Caffeic acid phenyl ester induces apoptosis in HT29 cells through the modulation of MAPK mediated cellular proliferation and heat shock proteins

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Abstract

Colorectal cancer is among the most prevalent epithelial origin gastrointestinal cancers in the world with over 2 million cases detected every year. Multiple factors contribute to the progression of colonic tumours. Emerging evidences suggest that nutrient deprivation leads to enormous oxidative stress, leading to diminished growth of cancer cells and maintenance of homeostasis. Colon cancer cells evade cell cycle check points and apoptosis leading to cellular proliferation. In this study, HT29 colorectal cancer cells were grown under serum supplemented and deprived condition and the effect of Caffeic acid phenethyl ester (CAPE) was tested. Cell cycle analysis revealed that CAPE could inhibit the cell cycle progression in G2/M phase. DNA fragmentation analysis and TUNEL assay were performed to evaluate the apoptosis inducing property of CAPE. Clonogenic and migration assays confirmed that CAPE could inhibit cancer stemness and invasiveness, respectively. p38 Mitogen activated protein kinase and JNK are notorious signalling mediators that directly links inflammation, cell death and expression of cancer signalling proteins. Western blot analysis reveal that CAPE inhibits inflammation and regulates the expression of p38 MAPK, JNK and ERK1/2, critical mediators of cancer cell progression. Heat shock proteins also known as molecular chaperones play a decisive role in colon cancer and facilitates maintenance of protein folding and homeostasis. In this study, CAPE regulates the expression of Hsp90, 70 and 40 in HT29 cells. The co-immunoprecipitation interaction between p38MAPK and HSP's validate the strong protein-protein interaction. The results of this study demonstrates that CAPE induces apoptosis in HT29 cells through the involvement of Hsp's and MAPK pathway.

1. Introduction

Colorectal cancer (CRC) is one of the notorious cancer with high mortality rate

and quiescent pathophysiology making it difficult to diagnose at the early onset [1]. CRC is characterised by the loss of colon mucosae, dysregulated absorption, bleeding and abrasion of the colon lining [2]. Earlier thought to be the western lifestyle dominant disease, however, CRC has seen a manifold increase in Asian countries for the last few decades, increasing the burden of cancer related deaths [3]. Cells usually maintain homeostasis by keeping tab on proliferation, transcription, survival, differentiation and apoptosis by different intra and extra cellular receptors and their downstream signal proteins. CRC remains dormant till metastasis by evading cell cycle checkpoints and immunogenic responses [4] Current treatment options are limited to resection and chemotherapy therefore, pursuit for affordable drug target molecules with less toxicity and nutraceutical benefits is still arguably best option for developing countries.

MAP kinase is one of the key signalling mechanism with surfeit biological functions in cell growth and differentiation [5] There are three main components in mitogen activated protein kinase signalling (p38, JNK and ERK) which majorly decide the response to external stimulus, growth factors, stress, G-protein coupled receptor antagonists and inflammatory cytokines. p38MAPK plays a critical role in inflammation, apoptosis and differentiation, while ERK1/2 downstream modulates growth and development [6] Stress

response protein p38-MAPK is proposed to have fundamental role cancer, since it mediates functions of antitumor agents like 5-FU and cisplatin and radiotherapy [7] Under physiological conditions, MAP kinases are vital in cell homeostasis and stress response, however mutations in MAPK signalling is precursor for metabolic stress, DNA methylation, protein misfolding and cancer [8, 9]. Therefore, altered MAP kinase signalling impinges normal cell functioning ensuing tumour progression [10]. p38α is an important isoform of p38MAPK poses dual nature in colon cancer progressions, it protects colonic epithelium against colitis damage, however under prolonged stress and inflammation it is associates with tumour progression [11] JNK1/2 which is universally presents in cytosol and nucleus is co-activated by dual phosphorylation of p38 under stress background [12] Oxidative stress and nutrient deprivation are considered driving forces for provoking MAPK response in CRC [10, 13], ERK is one of well comprehended mammalian MAPK pathway, Cancerous module of ERK is activated by mitogens and external growth factors, and overexpression of tyrosine kinase. In cancer, ERK is closely linked to phosphorylation of p53, facilitating apoptosis. [14] Zhaou and group propose that in colorectal cancer MAP kinase and apoptosis are inter-twined [15] Accumulating evidences suggest that during stress, heat shock proteins invoke activation of SAPK/p38[16] These findings collectively pave way for investigating deeper into individual roles of heat shock proteins and MAP kinases in colorectal cancer.

Heat shock family proteins also known as HSP's are molecular chaperones with plethora of functions in cell homeostasis, protein folding and ER redox, HSP's play a major part in cell proliferation and cancer metastasis [17]. Overexpression of HSP's is synonymous with tumour progression [18]. Growing evidences suggest that targeted inhibition of heat shock proteins has strong correlation to induce oxidative stress and autophagy [19]. HSP90 is amply present normal as well as cancer cells, however tumour phenotype HSP90 is more susceptible to drug targets with inhibitory potential [20] Calnexin is one of the prognostic markers of colorectal cancer and an ER stress regulating protein. Clinical studies suggest calnexin expression is intricately linked to stage advanced stage of multiple cancers [21]. Protein disulfide isomerase (PDI) is major contributor for proteostasis and unfolded protein response which critically takes part in maintenance of glucose metabolism, calcium signalling and organelle biogenesis [22, 23] BIP and HSP40 expressions are elevated in colorectal cancer [24, 25]. Recent studies strongly implicate that under stress condition, heat shock proteins intricately bind to MAPK signalling to mitigate inflammation [26]. These findings propose that inhibiting or regulating the expression of these key molecules by augmented receptor target therapy may contribute in development of new therapeutic leads in CRC.

In this study, we used Caffeic Acid Phenethyl Ester (CAPE) a natural polyphenolic compound, derivative of Caffeic acid and active compound of honeybee propolis holds plethora of benefits with therapeutic potential [27]. CAPE has been sought after with great interest during last few decades owing to its multifaceted pharmacological benefits like anti-diabetic [28] anti-cancer [29] anti-inflammatory [30] neuroprotective [31] anti-bacterial, hepatoprotective, and cardioprotective effects. [32, 33, 34]. CAPE is found to be involved in inhibiting the progression of ovarian cancer by suppression of inflammatory markers like NF-κB, as reported by Alexandra [35] CAPE exerted strong anti-oxidative response by modulating p38-MAPK pathway. CAPE is found to reduce metastasis in breast and gastrointestinal cells

[36]. In corroboration to the above findings, in this study we demonstrate that CAPE has role in MAPK and Heat Shock protein regulation, which potentially facilitates apoptosis in HT29 colorectal cancer cells.

2. Materials And Methods

2.1 Cell culture and antibodies

Human epithelial colorectal cells (HT29) were purchased from National Centre for Cell Science (NCCS) Pune India, High glucose DMEM and McCoy's 5A medium, Fetal bovine serum, 100x anti-anti solution was purchased from Gibco (USA), TPVG (1x) was purchased from Himedia labs Mumbai, India, ERK1/2, Beta-Actin(sc-47778) were purchased from SantaCruz biotech (USA), p38α (#9218), Heat Shock proteins was procured from Cell Signalling technology (USA). Alexa Fluor 488 was purchased from Jackson Immuno Research Laboratories (USA), Monarch® Genomic DNA Kit (T3010S) was purchased from BioLabs, New England. TUNEL assay kit (ECKA334) and p-ERK1/2 (E-AB-70292) and JNK (E-AB-60070) were purchased from Elabscience (USA). PMSF (Sigma-329986). FemtoLeucent (786003) enhanced chemiluminiscence kit was purchased from G-Biosciences. ProLong™ Gold Mounting medium with DAPI was purchased from Fisher scientific (USA). Caffeic Acid Phenethyl Ester (FC19630) was purchased from BioSynth CarboSynth (UK) and TCI chemicals (Japan), DCFH (4091-99-0) was purchased from Cayman Chemicals company MI (USA).

2.2 Serum starvation

The serum starvation protocol was done with slight changes in previously executed method of Nair [37]. HT29 cells were grown in 6 well plates or 90mm sterile culture dishes, on complete first cell division in 21 hours' media (10% FBS + DMEM) was replaced with 3% media until the end of treatment period.

2.3. Cell viability assay

Cell viability assessment was performed by widely used colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [38]. Briefly 5×10^3 cells were seeded evenly into flat bottom 96 well plate. At 70–80% confluence, cells were treated with variable concentration (10-100μM) of CAPE for 24 and 48hrs ± serum supplementation. At the end of the treatment period, drug laden media was aspirated followed by 90μl of serum free media + 10μl of 5mg/ml of MTT reagent. Incubated for 3–4 hours in dark at 37 c, with visible formazan crystals, media with MTT reagent was removed, followed by 100μl DMSO to each well, placed on orbital shaker for 3–5 minutes and reading was taken at 540nm with BioRad iMark™ Elisa plate reader.

2.4 Morphological studies

HT29 Cells were seeded on 22mm round coverslip in 6-well plate, after treatment with CAPE, media was removed and washed twice with fresh 1x PBS, morphological studies were recorded with Motic inverted microscope AE-31 with 20x magnification.

2.5 Cell Migration Assay

Cell migration was performed as previously done by Li [39]. Cells were seeded in 6 well plate left until it reached 80–90% confluence, followed by an artificial wound or scratch across the monolayer of the well in plus (+) contour. Images of control and CAPE treated cells were taken on 0 hour, 24 hours and 48-hour interval with Motic AE31 microscope and scratch dimensions were analysed by ImageJ software.

2.6 Clonogenic assay

This assay was performed with slight modifications as previously described [40, 41]. Briefly, around 100–200 cells were seeded into a 12 well plate left to grow for 7 days in growth medium, media was changed every 48hrs. The cells were treated with CAPE and post incubation time, the cells were washed with 1x PBS twice then fixed with ice cold methanol left at RT for 10–15 minutes, stained with 0.5% crystal violet solution, followed by multiple washes with distilled water until excessive dye washed off. The plates were then left to air dry. Colonies were analysed macro and microscopically, colonies were counted by particle extension in ImageJ software.

2.7 Assessment of intracellular reactive oxygen species

Determination of ROS was done by method previously described by Kim [42]. Briefly, HT29 cells with or without serum were seeded in coverslips in a 6 well plate and treated with CAPE for definite time points. A working concentration of 10mM of DCFH in DMSO was prepared fresh before use and was added into each well and incubated at 37°C for 30 min in dark. thereafter washed with PBS. The cells were imaged in a fluorescence microscope broad range FITC filter with excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.8 Analysis of Cell cycle by FACS

Cell cycle analysis was carried out by method of Yao [43] with slight variation in fixation procedure. Cells were seeded into a 6-well plate and treated with CAPE, washed once with PBS, centrifuged at 2000 rpm and discarded the supernatant and fixed cells by dropwise ice cold absolute ethanol, stored at -80 C for overnight for optimal fixation. Subsequently ethanol was replaced with 1x PBS, 0.15 TritonX 100, and 10mg/ml RNase-A, cocktail and 10µl of 1mg/ml stock of Propidium iodide was added to each tube incubated for 10-15mins in dark. FACS data was acquired by Beckman Coulter Cyto^{Flex} and analysed by CytExpert software.

2.9 DNA fragmentation assay

HT29 cells grown in 60mm culture plates followed by treatment with CAPE for 24 and 48 hours. Genomic DNA extraction was done according to manufacturer's protocol. Briefly 3×10^6 cells were collected by centrifugation at 1000×1g for 1 minute, followed by 1x Proteinase K, then 100µl of DNA lysis buffer was added to each tube, Incubated at 56ºC for 5 minutes, transferred to column inserted tubes, centrifuged at 1000×1g for 1 minute for optimal binding of genomic DNA, then centrifuged at 14000 rpm for 10 minutes, discarded the elute, and transferred column to fresh tube, centrifuged at 14000 rpm then transferred to DNAse free tubes with pre warmed DNA elution buffer, after final centrifugation at 14000

rpm gDNA was collected and quantified by A_{260}/A_{280} by spectrophotometer and then ran with a 1.5% agarose gel electrophoresis for 1 hour.

2.9 Tunel Assay

Tunel assay was performed according to manufactures instructions. Briefly, HT29 cells were grown on coverslips in 6 well plates, after treatment period cells were washed twice with 1x PBS for 5 minutes, followed by fixation with 4% neutral buffered formalin for 30 minutes, washed with PBS 3x, 100ul of 1x Proteinase-K was added to all control and treated samples, Washed cells with PBS 3x for 5 minutes each, added Tunel buffer and TdT enzyme mixture, Finally added 50µl of enhanced FITC-fluorescein mix and mounted on new slides with ProLong™ Gold mounting medium. The image acquisition was done using Zeiss confocal microscope with FITC and DAPI filter.

2.10 Immunofluorescence

Immunofluorescence was done with modification as per the method of Çağatay [44] Cells were seeded in 6 well plates on round cover slips, after experimental period, media was aspirated replaced with 2 x washes with PBS, fixed with ice cold methanol or 4% neutral buffered formalin and stored at -20 for future use. The fixed cells were blocked with 5% bovine serum albumin dissolved in PBST, followed by incubation with P38α antibody 1:300 overnight at 4°C, then slides were washed with PBST 3x for 5 minutes each, later incubated for 1 hour with Alexa Fluor® 488 conjugated secondary antibody for one hour, washed with PBST and mounted after evaporating extra moisture with ProLong Gold Mounting medium enhanced with DAPI. The images were captured using Nikon fluorescence microscope and merged using ImageJ software overlay extension.

2.11 Immunoblot analysis

Control and treated HT29 cells were harvested either by cell scrapper or trypsinization at end of the treatment period, followed by centrifugation at 1500rpm for 5 minutes, the pellet was washed 3 times with 1X PBS, supernatant was discarded and 500µl of RIPA lysis buffer fortified with 1mM PMSF protease inhibitor. Subsequently, cell samples were further centrifuged at 12000rpm at 4°C for 30 minutes. Supernatant was collected in a fresh tube and protein was quantified using Bradford's reagent with biophotometer (Eppendorf). 30–40µg of control and treated protein samples were run in the SDS-PAGE in BioRad Mini-Protean®, apparatus which was further transferred to Amersham Protran nitrocellulose membrane (Merck- GE10600002). Further the membranes were washed with PBST (1x PBS + 0.1% Tween 20) V/v and blocked with BLOT-QuickBlocker™ (G-Biosciences 786 – 011) for one hour at room temperature, followed by 4-12Hr incubation at 4°C with primary antibodies, then 4x wash with PBST once completed secondary antibody incubation for one hour, finally 3x wash with PBST, the blots were developed using femtoleucent ECL solution in dark and scanned using CanoScan LiDE-400.

2.12 Co-immunoprecipitation

Cells were lysed for 30 min on ice in IP lysis buffer (10 mm Tris-HCl, pH 8, 150 mm NaCl, 5 mm EDTA, pH 8, 0.5% (v/v) Triton X-100,) and 10ul RNAse A 10mg/ml. cell lysates of control and treated HT29 cells poured into the fresh tubes along with Protein A/G PLUS-Agarose (SantaCruz Biotechnology SC-2003) and 1mg/ml per tube of p-38α MAPK antibody in centrifuge tube placed on axis rotator overnight at 4°C, subsequently unbound fractions were removed by a washing with IP lysis buffer fortified with sodium ortho-vanadate followed by centrifugation at 12000 rpm for 5mins the bound factions were collect from supernatant and unbound factions in pellet were discarded, Further the samples were used to run SDS-PAGE and western blotting.

2.13 Bioinformatics studies

The structural activity relationship and molecular docking study was carried out using Glide/Schrödinger software between CAPE and 3D structures of HSP90 and p23 (PDB ID. 7RY1, IEJ) compared docking score and binding energy with widely known HSP inhibitor Geldanamycin.

Statistics

The statistical analysis was done using GraphPad Prism v 9, software. One-way ANOVA followed by students t-test whenever applicable Tukey's was used. P values less than 0.05 were considered as statistically significant. Data are represented as mean ± SD. and mean ± CV unless stated otherwise

3. Results

3.1 Effect of CAPE on cell viability and morphology of HT29 cells

To evaluate the cytotoxic effect of CAPE and to determine the optimal dosage IC_{50} value, HT29 cells were treated for 24 and 48 hours in presence and absence of serum. The cells were treated both time and dose dependent manner, CAPE at 45μM ± 1 could induce cell death in serum supplemented milieu and 40μM under serum starved condition respectively, at 48-hour time point [Fig. 1A], Further studies were carried out based on the IC_{50} values. HT29 cells were treated with IC_{50} values of CAPE for 24 and 48 hours under variable serum conditions and the changes in morphology of Control and CAPE treated group of cells was observed under phase contrast microscope at 20x magnification. Control cells exhibited morphology characteristics of epithelial cells with membrane integrity. However, CAPE treated cells show deformed and stunted growth [Fig. 1B] These results signify that CAPE has potent effect on general morphology and growth of colorectal cancer cells.

3.2 Cell migration assay

After optimising CAPE dosage, wound healing assay or commonly known as migration assay was performed to assess the migration or invasiveness of HT29 colon cancer cells [Fig. 2] represents the migration of HT29 cells from 0, 12, 24 and 48hour time points under serum supplied and limited

conditions. The control cells showed typical cancer cell behaviour and kept proliferating until end of experiment, CAPE treated cells under serum stressed condition exhibited diminutive change from the actual scratch and showed maximum effect at 48hours exposure with CAPE. The results of study conclude that CAPE has strong ability to impede the migration, therefore could be of therapeutic value in metastatic colorectal cancer.

3.3 Clonogenic assay

HT29 colorectal cancer cells per se has tendency to form clumps/spheroids [45], Briefly, 1×10^3 cells were seeded into 90mm sterile plate, followed by treatment with CAPE for 48 hours, the medium along with drug was replaced with fresh medium and cells were left to grow for 7 days with change of media every 2 days, the control cells kept forming colonies, however CAPE treated cells showed remarkable decrease in colony formation[Fig. 3], which emphasizes that CAPE has anti-clonogenic effect on colorectal cancer. Colonies were captured by optical microscope with 4x objective and counted by ImageJ, the data was normalised and plotted in Graphpad prism software.

3.4 Assessment of intracellular reactive oxygen species

The use of DCFH-DA is a sensitive method for determining intracellular ROS levels. This assay is based on the principle that presence of ROS converts DCFH ◊ DCF which emits green light on exposure to fluorescence. Reactive oxygen species are precursors for triggering antioxidant response elements [46] Cancer cells thrive on the basal levels of ROS, overexpression of ROS leads the cell to apoptosis or stress mediated cell death [47, 48]. CAPE is reported to induce ROS, however there are no reports about the ROS under serum starved environment. [Fig. 4] shows that treatment of CAPE in HT29 cells for 6h and 12h generates ROS production under both sera supplied and starved milieu. Further to validate this method of Katerji [49] was used to determine protein levels in control and treated samples.

3.5 CAPE induces G2/M phase arrest in HT29 cells.

CAPE is reported to induce cell cycle arrest in PC12 prostate cancer cells [50]. In order to evaluate the role of CAPE in cell cycle progression, flow cytometry was employed to determine whether or not CAPE could induce arrest in HT29 cells. In this study, CAPE induced G2/M arrest in both sera supplemented and stressed conditions [Fig. 5]. G2/M arrest is associated with epithelial cell cycle arrest [51] also vouch that the G2/M arrest is stress mediated [52] The results of this study demonstrate that CAPE increased cell count arrest to 22% ± 0.5. compared to control 9.9% ± 1%. Combined with the above findings, our studies indicate that CAPE could be pivotal in inducing cell cycle arrest and subsequent cell death.

3.6 DNA fragmentation and Tunel assay

DNA fragmentation is the benchmark study of cells undergoing apoptosis [53] DNA fragmentation was carried out in Control and CAPE treated HT29 cells, CAPE treatment induced DNA breaks in both sera supplied and limited environment. Tunel assay is versatile measurement of apoptosis in cancer cells [54], In this study the HT29 cells were treated with CAPE for 24 and 48 hours under variable serum conditions. This assay is measure of DNA integrity and damage; DNA is nick end labelled by deoxy-nucleotidyl

transferase enzyme. Our results confirmed CAPE treatment increased the TUNEL positive nuclei in HT29 cells compared to control or untreated cells which showed negligible nuclear damage [Fig. 6] which further confirms that CAPE could potentially induces DNA damage in colorectal cancer.

3.7 Expression of MAPK by immunofluorescence

MAPK14 or p38α is crucial molecule in colorectal cancer chemotherapy [56] It is considered as mainstay of MAP kinase signalling cascade [57]. Its role is critical in cell functioning as it facilitates apoptosis under normal conditions, Studies suggest that inhibiting MAPK14 induces cell cycle arrest or stress mediated death in colorectal cancer [58, 59, 60]. Our studies[Fig. 7] endorse above findings CAPE could significantly reduce the protein expression of P38α under both sera supplied and limiting conditions.

3.8 CAPE regulated the expression of heat shock proteins in HT29 cells

As mentioned above heat shock proteins are overexpressed in colorectal cancer, under physiological conditions HSP's modulate protein folding, signalling and also take part in regulating metabolic stress [61] metabolic stress mediated CAPE treatment has not been reported yet, immunoblot analysis of control and CAPE treated HT29 cells show that CAPE regulated the expression in almost all the key molecular chaperones[Fig. 8]. CAPE decreased the expressions of Hsp90, 70 and 40 in HT29 cells, demonstrating its potential to intervene heat shock family of proteins. To validate this study, bioinformatics approach was carried out. Figure 11 show that CAPE binds more effectively with P23 and co-chaperone of HSP90[62] and has almost equal binding energy as Geldanamycin which is one of the well-known HSP inhibitor. The results of these study show that CAPE has inhibitory/regulatory potential on heat shock proteins.

3.9 Effect of CAPE on MAPK signalling

MAP kinase signalling is considered critical in colorectal cancer therapy. CAPE is implicated to modulate up and downstream effectors of MAP kinase signalling in prostate cancer and lung ailments [63]. Its role in metabolic stress induced colorectal cancer is still elusive. In this study, we sought to evaluate immunoblot analysis of control and CAPE treated HT29 cells under metabolic stress milieu, our results reveal CAPE significantly $p < 0.01$ reduced the MAPK14/p38α, ERK expression and phosphorylated p-Erk and JNK [Fig. 9] which are imperative in MAPK signalling pathway [64]. Collectively our results demonstrate that CAPE impedes expression of fundamental mitogen activated protein kinase proteins.

3.10 Co-iP of p38α with HSP's

Targeting MAPK and HSP is challenging but promising as advancement of novel approaches *invitro* and *in silico.* Mkaddem and group earlier reported protein-protein interaction between HSP96 with PP5 on activation of ERK1/2 signalling [65] studies suggest that HSP's acts as substrate for the p38[66] therefore, we carried out Co-immunoprecipitation to evaluate the protein-protein interaction between MAPK and Heat Shock Protein 90 and 40, [Fig. 10] shows the western blotting results of protein-protein interaction between p38α and critical heat shock proteins complex, The results of this study show there is

strong interrelation between the two, and gives novel avenues to the chemotherapeutic agents with combinatory effect on HSP's and MAPK combined.

3.11 CAPE and heat shock protein structure activity relationship by molecular docking

Geldanamycin and its derivatives are well known heat shock protein inhibitors [67, 68] Molecular docking studies[Fig. 11] of CAPE with HSP's reveal that CAPE binds to HSP90 at Gly-135 and Phe-138 with docking score of -12.5, Glycine decreases activation energy in protein dynamics and promotes folding [69] while as phenylalanine is known to take part in synthesis, CAPE binds to p-23 more efficiently than Geldanamycin which is critical in hydrolysing ATP and stabilizing HSP functioning [70] the hydrogen bonding occurs at Leu-89,96, Thr-90, Trp-8 and Trp106, these amino acids are pivotal in metabolic regulation of this small heat shock protein[71].

Combining the data obtained in this study, it is evident that CAPE exhibits anti-cancer effect in HT29 cells through modulation of heat shock proteins and MAPK signalling.

4. Discussion

Despite the various advanced treatment options available over the last few years, colorectal cancer (CRC) imposes a huge health burden, in developing countries.[72] In current study, we had two aims, one to evaluate the role of Caffeic acid phenethyl ester in mitigating colorectal cancer *in-vitro*, under metabolic stress condition, and second was to understand the molecular mechanism involved in colorectal cancer with primary focus on interplay between MAPK signalling and heat shock family proteins. To arrive at this hypothesis, HT29 cells were treated with CAPE and the cell cycle and apoptosis inducing effect of CAPE was evaluated. MTT assay revealed that CAPE induced cytotoxic effect and effectively impeded cell migration, clonogenicity in HT29 cells. Also CAPE implicated inhibitory potential in heat shock proteins corroborated by western blotting and molecular docking studies. There are reports of CAPE generating ROS in cancer cells [73]. To support this, in this study, CAPE generated ROS mediating killing of HT29 cells. Pharmacological, genetic and epidemiological data back the fact that inflammation is one of the main factor in cancer, in fact inflammatory bowel disease is one of the prime risk factors for colorectal cancer development [74] CAPE regulates inflammation by subsiding the expression of NF-kB and NLRP3 Inflammasome, which are contributing elements to CRC progression [75].

Cells trigger apoptosis under prolonged limited nutrient aura [76]. We sought to explore, whether treatment with CAPE under limited nutrient environment will prompt cell death. CAPE is known for G2/M module arrest in breast and prostate cancer [50, 51]. Our study confirmed above and CAPE effectively prompted G2/M arrest and induced apoptosis in HT29 cells. Among the other cancer signalling pathways, MAPK signalling plays a key role in instigating inflammation and metastatic potential in cancer cells. There are reports of natural compounds intervening MAPK cascade thereby reducing

inflammation and the burden of cancer. CAPE intervening MAPK has been addressed in oral and lung cancer [64, 78]. In this study fundamental proteins of MAPK like p38α which is known to be facilitate inflammation in colorectal cancer, CAPE could significantly reduce the expression in metabolic stress induced HT29 cell. Dysregulated MAPK downstream ensures phosphorylation of Erk and JNK which facilitate the proliferation and hinder apoptosis [79] CAPE treatment reduced the expression of p-ERK and JNK especially under serum starved condition.

Recent reports suggest that heat shock proteins at large take part in cancer progression and metastasis, under extra cellular stress background Hsp90 and Hsp70 along with matrix metalloproteinase take part in migration and metastasis [80] Ma and group [81] reported that knockdown of protein disulfide isomerase, an ER stress regulating molecular chaperone, induces apoptosis in CRC. Among the several Hsps, the role of Hsp40 or DNAJ is ambiguous in cancer however, recent reports [82] suggest that Hsp40 presented modulatory effect CRC. The studies above conclusively cue that heat shock proteins are vastly expressed in CRC, In our study a diminished expression of Hsp40 has been observed, which supports the previous findings (Ref). CAPE is reported to reduce the heat shock protein expression under hypoxic conditions [34, 83]. Key factors of MAPK pathway Erk, JNK that facilitate tumorigenesis were downregulated, cancer cells take control of glucose metabolism and protein folding, angiogenesis, heat shock proteins have plentiful role in cell function and proteostasis, there are compelling evidence about overexpression of heat shock proteins in cancer related incidents, our study corroborated with the previous findings and CAPE was able to inhibit the overexpression heat shock proteins.

External stress triggers crosstalk between MAP kinase signalling and heat shock proteins. Studies on rodent intestine, reveal that MAPK modulation prevents over expression of Hsp70 [84] Pawaria and group reported that MAP kinase immuno-precipitates with HSP’s in mouse lymphoma [85] Our studies confirmed that p38α-MAPK interacts with Hsp40 and Hsp90, this protein-protein complex potentially induces modulatory effect in colorectal cancer.

Conclusion

Taken together, the results of this study demonstrate that CAPE possesses anticancer potential through modulation of Heat shock proteins and MAPK. CAPE induces apoptosis in colon cancer cells, in part, through regulation of these factors. Further studies are in progress to demonstrate the mechanistic role of CAPE in modulating epithelial to mesenchymal transition mediated cancer signalling pathways (Fig. 12)

Declarations

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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Figures

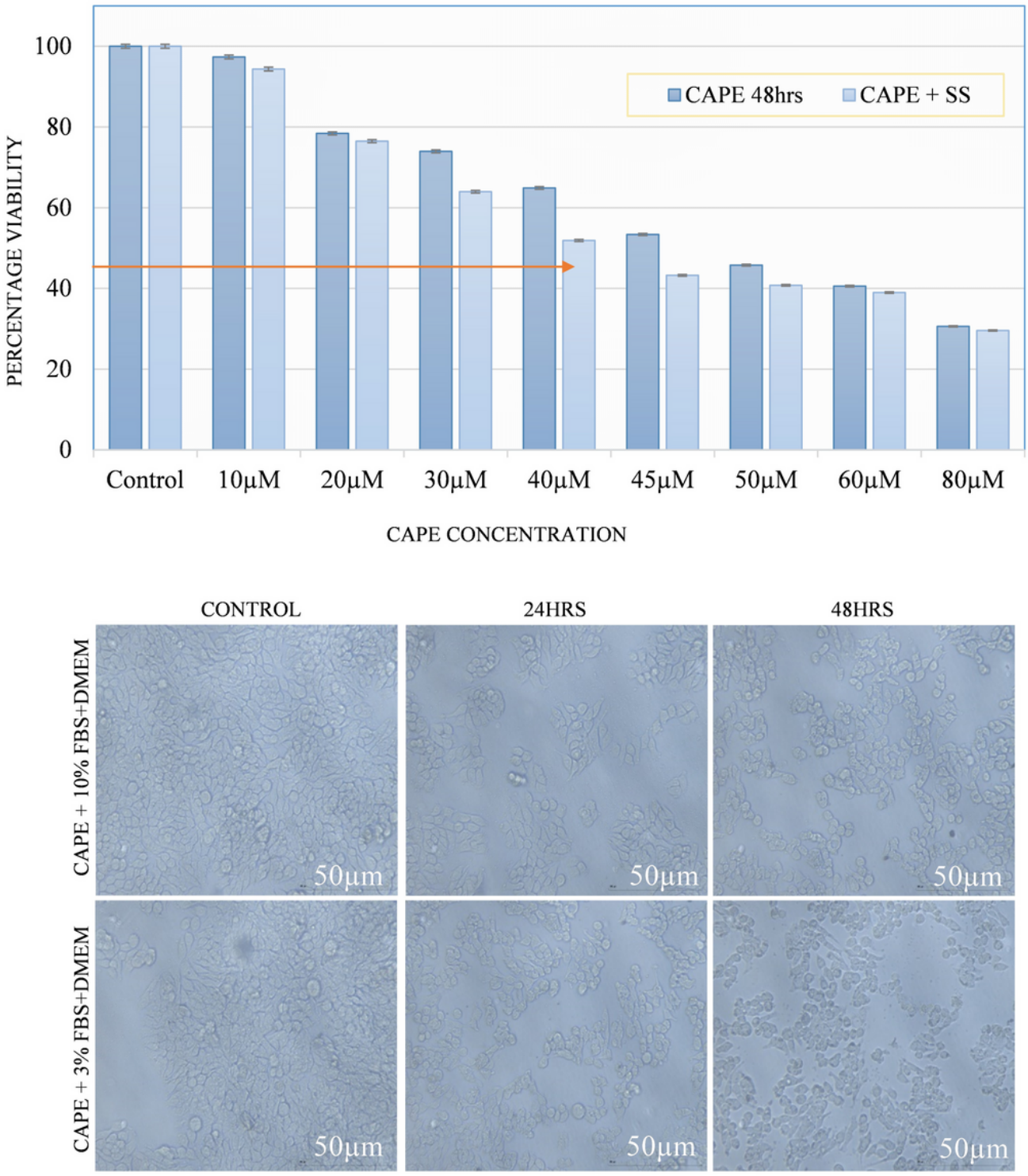

Figure 1

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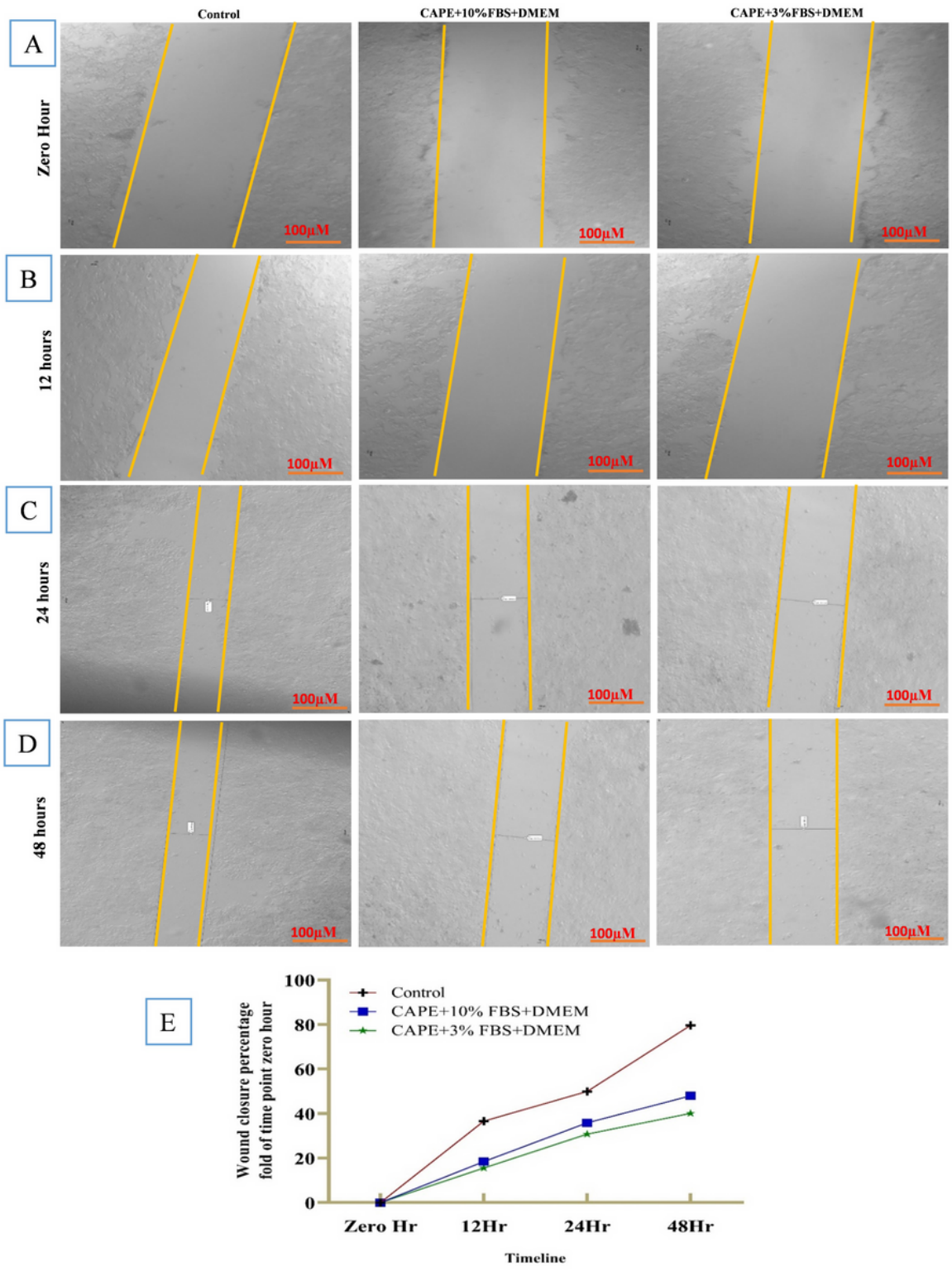

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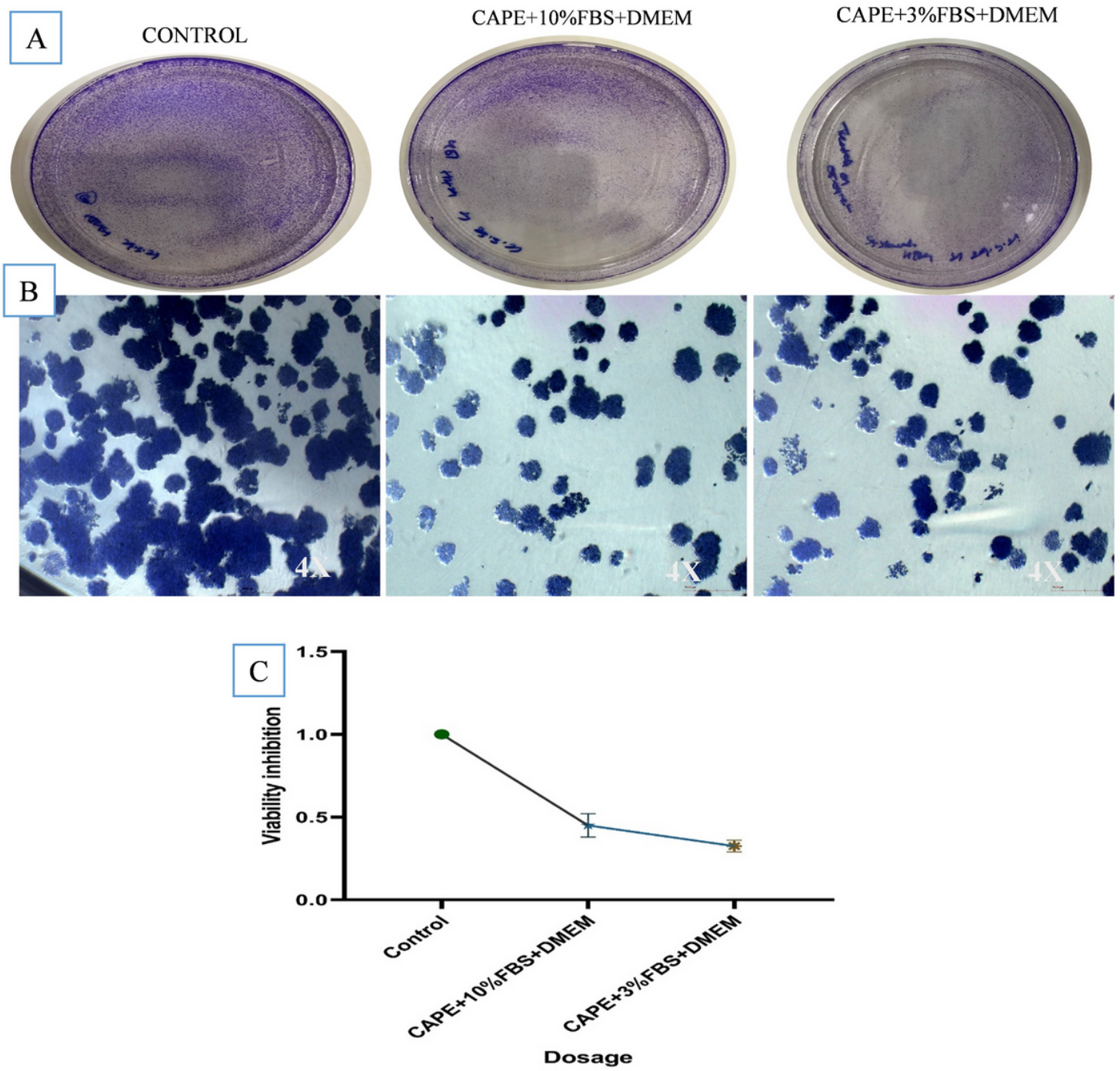

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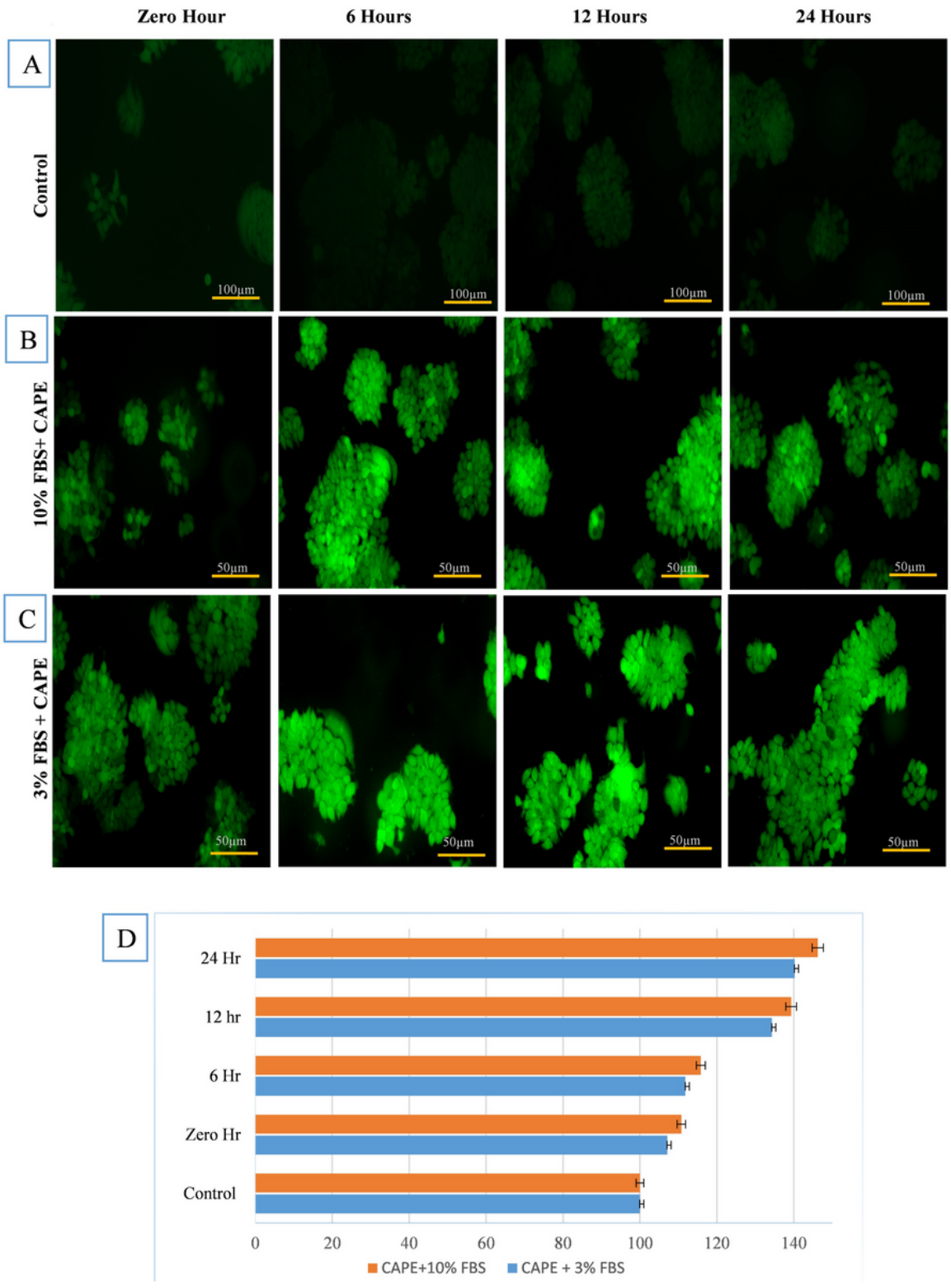

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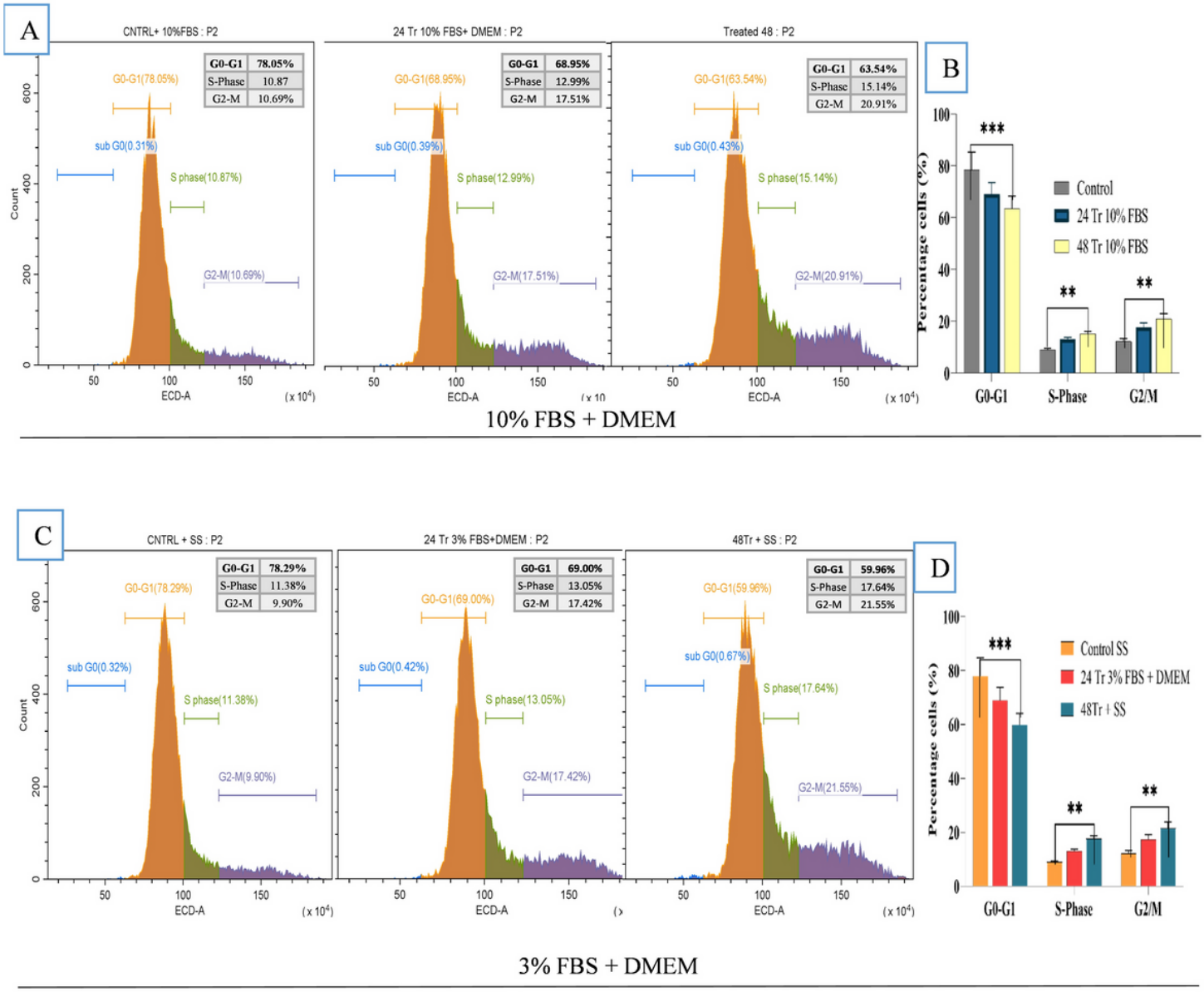

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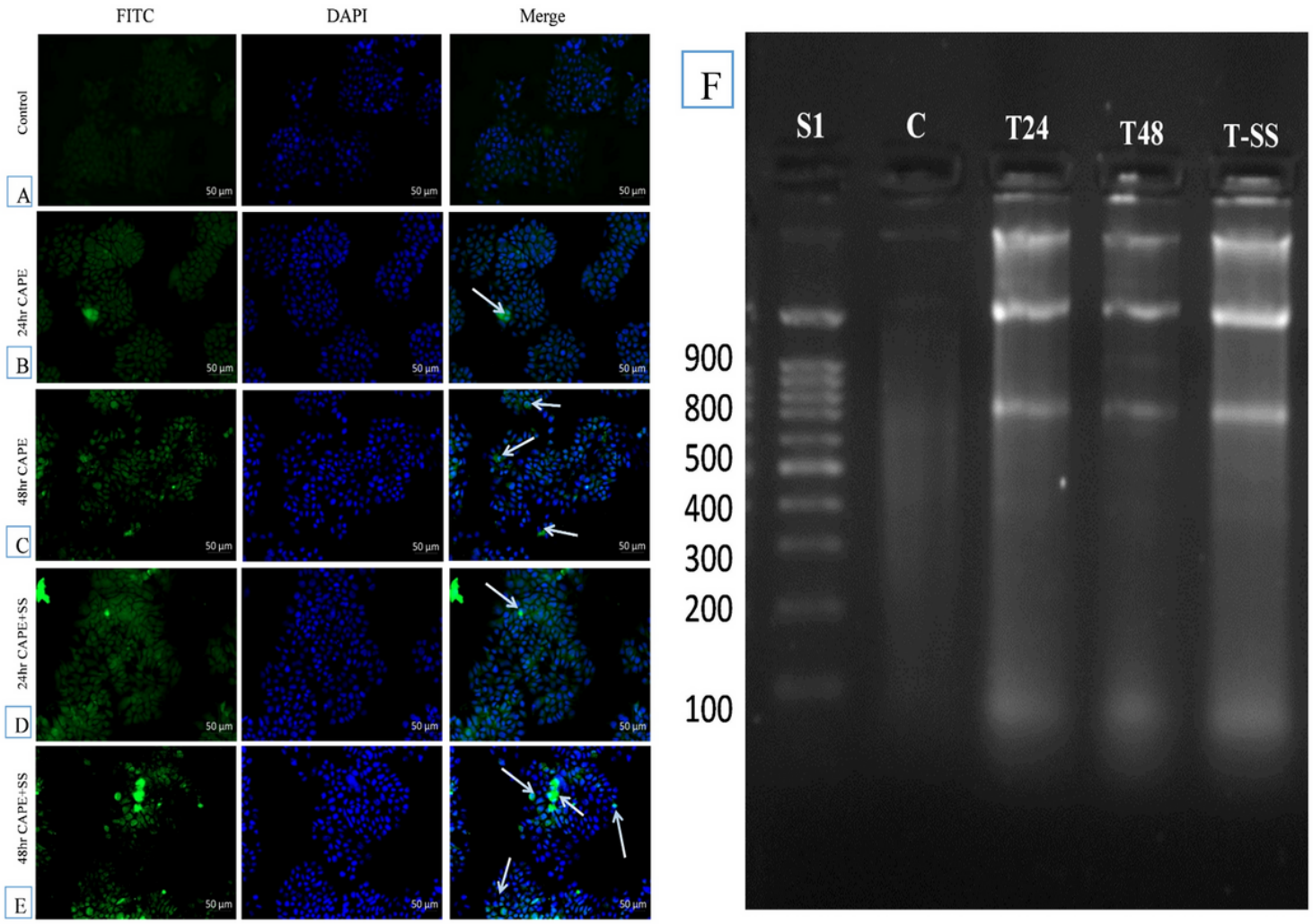

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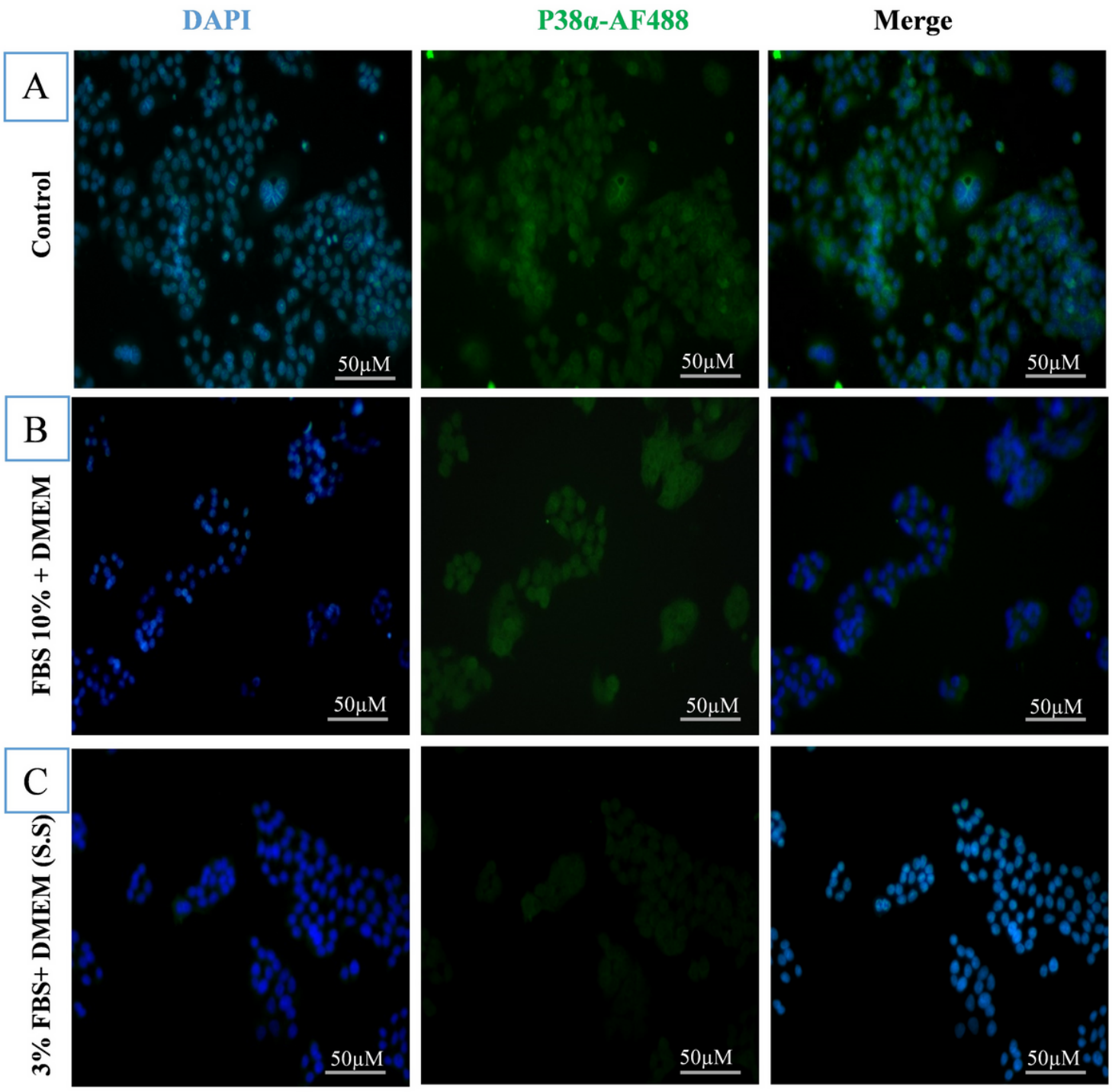

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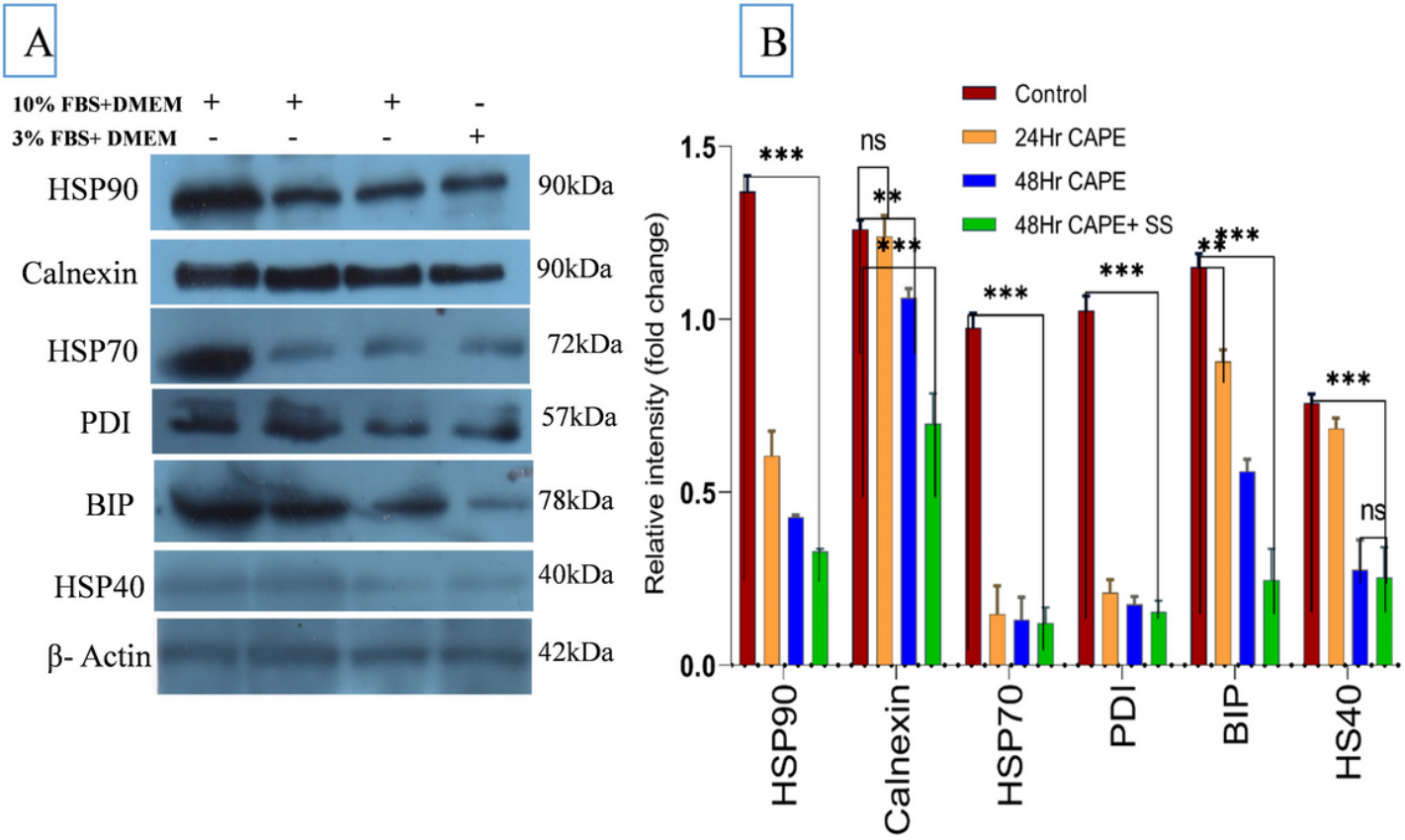

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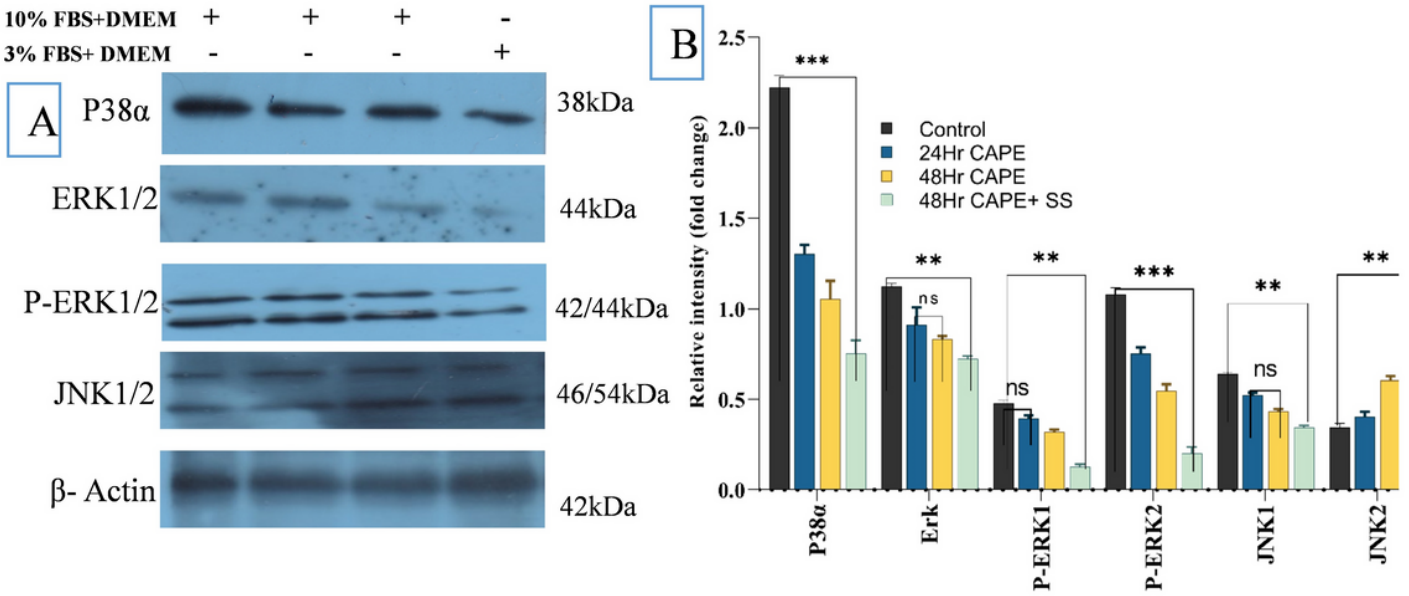

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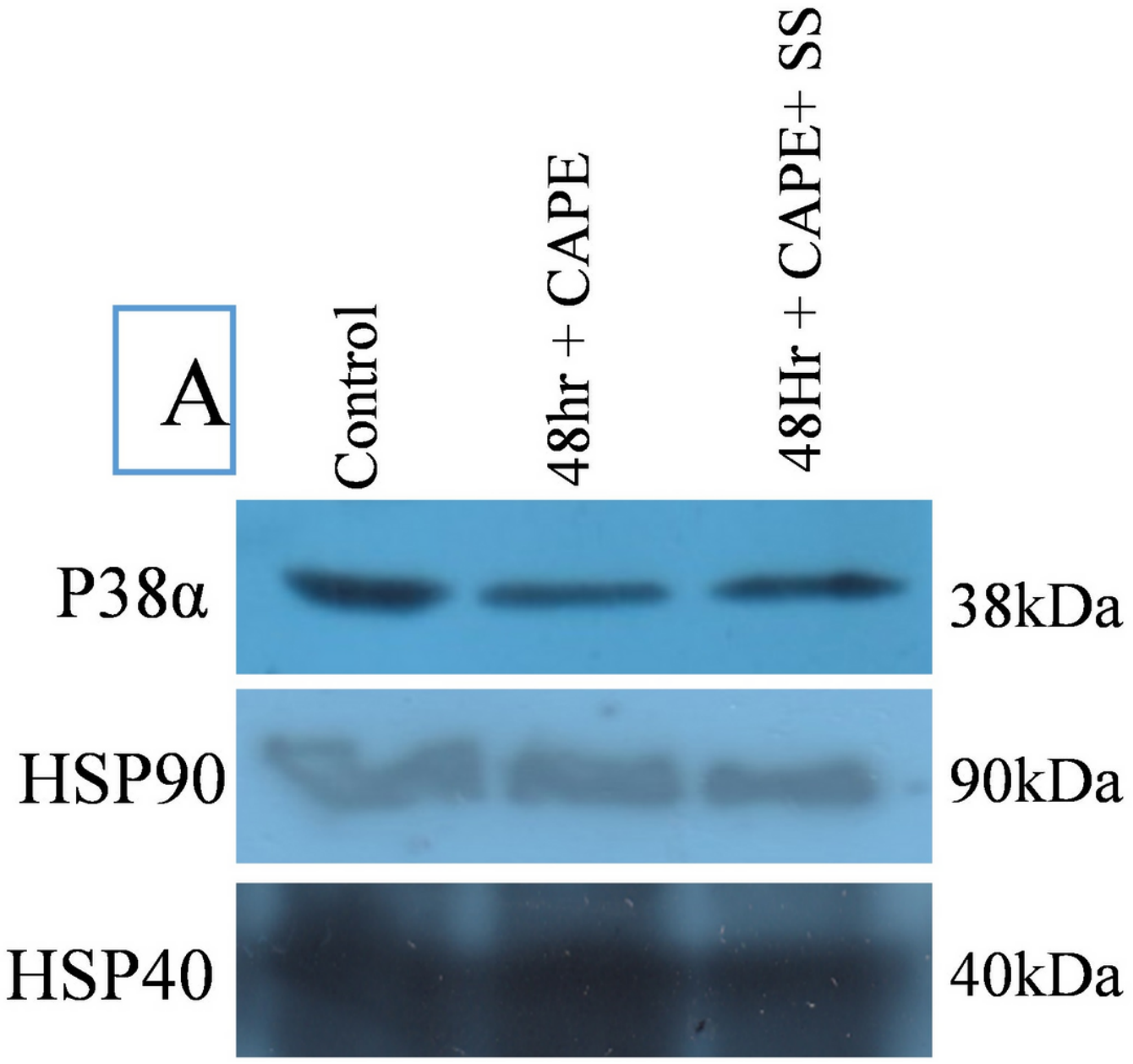

Figure 10

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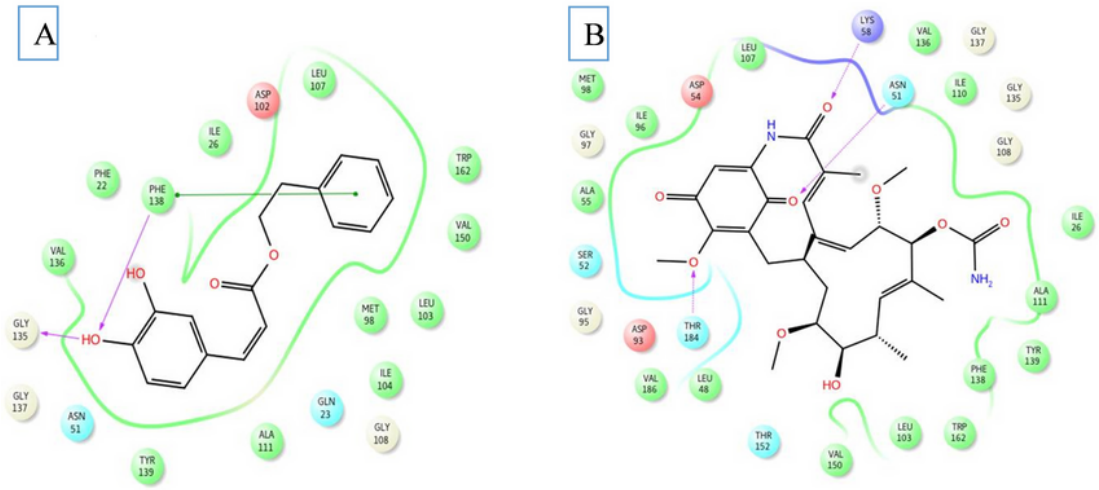
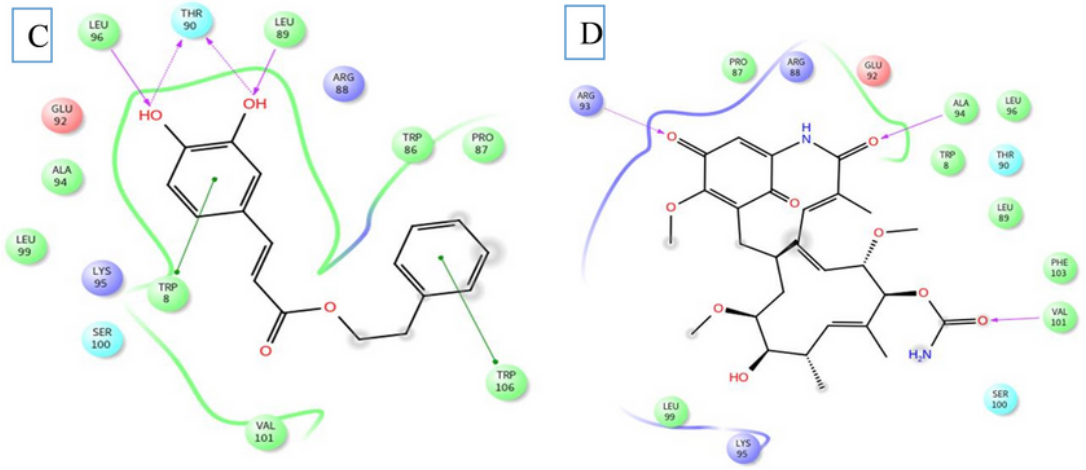


Drug	Docking score	Glide energy
Geldanamycin	-11.754	-73.002
Caffeic acid phenethyl ester	-12.549	-37.723



Drug	Docking score	Glide energy
Caffeic acid phenethyl ester	-9.59	-45.505
Geldanamycin	-6.235	-46.348

Figure 11

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Supplementary Files

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