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Original article

Methanolic extract of *Citrullus colocynthis* suppresses growth and proliferation of breast cancer cells through regulation of cell cycle

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ABSTRACT

Breast cancer is a major cause of cancer related deaths in women worldwide. Available treatments pose serious limitations such as systemic toxicity, metastasis, tumor recurrence, off-target effects, and drug resistance. In recent years, phytochemicals such as secondary metabolites due to their effective anti-cancer potential at very low concentration have gained attention. Aim of the study was to evaluate anti-cancer potential of *Citrullus colocynthis* and its possible molecular targets on MCF-7, a human breast cancer cell line. Methanolic extract of leaves was prepared and fractionated by solvents (n-hexane, chloroform, ethyl acetate and n-butanol) with increasing polarity. Bioassays and gene expression regulation was conducted to evaluate the anticancer activity, proliferation rate and cell cycle regulation of breast cancer cells treated with extract and its fractions, separately. Results showed a significant anticancer activity of methanolic extract of *C. colocynthis* and two of its fractions prepared with chloroform and ethyl acetate. Bioassays depicted significant decrease in proliferation and growth potential along with cell cycle arrest of treated cells compared to control untreated cells. Expression regulation of genes further confirmed the cell cycle arrest through significant upregulation of cyclin-CDK inhibitors (p21 and p27) and cell cycle checkpoint regulators (HUS1, RAD1, ATM) followed by downregulation of downstream cell cycle progression genes (Cyclin A, Cyclin E, CDK2). It is concluded that *C. colocynthis* arrests cell cycle in human breast cancer cells through expression regulation of cyclin-CDK inhibitors and with further research can be proposed for therapeutic interventions.

1. Introduction

Breast cancer is a heterogenous disease, cases taking aggressive growth while others having slow clinical course with excellent prognosis. Breast cancer is one of the frequently occurring and main cause of cancer related deaths in women worldwide with diagnosis rate of 1.7 million cases per year. It has been predicted that by 2050, this rate will increase to nearly 3.2 million cases per year. This huge magnitude of occurrence severely impacts people worldwide and demands potential new strategies for its prevention and cure (Tao et al., 2015). Availability of equal screening opportunities and effective treatments for all women besides socio economic problems can reduce the worldwide burden of breast cancer. However, previously used chemotherapeutic drugs are not only known to cause several side effects such as peripheral neuropathy, bone health, fatigue, anxiety, depression, cognitive impairment, cardiotoxicity and lymphedema but also face issues like drug resistance and tumor recurrence (Neil-Sztramko et al., 2019). Therefore, one of the main challenges in breast cancer research is to look for new potential, effective and less expensive therapeutic options to control morbidity and mortality rate. Medicinal plants are rich source of anticancer secondary metabolites. Research on phytochemicals is gaining attention as they are potent anticancer agent at a very low dose with minimal side effects.

Citrullus colocynthis is a well-known and traditional medicinal plant. It is commonly known with many names that are desert gourd, egusi, colocynth or bitter apple. It belongs to Cucurbitaceae family of plants which is mainly distributed in desert regions of

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Mediterranean Basin and Asia including Pakistan (Sultan et al., 2010). Due to medicinal and nutraceutical values of *C. colocynthis* its cultivation rate has been increased in many regions in the last decade. Its largest producers include China, India, Russia, USA, Egypt and Iran (Zaini et al., 2011). Many studies have been conducted on its pharmacology, toxicology and phytochemistry. In 2010, Australian New Crop website demonstrated that the research publications on this plant are getting high in number (Hussain et al., 2014). Crude extracts of its leaves, fruits and roots are rich in therapeutic phytochemicals. These include saponins, phenols, tannins, steroids, flavones, flavonoids, alkaloids, glycosides and terpenoids (Ahmed et al., 2019). This plant is also reported as a traditional cure for various respiratory, cardiovascular, neurological, musculoskeletal and gastrointestinal problems (Akhzari et al., 2015; Al-Snafi, 2016; Alhawiti, 2018; Chen et al., 2019; Dhakad, 2017; Genwa et al., 2017; Gill et al., 2011; Marzouk et al., 2012; Mehrzadi et al., 2016).

C. colocynthis is a potent free radical scavenger and acts as an antioxidant. Due to this property it can cope free radical damage and treat oxidative stress-related diseases (Limem et al., 2016). Its antioxidant potential is important as ROS contributes to cancer, inflammation, tissue injury and other diseases. Different parts *C. colocynthis* such as fruit, stem, roots and leaves has shown anticancer potential against many cancers in numerous studies. Its alkaloid rich fruit extract has shown promising anticancer activity in breast and liver cancer cells (Mukherjee and Patil, 2012). Its whole extract has also shown effective anticancer effect in larynx cancer cells (Tavakkol Afshari et al., 2005). A study also reported inhibition of proliferation and metastasis potential of breast cancer cells treated with *C. colocynthis* fruit extract (Chowdhury et al., 2017). However, till to date no study is documented to evaluate anticancer potential and cell cycle regulation of methanolic extract of *C. colocynthis* leaves and its fractions in breast cancer cells. The present study was conducted to evaluate anticancer potential of methanolic extract of *C. colocynthis* leaves and its fractions on MCF-7 breast cancer cell line. Furthermore, its effect on cell cycle regulation was also investigated through bioassays and gene expression analysis.

2. Materials and methods

2.1. Cell culture

MCF-7, a human breast cancer cell line was obtained from cell culture bank of School of Biological Sciences, University of the Punjab, Pakistan. The Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) media supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) was used to culture the cells in humidified incubator at 37°C with 5% CO_2. Standard trypsinization procedure was used for harvesting cells using Trypsin-EDTA.

2.2. Preparation of plant extract

C. colocynthis used in the study was collected in September 2018 from Dera Ghazi Khan located in northern parts of Punjab Province, Pakistan. The leaves were washed and dried with liquid nitrogen and crushed with pestle and mortar. Fine powder was obtained by grinding in electric grinder. Methanolic extract of the leaves powder was prepared by dissolving 100 g of powder in 400 ml of absolute methanol and kept for 72 h on an orbital shaker at 37○C. The plant materials were settled down and the supernatant containing dissolved plant chemicals was separated and filtered. Supernatant was further resuspended in 300 ml methanol and kept for 72 h on the orbital shaker at 37°C. Supernatant was again separated, filtered and dissolved in 200 ml methanol for 72 h on orbital shaker at 37°C. Finally, separated and filtered supernatant was allowed for rotary evaporation of methanol and crude methanolic extract was obtained.

2.3. Preparation of fractions

The methanolic extract (CC-Met) was further fractionated by solvents with increasing polarity to prepare n-Hexane (CC-Hex), Chloroform (CC-Chl), Ethyl Acetate (CC-EA) and n-Butanol (CC-But) fractions by solvent-solvent extraction method. Stock solutions 100 mg/ml of the methanolic extract and all fractions were prepared in sterile DMSO and were syringe filtered by 0.22 μ filter. All the stocks were stored at −20○C. Working solutions of 1 mg/ml were prepared in DMEM and used for further dilutions used in experiments.

2.4. MTT cell viability assay

MTT Assay was performed to determine the LC_{50} value of the methanolic extract of *C. colocynthis* and its fractions. In a 96 well plate, 10,000 cells/well were seeded in 200 μl media. Cells were grown till 70% confluency was achieved. The media was removed, and each well was washed with 200 μl 1X PBS. 200 μl media containing different concentrations of plant extract were added in triplicates. Similarly, control wells were also incubated with equal volume of the culture media in triplicate. The plate was incubated for 72 h. After 72 h, media was removed and DMEM containing 10% MTT reagent (5 mg/ml) was added in each well and incubated for 4 h. After 4 h, Formazan crystals were dissolved in 150ul DMSO. The suspension was transferred to an ELISA reader plate and the absorbance was taken at 570 nm. The percentage viability was calculated by using formula:

$$\text{Percentage Viability} = \frac{\text{Absobance of experimental well}}{\text{Absorbance of Control well}} \times 100$$

Table 1
List of primers.

Gene name	Primer Sequence (5′-3′)
CD24	Forward GTCCAGAAAGGAGAATACAG
	Reverse GAAATGGTGCTGGAGATAA
CD29	Forward CAGAGGCTCCAAAGATATAA
	Reverse GAGTAAGACAGGTCCATAAG
CD44	Forward GTCCAGAAAGGAGAATACAG
	Reverse GAAATGGTGCTGGAGATAA
CD133	Forward CCCTGTTCTTGATGAGATTA
	Reverse CAGTTCAGGGTTGCTATT
p53	Forward GTTCCGAGAGCTGAATGAGG
	Reverse TTATGGCGGGAGGTAGACTG
p27	Forward GCCCTCCCCAGTCTCTCTTA
	Reverse TCAAAACTCCCAAGCACCTC
p21	Forward GGAAGACCATGTGGACCTGT
	Reverse GGCGTTTGGAGTGGTAGAAA
Ki67	Forward CCTGACAGTGGAAAACCTCT
	Reverse CACAATTTCCTCTGGTGCTG
CDK2	Forward ACCAGCTCTTCCGGATCTTT
	Reverse TAGGGTCGTAGTGCAGCATT
Cyclin A	Forward GATGCTGACCCATACCTCAA
	Reverse GGTTGAGGAGAGAAACACCA
Cyclin E1	Forward CAGAGACAGCTTGGATTTGC
	Reverse ACTGTCTTTGGTGGAGAAGG
HUS1	Forward GACGCTTTTCTGTTACCCAC
	Reverse CCAGCTTGTCACAAAGGATG
RAD1	Forward CGGACAGATAACAGAGGCTT
	Reverse TTCCCCATTGTGCTTCTTCT
ATM	Forward AGAAACTCTTGTCCGGTGTT
	Reverse ACGTGTACATAGCTGCATCA

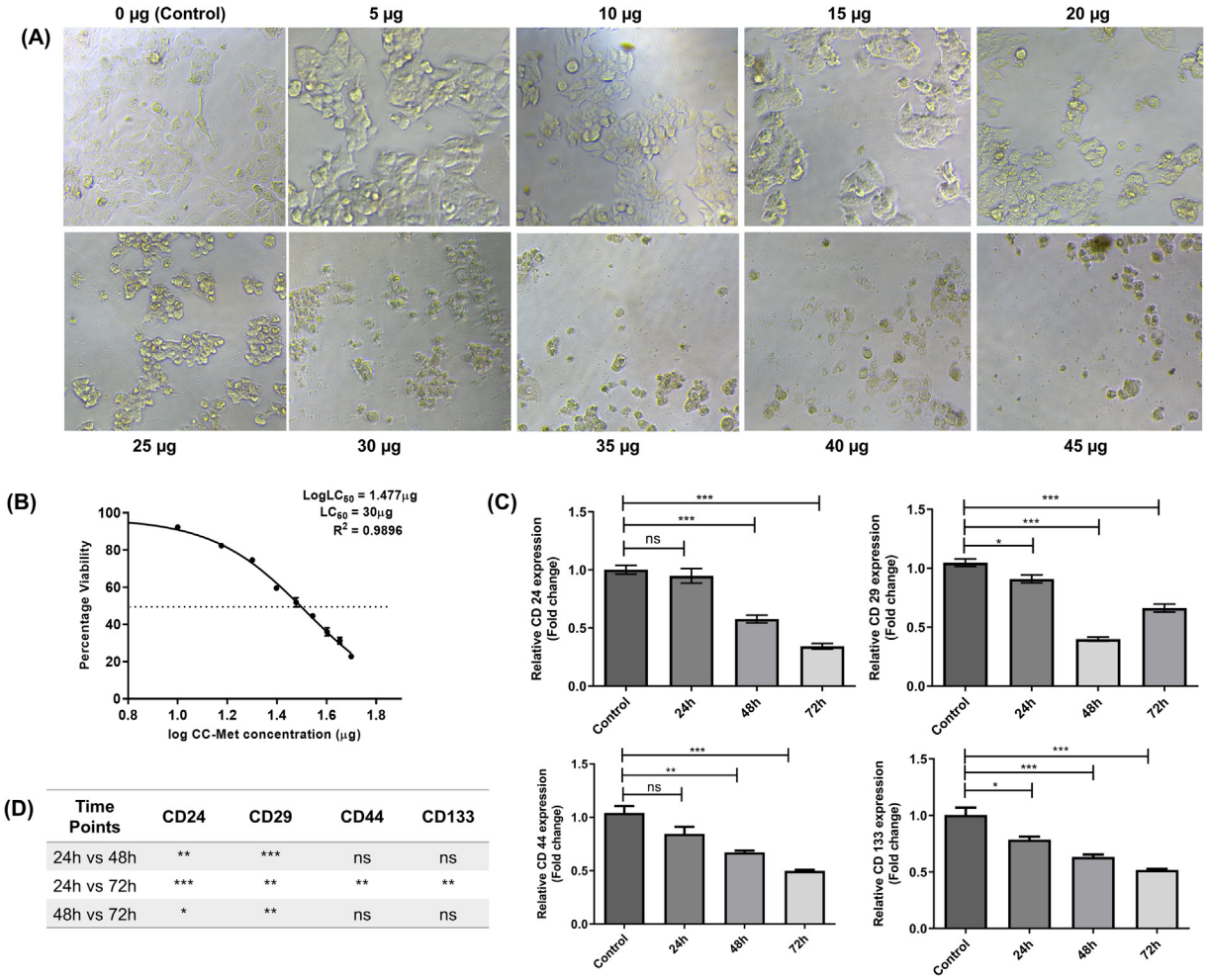

Fig. 1. (A) CC-Met exhibit cytotoxic effect on MCF-7 in dose dependent manner. (B) MTT assay was done to measure percentage viability of cells after treatment with various concentration of CC-Met for 72 h. (C) Expression analysis of CSCs genes in CC-Met treated cells show significant decrease in time dependent manner. (D) Statistical analysis of CSC genes expression between different time points. (*P < 0.05, **P < 0.01, ***P < 0.001).

2.5. Colony formation assay

In order to access the growth inhibition potential of *C. colocynthis* methanolic extract and the fractions, colony formation assay was performed. 1000 cells/well were seeded in a six well cell culture plate in 2 ml media containing LC_{50} doses of methanolic extract and of each fraction separately. Each experimental and the control well was run in duplicates. Both treated and control cultures were incubated at a humidified atmosphere of 5% CO_2 at 37 °C for one week. After one-week incubation, media was removed, washed with 1X PBS followed by colony fixation with methanol and acetic acid in 3:1 ratio. Fixed colonies were further stained with 0.5% crystal violet dissolved in methanol for 20 min. Stain was removed and washed with plenty of water. The colonies were observed and photographed under the bright field of Olympus fluorescence microscope.

2.6. Spheroid formation assay

Spheroid formation assay was done to analyze the effect of *C. colocynthis* methanolic extract and its fractions on 3D culture potential of breast cancer cells. Briefly, 1.5% agarose was prepared in 1X PBS and mixed with equal volume of DMEM culture media. 1 ml of agarose with DMEM mixture was uniformly spread in a six well cell culture plate and allowed to solidify. 10,000 cells/well were seeded in each well in media with LC_{50} concentration of methanolic extract and the fractions separately. The plate was further incubated in a humidified chamber with 5% CO_2 and 37 °C for 10 days. Experiment was carried out in duplicates. By the end of experimental period, morphology of spheroids was observed, their size was measured using cellSens (Ver.1.18) and were photographed under the bright field of Olympus fluorescence microscope.

2.7. Gene expression analysis

Breast cancer cells treated with LC_{50} concentration of the methanolic extract for the time interval of 24, 48 and 72 h separately were collected for RNA isolation. Cells were also treated with LC_{50} concentration of each fraction for 72 h in independent experiments and allowed to isolate RNA. Cells treated with normal culture media were kept as control. RNA was isolated from these cells by standard trizol method. RNA quantity and purity were checked by DeNovix DS-11 FX + spectrophotometer. cDNA was prepared using 2 µg RNA by Thermo Scientific™ RevertAid First Strand cDNA Synthesis Kit (Catalog No. K1622). For RT-PCR, Thermo Fisher Syber Green master mix was used. RT-PCR was done in PikoReal qPCR (Thermo Scientific) system in triplicates with cancer stem cells markers (CD-24, CD-29, CD-44, CD-133), proliferation marker Ki67, cyclin/CDK inhibitors (p21 and p27), tumor suppressor gene

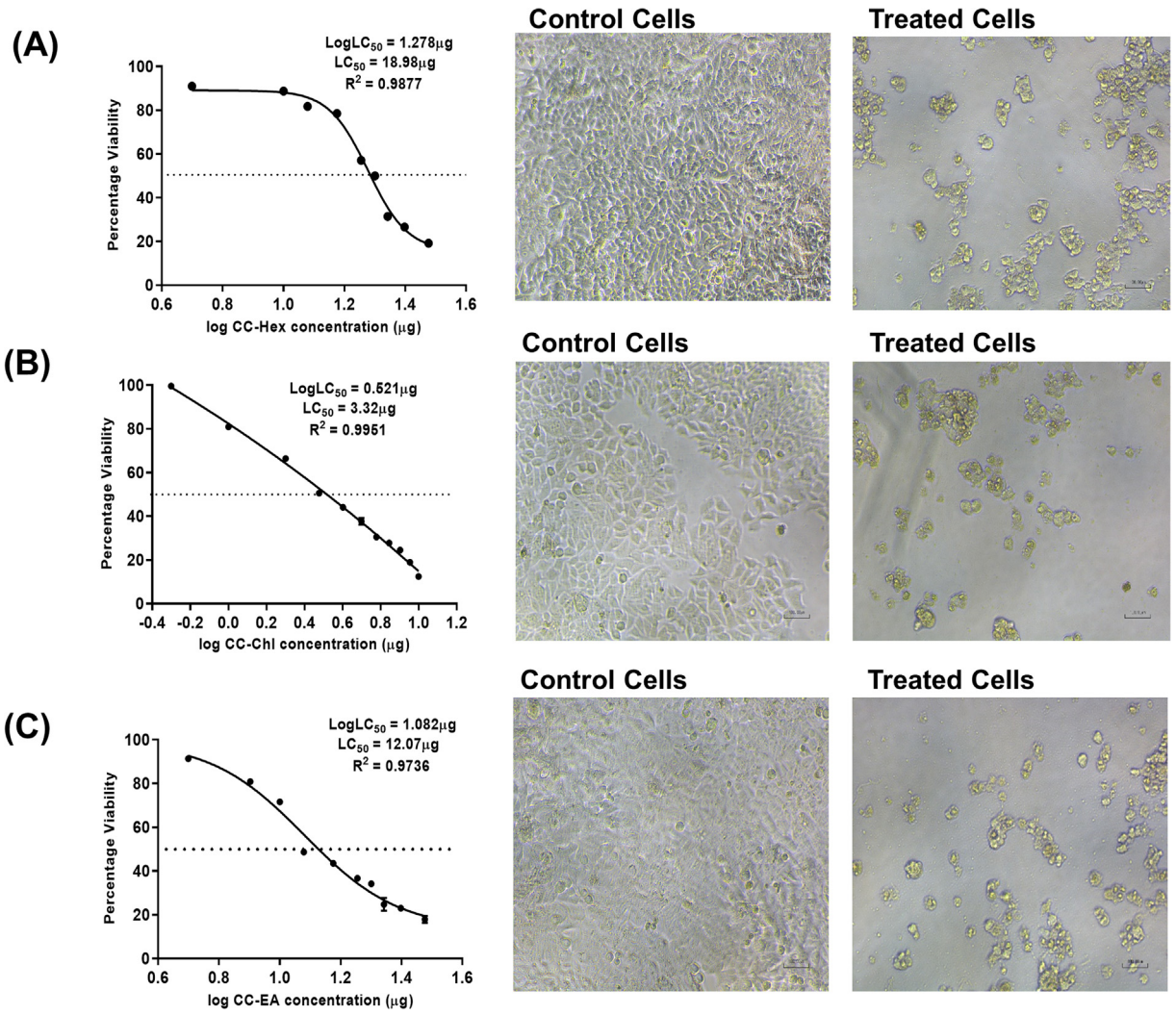

Fig. 2. (A) After 72 h percentage viability of MCF-7 cells treated with various concentrations of CC-Hex, control cells and treated cells at its LC_{50} dose 20 µg. (B) After 72 h percentage viability of MCF-7 cells treated with various concentrations of CC-Chl, control cells and treated cells at its LC_{50} dose 03 µg. (C) After 72 h percentage viability of MCF-7 cells treated with various concentrations of CC-EA, control cells and treated cells at its LC_{50} dose 12 µg.

(p53), cell cycle progression genes (Cyclin A, Cyclin E, CDK2), and cell cycle checkpoint regulators (HUS1, RAD1, ATM). Housekeeping gene GAPDH was used to normalize the expression level of target genes. Primers used are provided in Table 1.

2.8. Statistical analysis

The data was statistically analyzed by GraphPad Prism 5. To calculate LC_{50} dose, log dose-response curves were assessed using nonlinear regression with variable slope. To analyze groups data, independent *t*-test and one-way ANOVA with Tukey's as post hoc test was used. $P < 0.05$, $P < 0.01$& $P < 0.001$ was held as significant. The results were shown in mean ± standard error of mean (SEM).

3. Results

3.1. CC-Met and its fractions inhibit the growth of breast cancer cells

Methanolic extract of *C. colocynthis* leaves (CC-Met) exhibited significant anticancer activity in a dose dependent manner. Microscopic examination revealed that the cell morphology was changed from normal elongated form to round with the increase in concentration of extract. MTT assay further showed that with an increase in plant extract dose from 0 µg to 50 µg, the cell viability was decreased from 100% to 23% (Fig. 1a). The LC_{50} calculated from MTT assay was 30 µg ± 4.15 (Fig. 1b). Expression analysis by RT-PCR elucidated that the expression of CSC genes; CD-24, CD-29, CD-44 and CD-133 were significantly downregulated in treated cells relative to the control. Significant difference in the expression of these genes in 24, 48 and 72 h treated cells was confirmed by statistical analysis relative to the control (Fig. 1c). Statistical analysis of between different time points revealed that CSC genes expression is also regulated in time dependent manner (Fig. 1d).

Fractions of CC-Met also possess potent anticancer activity. Morphological analysis and MTT cell viability assay exhibited reduced percentage in cell viability in treated cells with CC-Met fractions in a dose dependent manner. Here, CC-Hex gave LC_{50} at 20 µg ± 0.74, CC-Chl at 03 µg ± 1.14 and CC-EA at 12 µg ± 0.46 (Fig. 2). CC-But fraction did not show any significant effect on the viability of cells in the range of 5.0 µg-30 µg. Therefore, it was excluded from further experiments.

3.2. Reduce proliferation rate and growth potential of breast cancer cells

Colony formation assay revealed that CC-Met and its fractions inhibit colony formation potential of MCF-7 cells. Significant

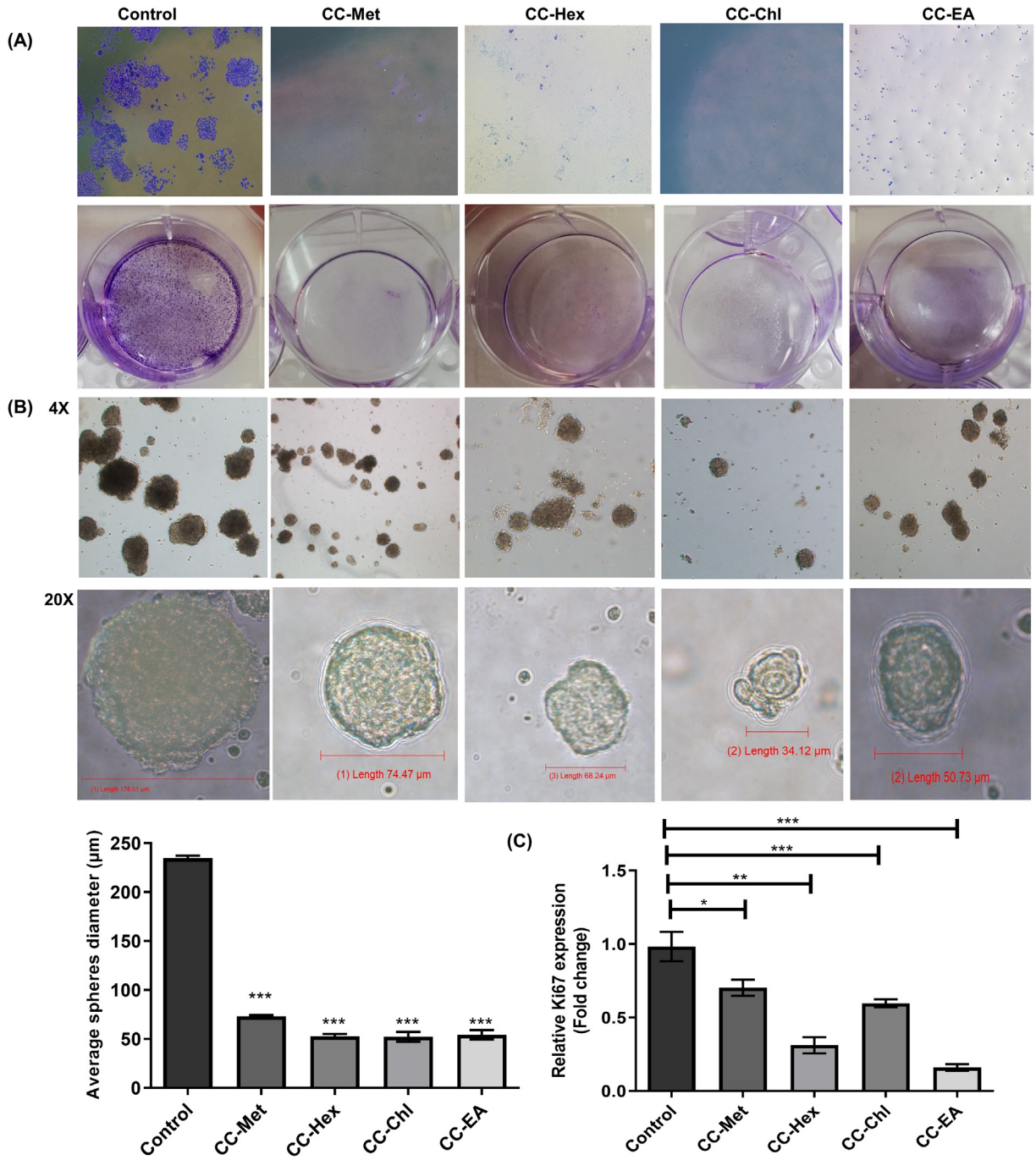

Fig. 3. (A) Effect of CC-Met and its fraction on clonogenic potential of MCF-7 cells. (B) Effect of CC-Met and its fraction on 3D growth potential of cells as compare to control. (C) mRNA level of Ki67. (*P < 0.05, **P < 0.01, ***P < 0.001).

decrease in survival and proliferation of the cells was observed relative to the control. In all the experimental groups cells were unable to grow and exhibit clonogenicity whereas in control group cells were proliferated and formed healthy colonies (Fig. 3a). 3D growth potential of MCF-7 breast cancer cells was significantly reduced when treated with CC-Met and its fractions relative to the control. CC-Chl reduced maximum spheroid formation potential of the cells as compare to other groups (Fig. 3b). Downregulated expression of proliferation marker Ki67 was observed in all the groups relative to the control (Fig. 3c).

3.3. Upregulation of cyclin/CDK inhibitors and checkpoint regulators gene expression

Cyclin/CDK inhibitors p21 and p27 belongs to Cip/Kip family of inhibitors. They bind to CDK-cyclin complexes and inhibit their activity. In expression analysis of cyclin/CDK inhibitors mRNA levels, p21 and p27 expression was upregulated in all the groups relative to the control with maximum upregulation in CC-EA group (Fig. 4a). p53 is a tumor suppressor gene. It controls p21 expression through transcription regulation. Its binding site is present on p21

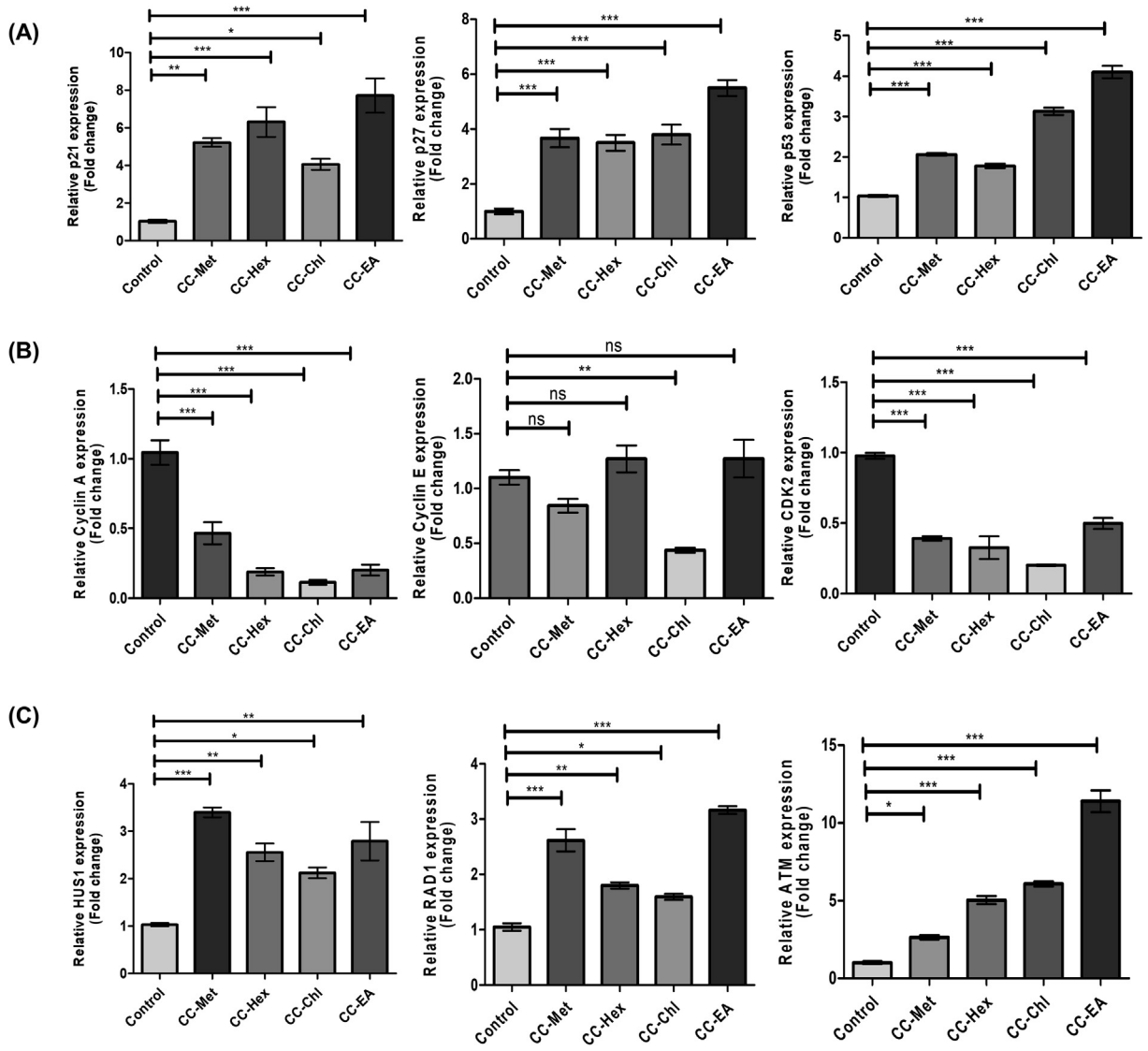

Fig. 4. Effect of CC-Met and its fractions on relative levels of mRNA. (A) Cyclin-CDK inhibitors p21 and p27 and tumor suppression gene p53. (B) Cell cycle progression genes cyclin A, cyclin E, CDK2. (C) Cell cycle checkpoint regulators HUS1, RAD1 and ATM. (*P < 0.05, **P < 0.01, ***P < 0.001).

gene promoter. Relative mRNA expression level of p53 was also upregulated in all the groups (Fig. 4a).

Expression of downstream targets of cyclin/CDK inhibitors was also evaluated. These targets include cell cycle progression genes. During cell cycle, a cyclin binds and activate its specific CDKs to progress the cell cycle. Cyclin A, cyclin E and CDK2 expression was downregulated in all the groups with maximum downregulation in CC-Chl group. Change in relative expression of Cyclin E was non-significant in CC-Met, CC-Hex and CC-EA group indicating that its expression is not affected in these groups (Fig. 4b).

Cell cycle checkpoint regulators, HUS1, RAD1 and ATM gene expression is increased in DNA damaged cells. HUS1 and RAD1 encircle damaged DNA to activate downstream cell cycle checkpoint pathway. ATM gene encodes ATM kinase which phosphorylate p53 protein and activate it (Maclaine and Hupp, 2009). HUS1, RAD1 and ATM relative mRNA level was increased in all the groups. Maximum HUS1 expression was in CC-Met group whereas maximum RAD1 and ATM expression was observed in CC-EA group (Fig. 4c).

4. Discussion

Breast cancer is the most prevalent and commonly diagnosed cancer with maximum cancer related death rate in women each year across the globe (Siegel et al., 2019). Medicinal plants are known as rich source of secondary metabolites and their potential to treat cancer is getting immense attention (Bernstein et al., 2018). The aim of the present research was to explore the anti-cancer potential of *Citrullus colocynthis* leaves that are traditionally used for their medicinal attributes and to study cell cycle regulation in MCF-7 breast cancer cells.

CC-Met was prepared and further fractionated by solvents with increasing polarity. No previous study has reported such fractions of CC-Met. In the present study, methanolic extract of *C. colocynthis* leaves and its fractions showed significant anticancer potential against MCF-7 breast cancer cells in a dose dependent manner. Characterization of cancer stem cell markers in CC-Met treated cells showed significant downregulation indicating that this extract is very potent in reducing stemness. In 2017, a study on *C. colocynthis* ethanol and acetone fruit extract treated MCF-7 cells

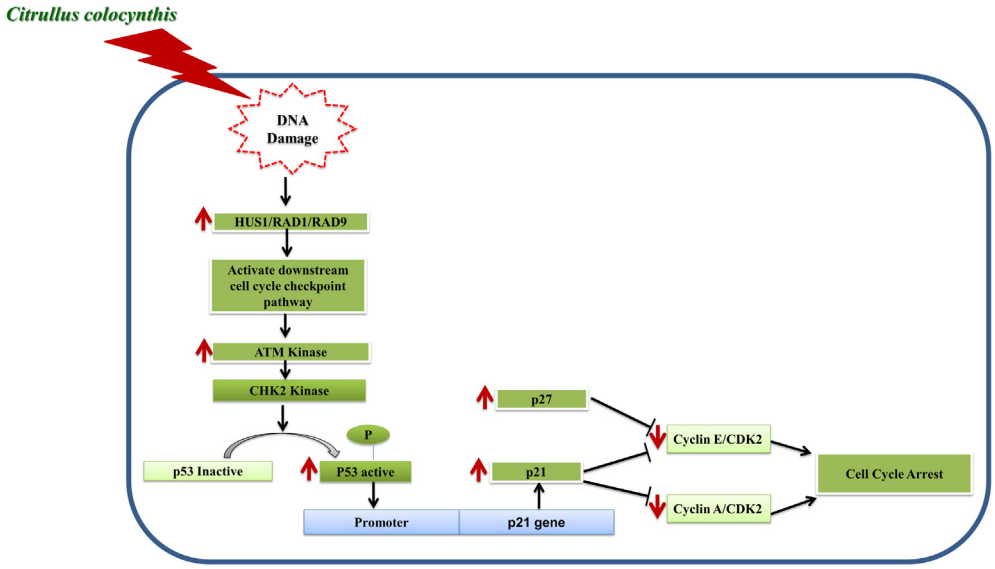

Fig. 5. Proposed molecular mechanism of cell cycle arrest induced by *Citrullus colocynthis*. Red arrows indicate expression regulation of mRNA transcripts.

for 24 h reported its LC_{50} 105 ± 8.3 and 94 ± 6.4 respectively (Chowdhury et al., 2017). Furthermore, cell viability assay of fractions revealed very low LC_{50} dose as compare to CC-Met. Very low LC_{50} dose of CC-Chl as compare to others show that very potent anticancer agent is present in this fraction.

Cell cycle regulation was studied at LC_{50} dose of methanolic extract and its fractions in MCF-7 treated cells. In cancer patients, metastasis is one of the major threats. Our results of colony formation assay and spheroid formation assay depicted notable decrease in clonogenicity, proliferation, growth potential of MCF-7 cells. On molecular level downregulated expression of proliferation marker Ki67 exhibited that the growth and proliferation potential of breast cancer cells was significantly reduced. Ki67 is a nuclear antigen involves in rRNA transcription. In 2019, a study in breast cancer patients treated with neoadjuvant endocrine therapeutic approaches reported significance downregulation in Ki67 expression (Gianni et al., 2019).

Gene expression of cyclin-CDK inhibitors (p21 and p27) and tumor suppressor gene p53 was upregulated whereas expression of downstream cell cycle progression genes (cyclin A, cyclin E and CDK2) was significantly downregulated. p53 is transcriptional regulator of p21 gene expression as it binds to p21 gene promotor region and initiate its transcription. Cyclin-CDK inhibitors (p21 and p27) binds with Cyclin E-CDK2 and cyclin A-CDK2 complex and inhibit their activity. During cell cycle, cyclin/CDK complex through phosphorylation activates number of proteins essential for cell cycle progression. Cyclin E-CDK2 complex progresses the cell cycle from G_1 to S phase. In S-phase cyclin E is replaced by cyclin A and cyclin A-CDK2 complex is formed which initiate and complete DNA replication (Vermeulen et al., 2003). Cyclin E-CDK2 and cyclin A-CDK2 both regulates S-phase (Kamenz and Ferrell, 2017). Increase expression of cyclin-CDK inhibitors and decrease expression of their downstream targets that are cell cycle progression genes suggest that cell cycle is arrested in treated breast cancer cells. In 2009 a study on prostate cancer reported that it can be treated by downregulation of cell cycle progression genes involve in G_1, S and G_2 phase and upregulation of genes involve in cell-cycle arrest (Padua and Hansen, 2009). It was reported in a study in 2014, that the expression of cell cycle progression genes cyclin A, cyclin B1 and CDK1 is downregulated in colon cancer cells treated with phytochemical cucurbitacin-I isolated from Cucurbitaceae (Kim et al., 2014). A study in 2016, reported upregulation of p21 and p53 in melanoma cells treated with tetrahydro anthraquinone derivative (Genov et al., 2016). A study in 2017, on MCF-7 cells treated with combination of evodiamine and berberine reported decrease expression of cell cycle progression proteins (cyclin D1, cyclin E, CDK4, and CDK6) and increase expression of cyclin-CDK inhibitors (p21, p27, 53) resulting in cell cycle arrest (Du et al., 2017). Similarly another study in 2017 reported upregulation of cyclin-CDK inhibitors in resveratrol with docetaxel treated pancreatic cancer cells (Singh et al., 2017).

Cell cycle checkpoint genes (HUS1, RAD1, ATM) expression was significantly upregulated. During cell cycle, HUS1 interacts with RAD1 and RAD9 to form a trimeric complex which recognize DNA damage and encircle damaged DNA. As a result, ATM/CHK2/p53 pathway is activated to inhibit cell cycle (Fig. 5). This pathway phosphorylates p53 and activates it which further binds to promoter region of p21 and initiate its transcription thus arresting cell cycle (Bermudez et al., 2003). This suggest that CC-Met and its fractions has promoted DNA damage in breast cancer cells. A study in 2015, suggested that cell cycle checkpoints can be targeted to kill cancer cells (Benada and Macurek, 2015).

In conclusion, current research highlights the anticancer potential of C. *colocynthis* leaves extract though inhibition of growth and stemness of breast cancer cells. Growth and proliferation assays data together with expression regulation of various cell cycle genes further suggested that anticancer activity of C. *colocynthis* is through cell cycle arrest. However, additional comprehensive studies are required to identify, isolate and purify the most active compound involved in anticancer and cell cycle arrest potential of C. *colocynthis*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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