Journal of Biologically Active Products from Nature

ISSN: 2231-1866 (Print) 2231-1874 (Online) Journal homepage: https://www.tandfonline.com/loi/tbap20

Cell Cycle Arrest and Induction of Apoptosis in Human Breast Cancer Cells (T-47D) by *Annona squamosa* L. and *Thymus vulgaris* L. Ethanolic Extract

Ibrahim M.H. Al-seragy, Kiran R. Kharat & Arvind S. Dhabe

To cite this article: Ibrahim M.H. Al-seragy, Kiran R. Kharat & Arvind S. Dhabe (2019) Cell Cycle Arrest and Induction of Apoptosis in Human Breast Cancer Cells (T-47D) by *Annona squamosa* L. and *Thymus vulgaris* L. Ethanolic Extract, Journal of Biologically Active Products from Nature, 9:1, 47-56, DOI: 10.1080/22311866.2018.1554452

To link to this article: https://doi.org/10.1080/22311866.2018.1554452

Published online: 11 Mar 2019.

Taylor & Francis
Taylor & Francis Group

ISSN Print: 2231-1866
ISSN Online: 2231-1874

Cell Cycle Arrest and Induction of Apoptosis in Human Breast Cancer Cells (T-47D) by *Annona squamosa* L. and *Thymus vulgaris* L. Ethanolic Extract

Ibrahim M.H. Al-seragy [1], Kiran R. Kharat [2]*, Arvind S. Dhabe [1]*

[1] Department of Botany, Dr. Babasaheb Ambedkar
Marathwada University, Aurangabad 431005, India
[2] Department of Biotechnology, Deogiri College, Aurangabad 431001, India

Received 13 February 2018; accepted in revised form 23 November 2018

Abstract: *Annona squamosa* L. is belongs to the *Annonaceae* family. Similarly, *Thymus vulgaris* L. is a flowering plant in the mint family *Lamiaceae*. In this study, *Annona squamosa L. seeds ethanolic extract (ASEE)* and *Thymus vulgaris ethanolic extract (TVEE)* were prepared and used against the breast cancer cells. The Breast cancer cells (T-47D) death was analysed by Vybrant® MTT Cell Proliferation Assay Kit. The IC50 of the ASEE was found to be 8.72 ± 0.52 µg/ml. The IC50 of the TVEE was found 7.30 ± 0.6 µg/ml. Membrane marker proteins of apoptotic cells were analysed by Attune flow cytometer. The cell cycle assay was quantified in ASEE and TVEE treated and untreated cells. The ASEE or TVEE treated T-47D cells were found green in color due to the binding of annexin v-FITC to phosphatidylserine (PS) on the membranes of apoptotic cells. This was supported by the upregulated p53 and 14-03-03 sigma expression in cells treated with the ASEE or TVEE. This indicates the inhibition of the sequestring CDK1 in the cytoplasm and inhibition of cell cycle at G2/M phase. The *Annona squamosa* L. ethanolic extract (ASEE) and *Thymus vulgaris L. ethanolic extract (TVEE)* were found effective against Breast cancer cells (T-47D).

Key words: *Annona squamosa* L., *Thymus vulgaris* L., T47D cells, apoptosis, cell cycle.

Introduction

In ancient and modern medicine practices, plants and plant derived compounds have been used as medicine in all the region on earth [1]. In modern medicine practices, few current cancer-chemo drugs were of plants origin [2-4]. Our hypothesis in present study is to use selected whole plant extracts according to ethno botanical sources of historical use might contain multiple molecules with antitumor activities that could be very effective in killing human cancer cells.

Annona squamosa L. (Magnoliales: *Annonaceae*) is a tropical plant with fruits. Previously, many papers reported its medicinally active compounds [5-9]. *A. squamosa* have been used in traditional medicine in Asia and Africa for the treatment of high fever, bacterial infections, diabetes with hypertension and many types of cancerous diseases. Major medicinal compounds like alkaloids, phenols and acetogenins compounds have been identified and isolated from this plant. In previous papers, by using *in vitro* studies, extracts and phytochemicals of *A. squamosa* were used as anticancer [5-9].

The second herb *Thymus vulgaris* L., *Lamiaceae* family mainly reported for various valuable biochemicals like proteins, oils and fats and vitamins like A, B and vitamin C. Thymol is the main oil product from this plant. *T. vulgaris L.* extracts were reported for the treatment of antitussive antimicrobial, asthma and bronchitis and antifungal, antioxidative, and antiviral [10-14]. The

*Corresponding authors (Kiran R Kharat; Arvind S. Dhabe)
E-mail: < krkharat@gmail.com; arvindsdhabe@gmail.com >

previously reported Annonaceous acetogenins are found to be cytotoxic, cell growth inhibitory in nature [15]. The main Squamocin found in seeds of *A. reticulata is* active against many cancer cell lines. The phytochemical and pharmacological activities of *Annona squamosa* L. components suggest a wide range of clinical application in lieu of cancer chemotherapy [16-19].

In this study, we tried to investigate how *Annona squamosa* L. and *Thymus vulgaris* L. extract affects cell cycle and induces apoptosis in Breast cancer cells (T-47D). The anticancer mechanism of compounds was also elucidated by analysis of phosphatidylserine by flow cytometry and fluorescence microscopy and expression of cell cycle markers such as TP53 and 14-3-3 sigma in cancer cells.

Materials and methods

Collection of plants and preparation of Annona squamosa L. extract

Seeds of *Annona squamosa* L. were collected from the Marathwada region of the Maharashtra State (India). The specimen (Voucher no. 2) were identified by Professor Arvind S. Dhabe, (Head, Department of Botany) and deposited at (BAMU) herbarium Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Shade dried seeds (60 g dry weight) were crushed separately into fine powder and used for the preparation of the extracts (5 g yield). The ethanolic extract was prepared by using the powder of the seeds macerated in dark color bottle with 300 ml absolute alcohol at room temperature and were shaken several time for one month. After one month the extract was concentrated to give a deep cream suspension. The crude extract was suspended in water and partitioned with n-hexane, ethyl acetate, and n-butanol. The ethanolic extract (ASEE) were dissolved in 10 % w/v DMSO for further experimentation.

Collection of plants and preparation of Thymus vulgaris L. extract

Leaves of *Thymus vulgaris* L. were collected from Sana'a city, Yemen. The specimen (Voucher no. 3) were identified by Professor Arvind S. Dhabe and deposited at (BAMU) herbarium Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Shade dried leaves (60 g dry weight) were crushed separately into fine powder and used for the preparation of the extracts (8 g yield). The ethanolic extract was prepared by using the powder of the seeds macerated in dark color bottle with 300 ml absolute alcohol at room temperature and were shaken several time for one month. After one month the extract was concentrated to give a deep cream suspension. The crude extract was suspended in water and partitioned with n-hexane, ethyl acetate, and n-butanol. The dried ethanolic extract (TVEE) were dissolved in 10 % w/v DMSO for further experiments.

Cell culture conditions

RPMI (RPMI 1640, Life Technologies , USA) was supplemented with low endotoxin Fetal Bovine Serum (FBS, Life Technologies, USA), 0.2 Units/ml Insulin, 100 M/ml Penicillin (Gibco), 100 µg/ml Streptomycin (Gibco), 2 M/ml - glutamine (Gibco), 25 mM Hepes buffer (Life Technologies, USA) and 1 mM sodium pyruvate.

Effect of plant extract on human breast cancer cells T-47D

The ASEE and TVEE was applied on T-47D cells and Vero cells. The Vero cells were used as a standard reference (nonmalignant) cells. Vybrant® MTT Cell Proliferation Assay (Thermo Inc. USA) was used to analyze the effect of extracts on cells. The procedure was adapted as described previously by Roham, *et al.* [7]. Briefly, ASEE or TVEE or 5-FU (0, 2, 4, 6,8,10 µg/ml) was added to each well containing the cells. After 24 hrs incubation, MTT (5 mg/ml in PBS) was added in each well and incubated for 4 hrs. The absorbance was recorded at 490 nm using Multiscan Ascent (Thermo Inc. USA). The inhibitory effect of ASEE or TVEE on cell growth was assessed as percent cell viability, where cells without treatment were considered 100 % viable [7].

Cell cycle in ASEE or TVEE -treated cells

A previously described method was used for cell cycle analysis [7]. Briefly, after adequate growth of cells in RPMI 1640, ASEE or TVEE was added. Then the floaters were collected from spent medium by centrifuging at 1000 x g for 5

min. These cells were mixed with adherent cells for the analysis of cell cycle in extract treated cells. The cells were washed twice with the PBS and fixed in 70 % (v/v) ice-cold ethanol at 4ºC for 24 hrs. The fixed cells were stained with propidium iodide (Thermo Inc. USA) then analyzed in an Attune flow cytometer (Thermo Inc. USA). Data are presented as means with 95 % confidence interval.

Phosphatidylserine exposure on membranes of apoptotic T-47D cells

For analysis of exposure of phosphatidylserine on membranes of cells, a method was adapted from previous paper [7]. ASEE or TVEE treated cells were incubated for 24 hours and centrifuged at 1000 × g, 5 min. The cells were stained with Annexin V, Alexa Fluor® 568 conjugate (Thermo Inc. USA) in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) at room temperature for 15 min. Extract treated apoptotic cells were quantified by Attune flow cytometer (Life technologies Inc. USA), and calculated by Attune cytometric software v2.1 (Life technologies Inc. USA) [7].

Detection of apoptosis by using the fluorescence microscopy

The human breast cancer cells were exposed to ASEE or TVEE (10 µg/ml) for 24 hours and the spent media were collected and spun at 1000 × g, 5 min. The cells were stained with Annexin V, Alexa Fluor® 568 conjugate (Life technologies Inc. USA) in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) at room temperature for 15 min. Annexin V, Alexa Fluor conjugate stained apoptotic cells were observed by Evos FL microscope with excitation/emission maxima of ~578/603 nm (Life technologies Inc. USA).

Expression of cell cycle marker genes in ASEE or TVEE treated cells

RNA transcripts were analysed by Real Time PCR as described previously [7]. Briefly, ~ 1×10^4 cells were plated in each well of 24 well plate containing 300 µL RPMI1640 medium with 10 % v/v FBS and 0.2 Units/ml insulin.

After preparation of RNA from the T-47D cells (ASEE or TVEE treated and untreated cells) using iScript RT-qPCR sample preparation reagent (Biorad, USA), first strand cDNA was reverse transcribed from total RNA using the first strand cDNA synthesis kit (Life technologies Inc. USA). cDNA was mixed with SYBR Green PCR master mix (Life technologies Inc. USA) and Primers shown in Table 1, and Real-time PCR analysis was carried out as reported prevously [7].

Results and discussion

Annona squamosa L., and *Thymus vulgaris* L. have been used as a traditional medicine. In this study, we report the mechanisms of cell cycle arrest in human breast cancer cells (T-47D). This is the first report of cell cycle arrest in breast cancer cells by these plants. In order to determine the effect of these plants in breast cancer cells, MTT assay was performed.

Determination of IC_{50}

Initially, breast cancer cells T-47D were treated with the ASEE or TVEE. The antiproliferative effect of ASEE and TVEE was evaluated by MTT assay. As shown in (Fig. 1a) a dose dependent decrease in the growth of cancer cells was observed with increasing concentration of the extracts. The IC50 of the ASEE was found to be 8.72 ± 0.52 µg/ml. The IC50 of the TVEE was found 7.30 ± 0.6 µg/ml. The growth of the cells was found arrested in the ASEE and TVEE treated cells. In addition to

Table 1. Primers sequences for the real time PCR of cell cycle regulating marker genes

Genes	Forward primer	Reverse primer
TP53	5′-TAACAGTTCCTGCATGGGCGGC-3′	5′AGGACAGGCACAAACACGCACC-3′
GAPDH	5′-CAGGGCTGCTTTTAACTCTG-3	5′-GATGATCTTGAGGCTGTTGTC-3
Sfn	5’- GTGTGTCCCCAGAGCCATGG-3’	5’- ACCTTCTCCCGGTACTCACG-3’

cancer cells, these extracts were added to the normal cells Vero for 24 hours. The Vero cells were found healthy in presence of these extracts. Growth appeared normal in the extract treated Vero cells. (Fig. 1b).

Arrest of cell cycle in ASEE or TVEE treated cells

To determine whether extracts induced apoptosis was related to arrest of cell cycle progression in breast cancer cells, flow cytometry was used to quantitate the cell cycle distribution under treatment with extracts. In normal healthy cells the cell cycle is strictly regulated whereas it is disrupted in the cancer or malignant cells. The search of drugs against cancer targets mainly on the cell cycle of the cancer cells. The inhibition of the cell cycle phases and control the growth of the cells are one the major strategies to control the cancer cells. In previous reports, Graviola fruit

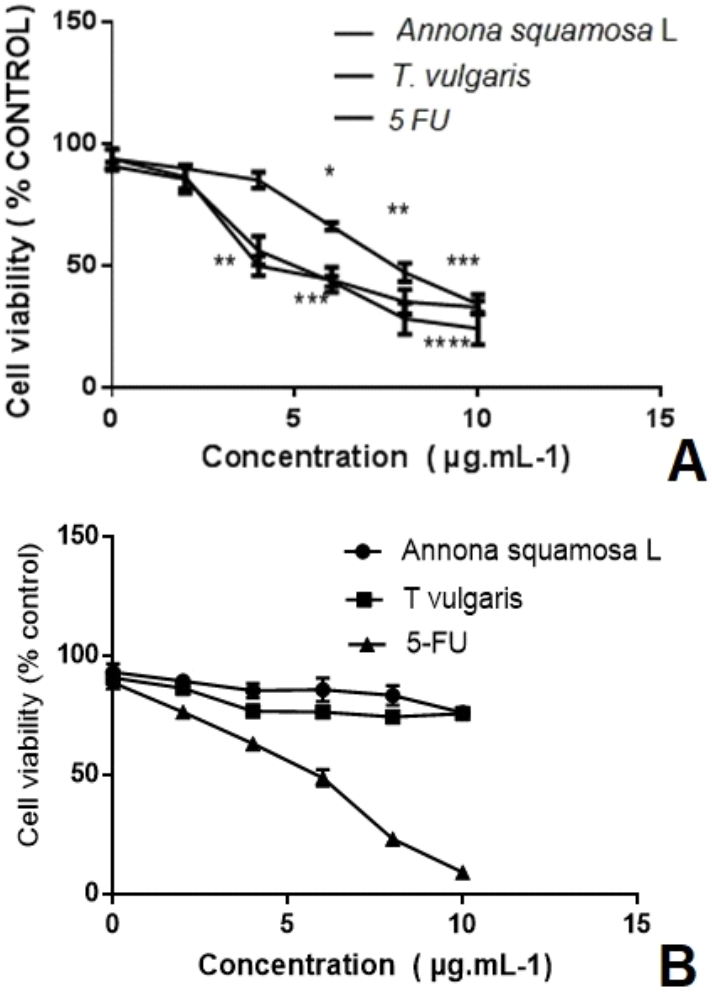

Fig. 1. Inhibition of growth in Human breast cancer cells. Where (A) T-47D cells were treated with ASME or TVME or 5-FU for 24 h. (B) Vero cells were treated with ASME or TVME or 5-FU for 24 h. Cell viability was monitored by MTT assay. The percentage of viability was calculated as the following formula: (viable cells) % = (OD of drug-treated sample/OD of untreated sample) ×100. Measurements were made in triplicate, and standard bars represent the standard deviation. The Student's two-tailed t-test was used to determine statistical significance of the differences between untreated cells and cells treated with the various concentrations of ASEE or TVEE OR 5-FU. Error bars indicate SD. (set as 100%; n = 3; ****P <0.0001, ***P <0.001, **P <0.01, *P < 0 .05, ns-not significant).

extract significantly down regulated EGFR mRNA expression, arrested cell cycle in the G0/G1 phase, and induced apoptosis in MDA-MB-468 cells [19]. The cell cycle was studied in extract treated and untreated T-47D cells. The number of cells in G2/M phase increased in ASEE extract treated cells, when the extract dose increased (Fig. 2). The cell population in ASEE (10 ug/ml) treated cells were found in G2/M phase of the cell cycle (30.8 %), whereas the cell population increases in ASEE (20 ug/ml) treated cells (45.9 %).

The TVEE arrest the cell cycle in S phase 77.6 % (10 µg/ml) and 80.1 % (20 µg/ml). This experimental finding implies that A. squamosa extract induced apoptosis in breast cancer cells via G2/M phase arrest. The result shows G1/M phase arrest in *A. squamosa* extract treated cell population. Annonacin from this plant has also been reported to arrest the cell cycle at G1 phase and Bax and Caspase 3 activation [20]. *T. vulgaris* extract arrest the cell cycle in S phase and induced apoptosis in breast cancer cells.

Detection of phosphatidylserine on membrane of the breast cancer cells T-47D

Morphological studies indicated that ASEE of TVEE induces apoptosis in the breast cancer cells- T-47D cells. The morphological changes during induction of apoptosis were detected with Annexin-V staining. Annexin-V binds to externalized phosphatidylserine (PS) of apoptotic cells. The healthy cell population was found 93 % in untreated control , which was reduced to 87.5 % in ASEE (10 ug/ml).

The apoptotic cell population was found increased to 12 % in ASEE (10 µg/ml) treated cells. Cell population 29.7 % was found apoptotic in ASEE (20 µg/ml) treated cells. The similar effect of the TVEE was found in 10 µg/ml concentration.The apoptotic cells population was found 16.1 % and 16.9 % in cells treated with TVEE 10 µg/ml and 20 µg/ml, respectively (Fig. 3). The majority of the untreated control cells were found to be viable (93 %). The viability of cells (93 %) decreases in treated cells.

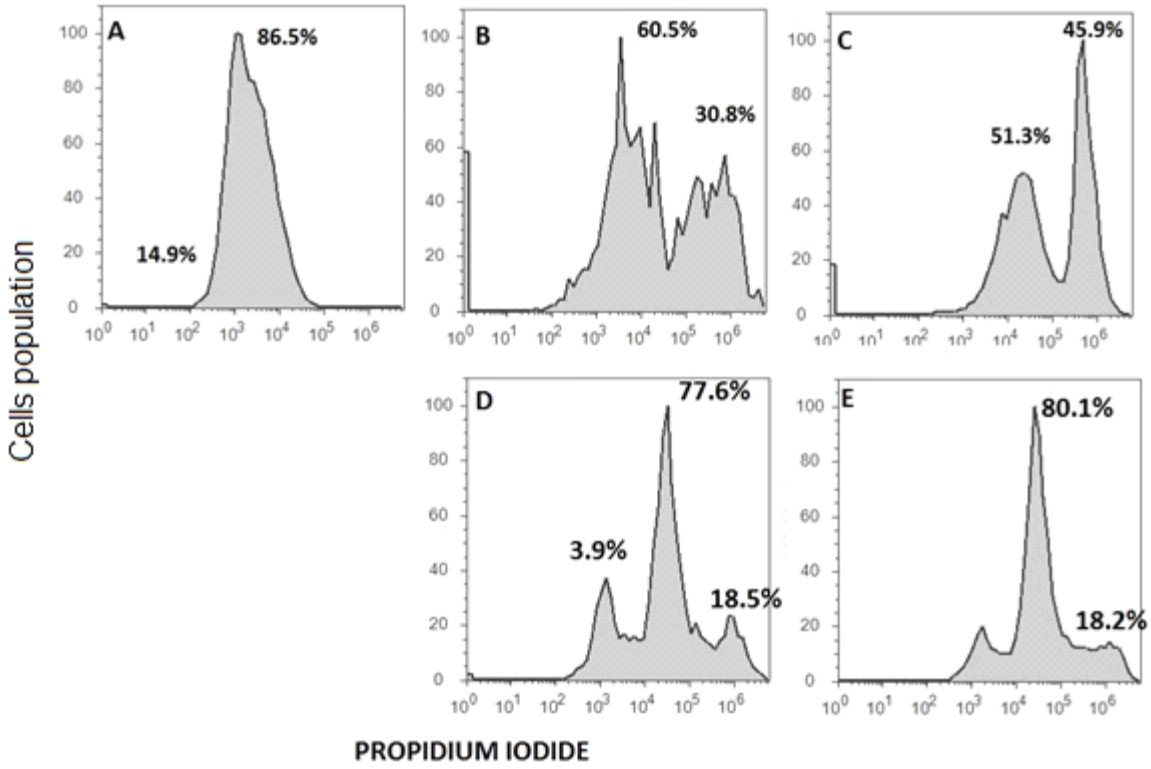

Fig. 2. Cell cycle analysis in AMEE or TVEE treated T-47D cells. Where, A) T-47D cells untreated control, B) AMEE 10 µg/ml., C). AMEE 20 µg/ml, D) TVEE L 10 µg/ml., E. TVEE 20 µg/ml. Cell cycle in T-47D cells was assessed after 24 h of treatment with extract by PI staining and measured by flow cytometry

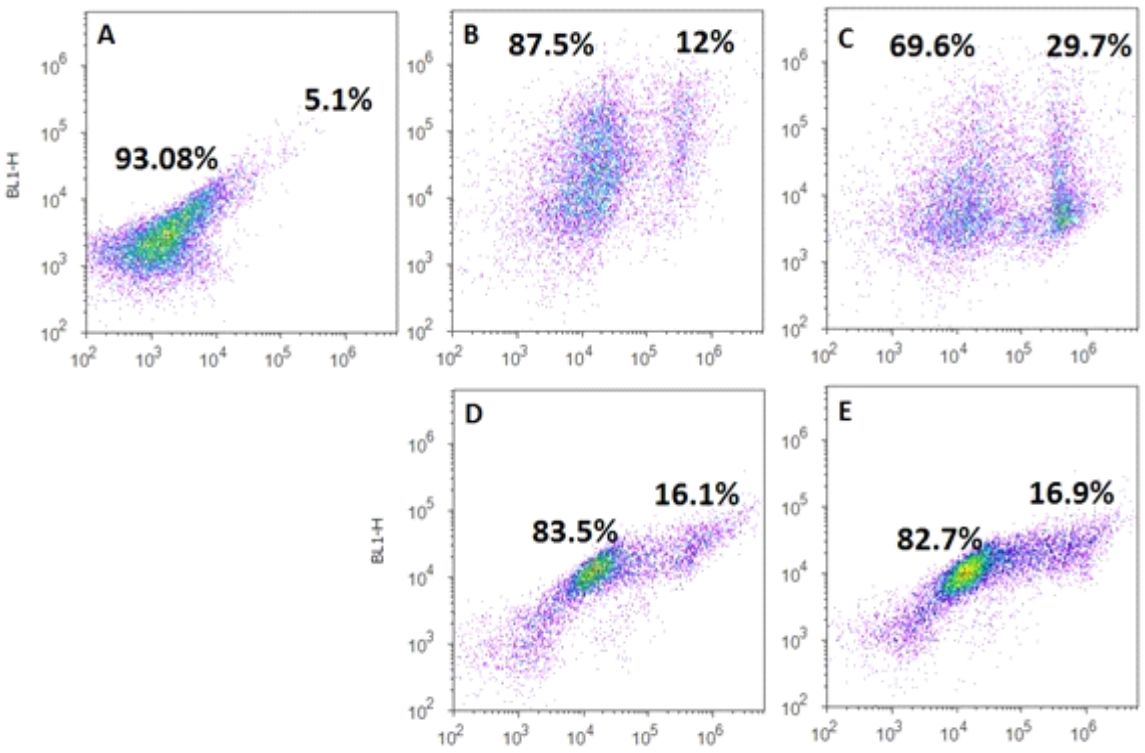

Fig. 3. Induction of apoptosis in human breast cancer T-47D cells. Where, A) T-47D cells untreated control, B) ASEE 10 µg/ml., C). ASEE 20 µg/ml, D) TVEE L 10 µg/ml., E. TVEE 20 µg/ml. Apoptosis in T-47D cells was assessed after 24 h of treatment with Extract by Annexin V, Alexa Fluor® 568 conjugate /PI binding and measured by flow cytometry analysis

Microscopy of apoptotic cancer cells

The phosphatidylserine (PS) on the extracellular plasma membrane [21] can be detected by annexin V proteins. The T-47D cells were exposed for 24 hours to the ASEE of TVEE for the analysis of apoptosis by Microscopy. After 24 hours, the cells were stained with apoptotic marker Annexin-V conjugated to FITC. Under light microscope 20x objective, the cells were found round and separated from the well surface (Fig. 4 A and C). Groups of cells were found in the wells. When observed under EVOS FL(Life technologies, USA), the cells were found green in color (Fig. 4B and D). This is due to the binding of annexin v-FITC to phosphatidylserine (PS) on the membranes of apoptotic cells. This confirms the induction of apoptosis in the T-47D cells due to ASEE or TVEE.

Expression cell cycle specific markers confirms the arrest at G2/M phase

The G2/M checkpoint of cell cycle inhibits the initiation of mitosis during DNA damage in G2. The G2 checkpoint controls the mitosis-promoting activity of the cyclin B-CDK1 complexes. The G2 checkpoint is depends on the p53, which induces cell cycle inhibitors like p21Cip1, GADD45, and 14-3-3σ proteins [22,23]. The cyclin B-CDK1 dissociated by directly binding of proteins GADD45 and 14-3-3σ, which results in separation of CDK1 from cyclin B (GADD45), and sequestering CDK1 in the cytoplasm (14-3-3σ), this induces halting of cell cycle at G2 phase [23].

In search of the effect of ASEE or TVEE on breast cancer cells T-47D cells, we analyzed cell cycle specific markers by using real time PCR (Fig 5 A-C). The transcription regulator p53 was found upregulated in cells treated with ASEE or TVEE (Fig.5 A). The proteins 14-03-03 sigma was found upregulated in the cells treated with ASEE or TVEE (Fig. 5B). This indicates the inhibition of the sequestering CDK1 in the cytoplasm and inhibition of cell cycle at G2/M phase.

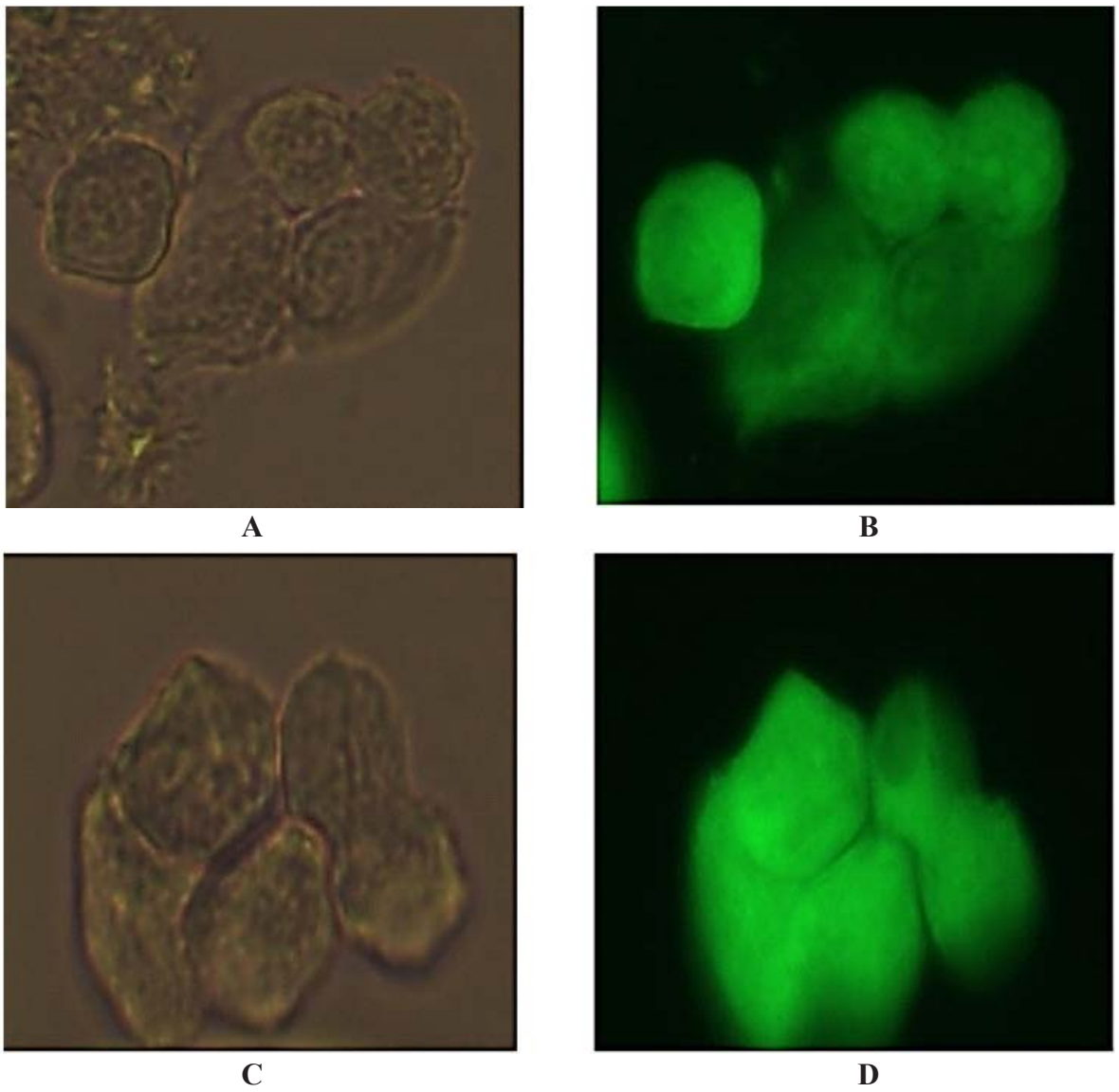

Fig. 4. Apoptosis in T-47D cells observed by Fluorescence microscope. Where, (A) ASEE treated T-47D cells observed under white light , (B) ASEE treated T-47D cells observed with excitation/ emission maxima of ~578/603 nm, (C) TVEE treated T-47D cells observed under white light , (D)) TVEE treated T-47D cells observed with excitation/emission maxima of ~578/603 nm. Annexin V, Alexa Fluor conjugate stained apoptotic cells were observed by Evos FL microscope with excitation/ emission maxima of ~578/603 nm. The green cells are apoptotic cells

Conclusion

Numerous studies have proved the medicinal effects of the of *Annona squamosa* L. and *Thymus vulgaris* L [5-12, 14-20]. The present work demonstrated antiproliferative action of *Annona squamosa* L.(ASEE) and *Thymus vulgaris* L.(TVEE) extracts on Human breast cancer cells T-47D. The work explored the molecular events in *Annona squamosa* L.(ASEE) and *Thymus vulgaris* L. *extracts* (TVEE) treated cells. This study reports the mechanisms of cell cycle arrest in human breast cancer cells (T-47D). The *Annona squamosa* L.*(ASEE)* and *Thymus vulgaris* L. *extract* (TVEE) arrests T-47D cells in G2/M phase leading to induction of apoptosis. This study proves the therapeutic use of the *Annona squamosa* L.(ASEE) and *Thymus vulgaris* L. *extract* (TVEE) in carcinoma treatment.

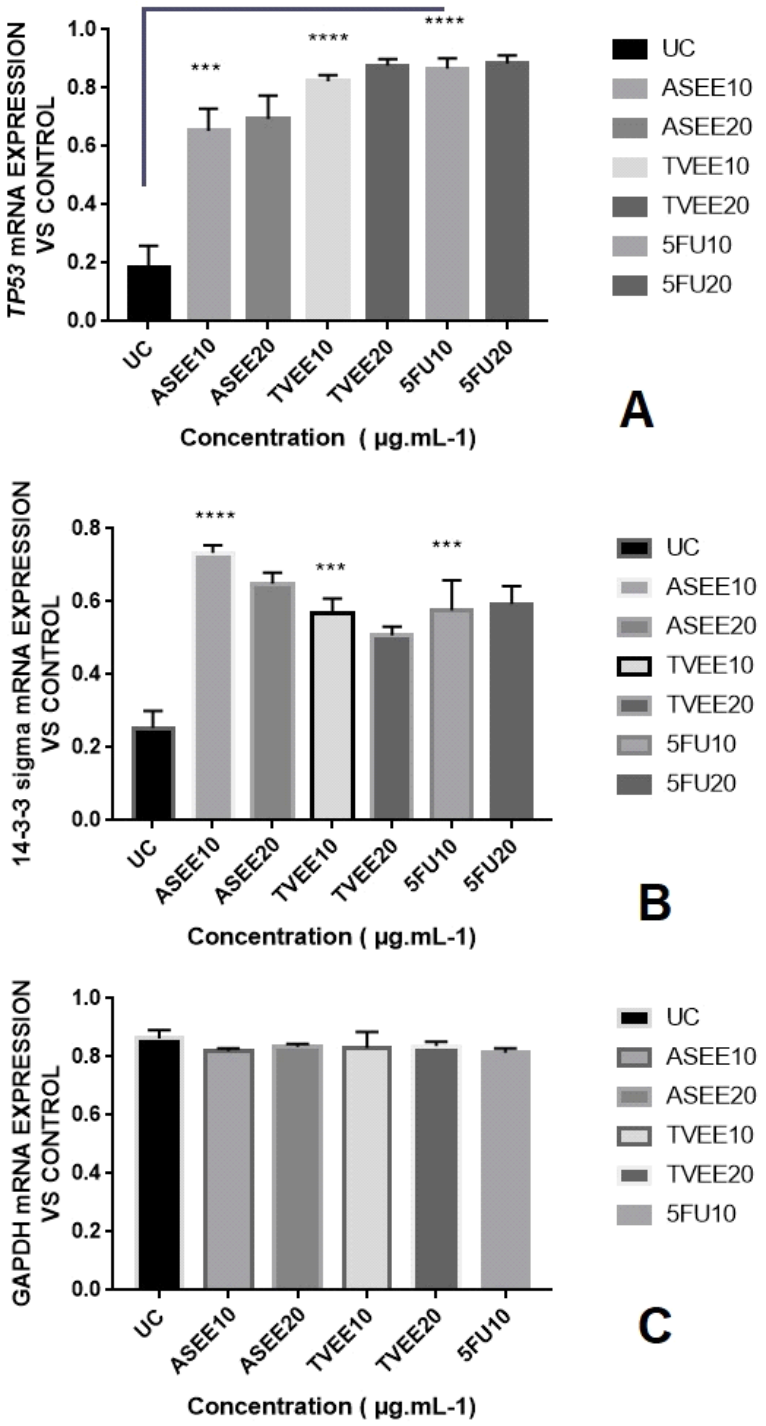

Fig. 5. Regulation of gene expression in T-47D cells . Where, (A) TP53, (B) 14-3-3 sigma and (C) GAPDH mRNA expression. T-47D cells were treated with ASEE OR TVEE (10 or 20 µg/Ml).The gene expression was studied by using Real Time PCR. The experiment was repeated three times and the above values of gene expression are mean of three replicates in three different experiments . Data are expressed as the mRNA copies in cells, where significance refers to the differences between ASEE or TVEE treated and control untreated cells (n = 3; ****P <0.0001, ***P <0.001, **P <0.01, *P < 0 .05, ns-not significant). Error bars indicate SD

References

1. **Elisha, S., Michal, L., Sarah, S., Helena, P., Elaine, S., Haya, L.G. (2014).** Evaluating Medicinal Plants for Anticancer Activity. The Scientific World Journal. 2014: 1-12.
2. **Newman, D.J. (2008).** Natural products as leads to potential drugs: an old process or the new hope for drug discovery. Journal of Medicinal Chemistry. 51(9): 2589-2599.
3. **Newman, J., Cragg, G.M., Snader, K.M. (2003).** Natural products as sources of new drugs over the period 1981-2002. Journal of Natural Products. 66(7): 1022-1037.
4. **Gordaliza, M. (2007).** Natural products as leads to anticancer drugs. Clinical and Translational Oncology. 9(12): 767-776.
5. **Tsabang, N., Tsouh, F.P.V., Yamthe, T.L.R., Noguem, B., Bakarnga-Via, I., Dongmo, N.M.S., Boyom, F.F. (2012).** Ethnopharmacological survey of *Annonaceae* medicinal plants used to treat malaria in four areas of Cameroon. J. Ethnopharmacol. 139: 171-180.
6. **Zorofchian, M.S., Karimian, H., Rouhollahi, E., Paydar, M., Fadaeinasab, M., Abdul Kadir, H. (2014).** *Annona muricata* leaves induce G(1) cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-29 colon cancer cells. Journal of Ethnopharmacology. 156: 277-89.
7. **Roham, P.H., Kharat, K.R., Mungde, P., Jadhav, M.A., Makhija, S.J. (2016).** Induction of Mitochondria Mediated Apoptosis in Human Breast Cancer Cells (T-47D) by *Annona reticulata* L. Leaves Methanolic Extracts. Nutrition and cancer. 68(2): 305-311.
8. **Hamizah, S., Roslida, A.H., Fezah, O., Tan, K.L., Tor, Y.S., Tan, C.I. (2012).** Chemo-preventive potential of *Annona muricata* L. leaves on chemically-induced skin papillomagenesis in mice. Asian Pacific Journal of Cancer Prevention: APJCP. 13(6): 2533-39.
9. **Zorofchian, M.S., Rouhollahi, E., Karimian, H., Fadaeinasab, M., Firoozinia, M., Ameen Abdulla, M., Abdul Kadir, H. (2015).** The chemopotential effect of *Annona muricata* leaves against azoxymethane-induced colonic aberrant crypt foci in rats and the apoptotic effect of Acetogenin Annomuricin E in HT-29 cells: a bioassay-guided approach. PloS One. 10(4): e0122288.
10. **Eqbal, M.A., Dauqan, Aminah Abdullah. (2017).** Medicinal and Functional Values of Thyme (*Thymus vulgaris L.*) Herb. Journal of Applied Biology & Biotechnology. 5(02): 17-22.
11. **Sharangi, A.B., Guha, S. (2013).** Wonders of leafy spices: Medicinal properties ensuring Human Health. Science International. 312-317.
12. **Alireza, K., Faeghe, H., Siamak, S., Negar, B. (2015).** Study of the effect of extract of *Thymus vulgaris* on anxiety in male rats. Journal of Traditional and Complementary Medicine. 1-5.
13. **Aksel, B. (2008).** Bioactive compounds in plants - benefits and risks for man and animals. The Norwegian Academy of Science and Letters, Oslo.
14. **Ocana, A., Reglero, G. (2012).** Effects of Thyme extract oils (from *Thymus vulgaris*, *Thymus-zygis* and *Thymus hyemalis*) on cytokine production and gene expression of oxLDL-Stimulated THP-1- Macrophages. Journal of Obesity. 1-11.
15. **Moghadamtousi, S.Z., Fadaeinasab, M., Nikzad, S., Mohan, G., Ali, H.M., Kadi, H.A. (2015).** *Annona muricata* (Annonaceae): A Review of Its Traditional Uses, Isolated Acetogenins and Biological Activities. Battino M, ed. International Journal of Molecular Sciences. 16(7): 15625-15658.
16. **Yang, C., Gundala, S.R., Mukkavilli, R., Vangala, S., Reid, M.D., Aneja, R. (2015).** Synergistic interactions among flavonoids and acetogenins in graviola (*Annona muricata*) leaves confer protection against prostate cancer. Carcinogenesis. 36(6): 656-665.
17. **Matsushige, A., Matsunami, K., Kotake, Y., Otsuka, H., Ohta, S. (2012).** Three new megastigmanes from the leaves of *Annona muricata*. J. Nat. Med. 66: 284-291.
18. **Nawwar, M., Ayoub, N., Hussein, S., Hashim, A., El-Sharawy, R., Wende, K., Harms,**

M., Lindequist, U. (2012). Flavonol triglycoside and investigation of the antioxidant and cell stimulating activities of *Annona muricata* linn. Arch. Pharm. Res. 35: 761-767.

19. **Dai, Y., Hogan, S., Schmelz, E.M., Ju, Y.H., Canning, C., Zhou, K. (2011).** Selective growth Inhibition of human breast cancer cells by graviola fruit extract *in vitro* and *in vivo* involving downregulation of EGFR expression. Nutr. Cancer. 63: 795-801.
20. **Ko, Y.M., Wu, T.Y., Wu, Y.C., Chang, F.R., Guh, J.Y., Chuang, L.Y. (2011).** Annonacin induces cell cycle-dependent growth arrest and apoptosis in estrogen receptor-alfa-related pathways in MCF-7 cells. J. Ethnopharmacol. 137: 1283-1290.
21. **Schutters, K., Reutelingsperger, C. (2010).** Phosphatidylserine targeting for diagnosis and treatment of human diseases. Apoptosis. 15: 1072-1082.
22. **Nyberg, K.A., Michelson, R.J., Putnam, C.W., Weinert, T.A. (2002).** Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet. 36: 617-56.
23. **Taylor, W.R., Stark, G.R. (2001).** Regulation of the G2/M transition by p53. Oncogene 20(15): 1803-15.