to MDA-MB-231 cells in presence of TNF- α and it is reduced further in presence of aqueous extract causing mitochondrial dysregulation. The dysfunction of mitochondria sends the signals for cell death pathways to be activated. Hence, experiments were further performed to decipher the molecular mechanism of cell death in the ER/PR +ve, MCF-7 and ER/PR -ve MDA-MB-231.

6.8 Effect of aqueous extract on the cell death in breast cancer cell lines.

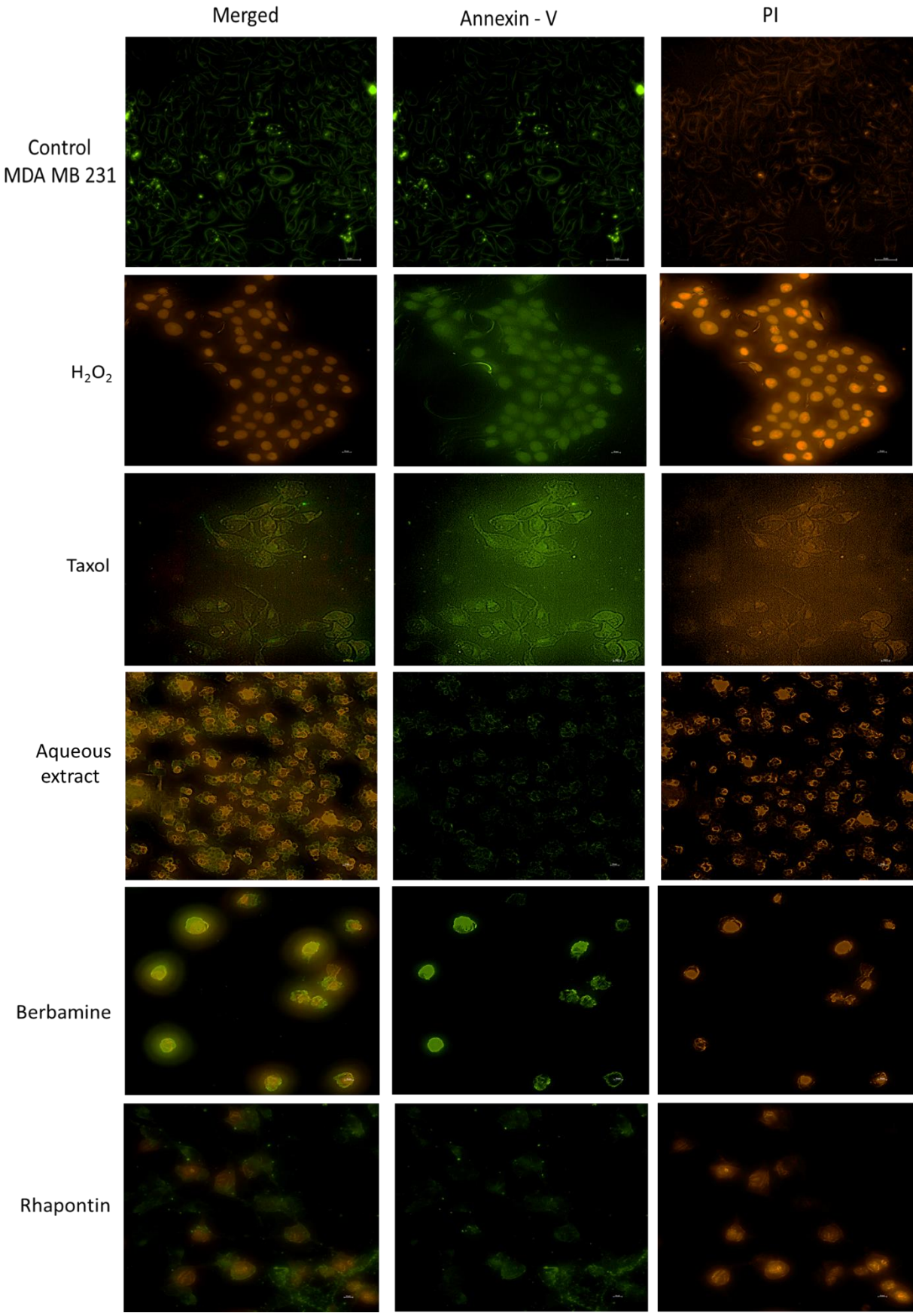
The breast cancer cell lines MCF-7 and MDA-MB-231 were exposed to aqueous extract and different standards (Berbamine, Rhapontin and Papaverine) individually and in combination. Taxol and H_2O_2 were taken as control.

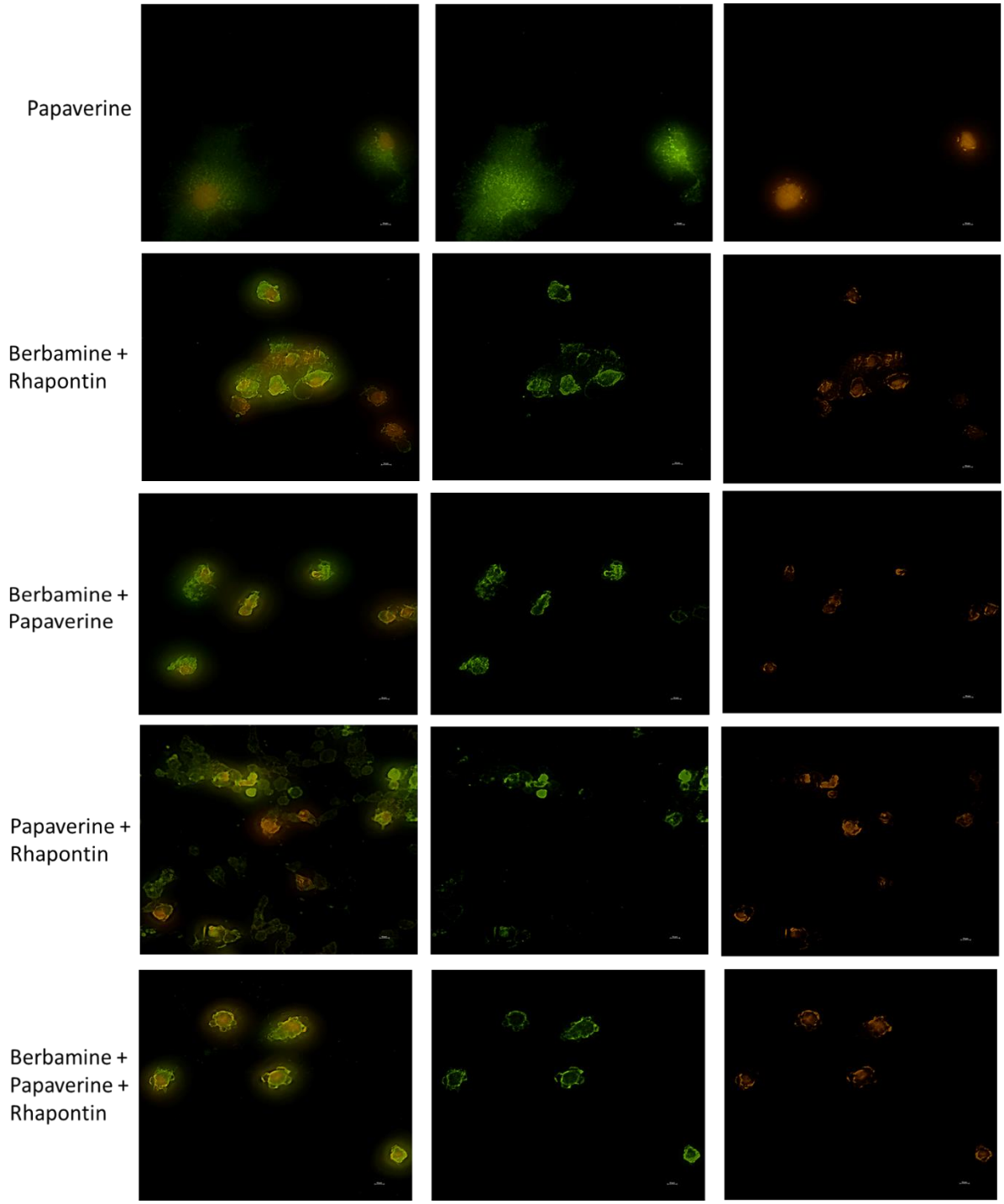
a)


Merged
Annexin - V
PI
Control MCF-7
H_2O_2
Taxol
Aqueous extract
Berbamine
Rhapontin

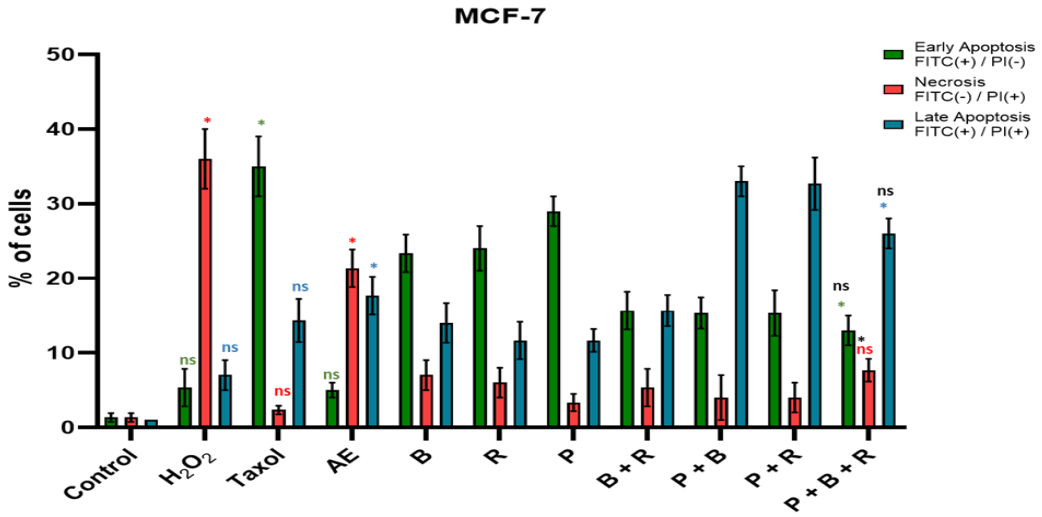

Papaverine
Berbamine + Rhapontin
Berbamine + Papaverine
Papaverine + Rhapontin
Berbamine + Papaverine + Rhapontin

b)


Merged
Annexin - V
PI
Control
MDA MB 231
H_2O_2
Taxol
Aqueous
extract
Berbamine
Rhapontin


Papaverine
Berbamine + Rhapontin
Berbamine + Papaverine
Papaverine + Rhapontin
Berbamine + Papaverine + Rhapontin

c)



d)

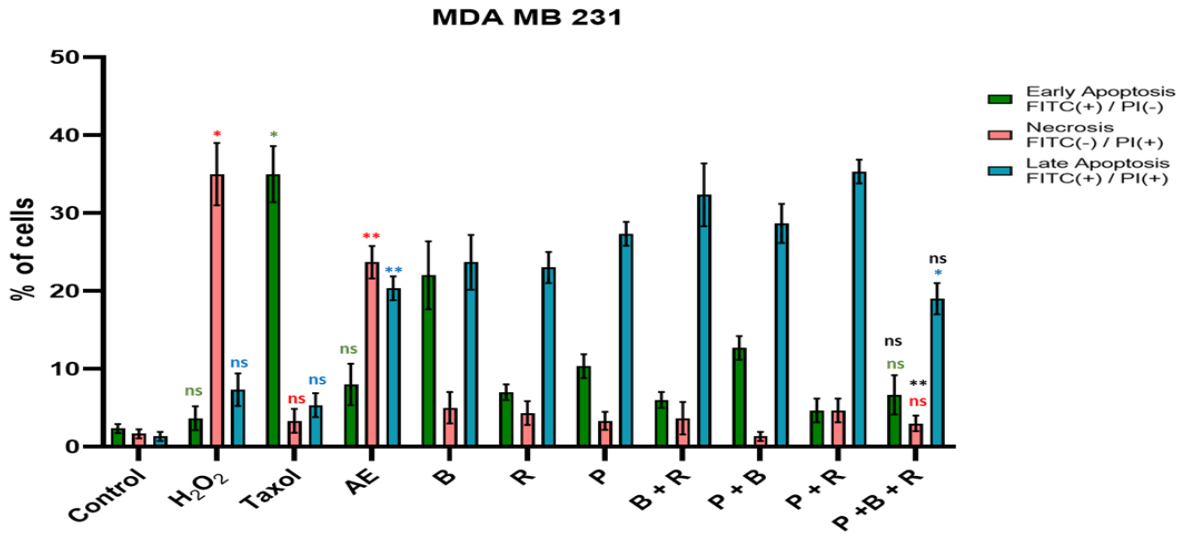


Figure 6.6: Cell death analysis by Annexin V - PI in breast cancer cell lines exposed to aqueous extract of *Bauhinia variegata* L. and different standards. a) MCF-7 and b) MDA-MB-231 breast cancer cells at 24 hours. c) Bar graph of apoptosis and necrosis for the breast cancer cells stained by AnnexinV-PI. Red, Green and Blue colour represents significance in FITC(+)/PI(-), FITC(-)/PI(+), and FITC(+)/PI(+) when compared to control cells respectively. Black color represents comparison between different groups between aqueous extract and combination of

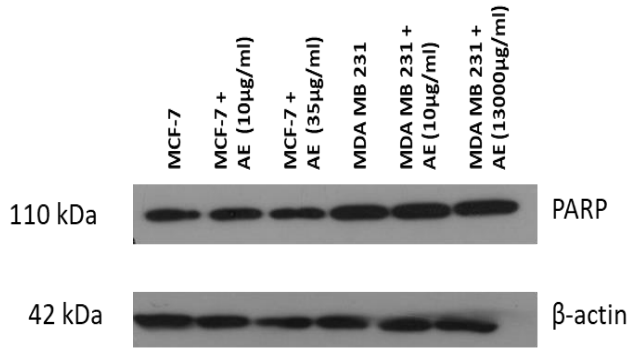
papaverine, Berbamine and Rhapontin group. p value indicates p > 0.05 (ns), p ≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.005 (***), p ≤ 0.001 (****)

It was observed that in MCF-7 and MDA-MB-231 untreated cells, no cell death was observed. In H_2O_2 and Taxol treated cells, there was clear indication of cell death through necrosis and apoptosis, respectively. In MCF-7, the aqueous extract showed presence of mixed typed of cells PI (+ve) and FITC(+ve) or (-ve) suggesting necrosis and late apoptosis responsible for the cell death. It showed significant increase as compared to control cells. In the combination group, cell death was not significant, except when FITC(-ve)/PI(+ve) cells were compared to the that of aqueous group. In MDA-MB-231 also, similar results were obtained (figure 6.6). This suggests that the mode of cell death caused by the aqueous extract of *Bauhinia variegata* L. might be similar in both the breast cell lines MCF-7 and MDA-MB-231, and irrespective of its hormonal and p53 status.

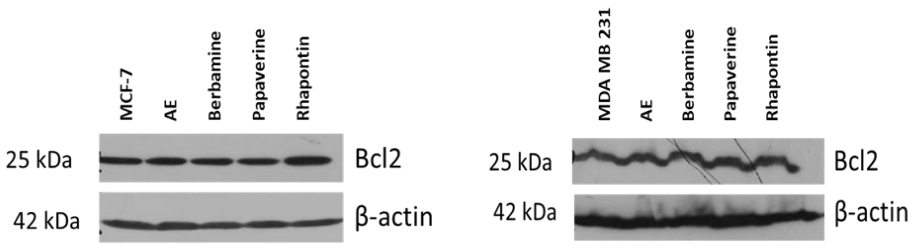
6.8.1 To assess the effect of aqueous extract on PARP, Caspase 8 and Bcl2 expression

During apoptosis process, the caspases that gets activated, cleaves and inactivates PARP, an essential DNA repair protein. The presence of cleaved PARP indicates the caspase activation which is marker of apoptosis process (Chaitanya, Steven, & Babu, 2010; D'Amours, Sallmann, Dixit, & Poirier, 2001). The breast cancer cells were treated with the aqueous extract and PARP cleavage, Caspase 8 cleavage and Bcl2 levels were monitored using western blot analysis.

(a)



(b)



(c)

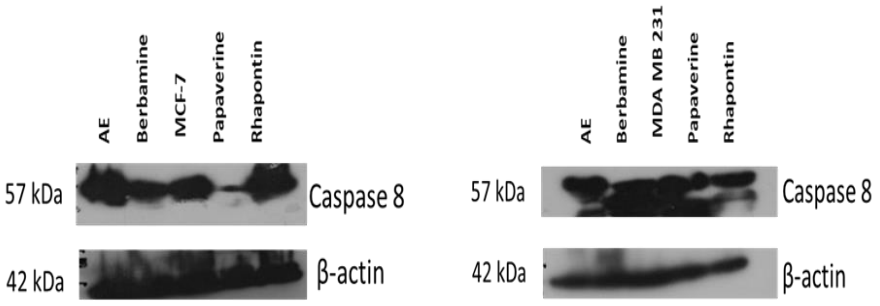


Figure 6.7: Effect of aqueous extract was analysed on PARP, Caspase 8 and Bcl2 in breast cancer cell lines. a) shows western blot analysis of PARP, b) shows western blot analysis with Bcl2 and c) shows western blot analysis with Caspase 8.

MCF-7 cells were treated with aqueous extract at two concentrations of 10 μg/ml and 35 μg/ml. Similarly, MDA-MB-231 cells were treated with aqueous extract at different concentrations of 10 μg/ml and 1300 μg/ml. The band of 110 kDa corresponding to PARP was observed in both the breast cancer cell lines. The band corresponds to the uncleaved form of PARP. This suggests that Caspases are not activated to cleave PARP during the cell death induced by aqueous extract suggesting the necroptosis mode of cell death. Further, the analysis of Bcl-2 and Caspase8 was also analysed in MCF-7 and MDA-MB-231 cells treated with extract (35 μg/ml and 1300 μg/ml for MCF-7 and MDA-MB-231 respectively). Here different standards along with the aqueous extract were used. The band of 57 kDa was detected corresponding to Caspase 8 by the western blot analysis in both the cell lines. Interestingly papaverine in MCF-7 showed decreased level of pro- form of Caspase-8. Similarly, the decreased expression is also observed in MDA-MB-231 cells. MCF-7 cells also showed increased expression of Caspase-8 in

aqueous extract and Rhapontin treated conditions. Further, the expression levels were unaltered in all the other treated groups in MCF-7 and MDA-MB-231 cells. The band of 25 kDa was detected corresponding to Bcl2 by the western blot analysis in both the cell lines. The expression was observed in all the treated groups; however, no alterations was seen in any of the treated groups (figure 6.7).

In the view of the above results, aqueous extract and other compounds show the phenotype of necroptosis or enhanced rate of apoptosis. Also, it was observed that the aqueous extract was more effective than any individual standard or their combination in all of the above experiments. Thus, the aqueous extract acts by modulating TNF-α induced ROS generation and TNF-α regulated mitochondrial function in breast cancer cells.

6.9 Discussion

Cancer cells have increased oxidative stress due to increased ROS generation, increased mitochondrial activity, alteration in factors maintaining redox status, proliferation and metabolic reprogramming as a survival strategy (Toyokuni, Okamoto, Yodoi, & Hiai, 1995). Cancer cells balance high level of ROS production by increasing the anti-oxidants activity like using anti-oxidant enzymes and diverting cell metabolism to counteract the excess oxidative stress. ROS at high levels is detrimental to biomolecules like lipids, proteins, and DNA (Liao et al., 2014). During chronic inflammation, ROS produced by myeloid cells stimulates invasive growth (Canli et al., 2017). ROS also helps cancer cells to escape immune surveillance by inhibition of T cells and natural killer cells and recruitment of M2 polarized macrophages (Griess, Mir, Datta, & Teoh-Fitzgerald, 2020). The role of ROS in mitochondrial damage leading towards decrease in the mitochondrial membrane potential and ultimately, apoptosis is well established (Ting et al., 2015); (Kocyigit & Guler, 2017).

In this study, it was found that the aqueous extract of *Bauhinia variegata* L. leaves had good anti-oxidant activity compared to other extracts, even though it showed increased ROS generation on breast cancer cell lines. Several studies carried out on Curcumin, a molecule known to possess a high anti-oxidant activity, showed significant ROS generation in MCF-7 cells (Patel, Thakkar, & Patel, 2015); (Syng-Ai, Kumari, & Khar, 2004) and MDA MB231 cells in a dose-dependent manner. It could be due to activation

of anti-oxidation system with the decrease in the total GSH level. Apoptosis may be due to combined effects of generated ROS and basal ROS, formed by the pro-oxidant activity of active constituents in cancer cells (Patel et al., 2015). An earlier study in T47D carcinoma cell lines, showed that the intracellular ROS produced by TNF-α were eliminated when the antioxidant enzyme seleno-glutathione peroxidase (GSHPx) was overexpressed (Mehlen et al., 1995). The antioxidants N-acetylcysteine, reduced glutathione, lipoic acid and ascorbic acid markedly reduced the enhancing effect of the hormone on TNFα-induced caspase activation (Gregory E. Weitsman, (2003)). It has been shown in MCF-7 cells that caspase activation by TNFα depends on stimulation of superoxide ion production in treated cells and is abolished by overexpression of superoxide dismutase (Sunil K. Manna, 1998). There are several reports suggesting the role of ROS in TNFα-induced cell death via both caspase-dependent and caspase - independent pathways (Goossens, Grooten, De Vos, & Fiers, 1995); (Goossens et al., 1999); (Chandel, Schumacker, & Arch, 2001).

TNFα-induced cytotoxicity can occur via two distinct pathways: caspase-dependent and caspase-independent (Kitanaka & Kuchino, 1999) where mitochondrial damage results in excessive ROS production. It is also found that ROS accumulation causes sensitization of the MCF-7 cells towards the cytotoxic effects of TNF-α (Ghandadi, Behravan, Biabani, Abbaspor, & Mosaffa, 2019). Recent studies have shown that there was significant increase in ROS levels in both the breast cancer cell lines MCF-7 and MDA-MB-231, where MDA-MB-231showed an higher ROS production compared to MCF-7 in presence of TNF-α (Shinde et al., 2021). This is in agreement with the ROS results obtained in this study with both the breast cancer cell lines MCF-7 and MDA-MB-231 where in presence of TNF-α, ROS levels were more in MDA-MB-231 cell line compared to MCF-7 cell line. In mitochondrial membrane potential assay by TMRM staining, it was found that in the aqueous extract group there was decrease in fluorescence intensity compared to the control cells in both the cell line. In presence of TNF-α, the mitochondrial membrane potential further decreased, suggesting TNF-α induced mitochondrial mediated cell death in MCF-7 and MDA-MB-231. Report by (Shinde et al., 2021), sheds light on TNF-α altered mitochondrial functionality and bioenergetics in ER/PR +ve (MCF-7) and triple negative (MDA-MB-231) cell lines.

Papaverine has shown aberrant morphological features in MDA-MB-231 with the cells showing changes like being shrunken, rounded, decrease in cell density, increase in cell debris, lamellipodia like cellular protrusions. The results are consistent with the observations of the present study. Papaverine did not affect ROS generation till 48 hrs in MDA-MB-231 whereas at 72 hrs it showed significant decrease in fluorescent intensity by DCFDA assay (Gomes, Joubert, & Visagie, 2021). Papaverine has ability to reversibly inhibits mitochondrial complex I and sensitizing EO771 breast tumor cells to radiation therapy (Benej et al., 2018). Literature suggest that Papaverine exhibited cytotoxicity through apoptosis in T47D breast cancer cells by annexin-V assays. Yet, it did not detect any increase in caspase activity (Afzali et al., 2015); (Rubis et al., 2009) also reported this lack of caspase activity by papaverine, indicating caspase-independent apoptosis pathways

There are no studies on Rhapontin and cancer but some evidences with similar compound, Rhaponticin and Rhapontigenin exist. Rhaponticin treatment demonstrated abnormal morphological changes like cell shrinkage, rounding, unequal shape, and cell detachment in MG-63 osteosarcoma cell line. It also decreased mitochondrial membrane potential, and increased apoptosis along with cell necrosis in MG-63 cells (Mickymaray et al., 2021). Another study also, showed necrotic cell death to the HL60 leukemic cell lines exposed to Rhaponticin (Czop et al., 2019). Rhaponticin also displayed antioxidant activity by two mechanisms, reduction in the reactive oxygen species production as well as augmenting the ROS depletion (He et al., 2021). Rhapontigenin can enhance the antioxidant activity to protect Chinese hamster lung fibroblast (V79-4) cells from oxidative stress (R. Zhang et al., 2007).

BBM induces apoptosis of MDA-MB-231 and MCF-7 cells (Liu et al., 2021). Berbamine downregulates expressions of Bcl-2 and Bcl-xL genes,in pancreatic carcinoma (Fu et al., 2018); (Zhu et al., 2018); (X. Jin & Wu, 2014); (S. Wang et al., 2009). Berbamine also induces apoptosis through mitochondrial pathway in prostate cancer cells (Zhao et al., 2016). Berbamine exposure to SMMC7721 (human hepatoma cell line) and HepG2 cells resulted in loss on mitochondrial transmembrane potential and caspase activation followed by apoptosis (G. Y. Wang, Zhang, Lü, Xu, & Dong, 2007); (G. Y. Wang, Lv, Dong, Xu, & Dong, 2009). Apart from apoptosis, Berbamine can also act as an autophagy inhibitor restricting autophagosomes accumulation by

inhibiting autophagosome-lysosome fusion in human breast cancer cells (Fu et al., 2018).

The active fractions from ethanolic extract of *Bauhinia variegata* L. leaves has shown down-regulation of anti-apoptotic genes (TGF-β, COX – 2, iNOS, c-Myc, k-Ras, and β- Catenin) and up-regulation of pro-apoptotic genes (Bax and Caspase 9) against colon cancer cell line (COLO 320) (Gunalan & Vijayalakshmi, 2020). In present study, PARP, Caspase 8 and Bcl2 levels were monitored by western blotting in Breast cancer cell lines, MCF-7 and MDA-MB-231 treated with aqueous extract. Here, when these breast cancer cell lines were exposed to the aqueous extract of *Bauhinia variegata* L. leaves, the cancer cells showed late apoptosis by Annexin V-PI staining. Similar results were obtained by ID7 fraction (from ethanolic extract) from *Bauhinia variegata* Stem on 4T1 cells (murine mammary cells) indicating late apoptosis by AO/PI double staining method and also observed the morphological changes associated with apoptosis in treated cells (K. M. Santos et al., 2018). ID7 fraction from *Bauhinia variegata* stem also increased the expression of caspase-7 and caspase-8, PARP, TNF-R1 and RIP in 4T1 cells (K. M. Santos et al., 2018). Another study by the same group showed that *Bauhinia variegata* candida stem Fraction (FR3) activation of Caspase-3, RIP, and TNF-R1 inducing tumor cell death by apoptosis along with necroptosis, on human cervical tumor cells (HeLa). The ratio of PARPc/PARP expression showed no significant difference (K. M. Santos et al., 2018). In the present study also, no changes in PARP was observed. As Annexin positive and PI positive cells are observed suggesting that there can be an increase in the rate of cell death. Further no major changes in the level of Bcl-2 was observed. Further, Caspase-8 and Bcl2 levels were observed in both the breast cancer cell lines MCF-7 (positive hormone and Her2 negative) and MDA-MB-231 (triple negative) breast cancer cells treated with aqueous extract of *Bauhinia variegata* L. leaves. It is evident form the literature that the phytocomponents Berbamine and papaverine strongly induces loss in mitochondrial membrane potential which can be observed in results of present study also. Studies on the cell death induced by Rhapontin and Papaverine is also indicative of late apoptosis or necrosis. Thus, from this study it can be concluded that the *Bauhinia variegata* L. has good anti-cancer potential and the phytocomponents present in it results in necrosis or necroptosis which might be responsible for the cell death by the synergistic effect.