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Research Article

Phytotherapeutics for Better Cancer Therapy

Emblicanin-A Inhibits Cell Growth in Human Prostate Cancer Cells (PC-3) By Modulating Apoptotic Signaling Molecules

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Abstract: Cancer is a one of the leading causes of death in the world, continue to be worldwide dreadful disease. Prostate cancer is the most common cancer diagnosed in men. Multi-drug resistance (MDR) is a major problem with the current treatment options. It is now widely believed that many herbal dietary products are available as chemo-preventive agents against commonly occurring cancer types such as prostate cancer. Emblicanin-A, a hydrolyzable tannins has been isolated from the fruit of *Emblicaofficinalis.* The present study was aimedto find out whether emblicanin-A can inhibit growth of the prostate cancer cells (PC-3) through the regulation of apoptotic signaling mechanisms. Hence, PC-3 cells were treated with different concentrations of emblicanin-A (10, 25, 50, 100 and 150µM) for the analysis of B-cell lymphoma 2 (Bcl-2), Tumor suppressor protein (p53), Caspase-3 and caspase-9 mRNA expression in PC-3 cells. Cell viability was done using MTT in order to find the optimal dose. MTT assay exhibited that emblicanin-A showed cell death at the concentration of 100 and 150µM. It significantly *($p<0.05$)* decreased the mRNA expression of anti apoptotic proteins (Bcl-2) while it upregulated the p53, caspase-3 and cas-9 mRNA levels effectively ($p<0.05$) in PC-3 cells which clearly indicates that emblicanin-A induces apoptosis in PC-3 cells by modulating intrinsic signalling mechanisms. To best of our knowledge, the present findings are the first to report anticancer activity of a bioactive compound from *E.officinalis.* Our study concludes that emblicanin-A may serve as a potential chemotherapeutic agent for the treatment of prostate cancer. Further studies on the effect of Emblicanin-A on the protein expression of further downstream signalling mechanisms is warranted in order to ascertain its potential mechanisms action towards clinical utility.

Key words: Emblicanin-A; Prostate Cancer, PC-3 cells, Apoptosis

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1. INTRODUCTION

Worldwide, prostate cancer is the most commonly diagnosed male malignancy and the fourth leading cause of cancer death in men. In 2018, this was amounted to 1,280,000 newly diagnosed cases and 359,000 deaths around the world from this disease[1-3]. Prostate cancer risk factors include male gender, older age, positive family history, increased height, obesity, hypertension, lack of exercise, and persistently elevated testosterone levels[4].The high long-term survival in localized prostate cancer, metastatic prostate cancer remains largely incurable even after intensive multimodal therapy by the lack of therapeutic regimens capable of generating durable responses in the setting of extreme tumor heterogeneity on the genetic and cell biological levels[5]. Although great advancements have been made in the treatment and control of cancer progression, significant deficiencies and space for improvement remains. Compared with synthetic compounds, natural products provide inherent larger-scale diversity and have been the major resource of bioactive agents for new drug discovery. Natural products are rapidly being utilized as source for drug discovery and development, because of its comparatively safe and low cost. Anticancer agents from natural source have a long history in folk medicine and it has been combined into traditional and allopathic medicine. Emblicanin-A, hydrolyzable tannins has been found from the fruit of *Emblicaofficinalis*. The fruit extract of *E.officinalis* has been used in traditional medicine for generations to treat symptoms ranging from constipation to the treatment of many types of cancer[6]. Emblicanin-A, emblicanin-B, punigluconin and pedunculagin, isolated from the fresh juice or extracts of Emblica fruits have been reported to have a number of disparate properties including antioxidant, anti-inflammatory, proapoptotic[7-9]. It has been reported that *E.officinalis* containing tannoid active constituents such as emblicanin-A and B, punigluconin and pedunculagin exhibited potential antioxidant activity done in in vitro and in vivo models[10]. Pandey (2011)[11] reported that emblicanin-A and B showed a strong antioxidant and anti cancer activity. A study by Bhattacharya et al. (2002)[12] report shows that emblicanin-A (37%) and emblicanin-B (33%) isolated from fresh juice of E.officinalis significantly reduced ischemia-reperfusion induced oxidative stress in rat cardiac tissue and this study clearly indicates that emblicanin-A possesses considerable cardio-protective effect. However, the possible role of emblicanin-A on prostate cancer has not been studied so far. Hence, the present study was aimed at assessing the chemo-preventive effect of emblicanin-A in human prostate cancer cells PC-3. In the present study we have shown the modulation of apoptotic signalling molecules by theemblicanin-A of *E.officinalis*.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals and reagents used in this study were of extra pure and analytical grade. Emblicanin-A and dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Pvt., Ltd. (St. Louis, MO., USA), polyvinylidenedifluoride (PVDF) membrane was purchased from Millipore (Billerica, Massachusetts, USA). Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, Roswell Park Memorial Institute (RPMI) medium, and phosphate-buffered saline (PBS) were purchased from Gibco, United States. Primers of Bcl-2, p53, Caspase-3, caspase-9 and GAPDH were purchased from Sigma-Aldrich (Bommasandra-Jigani Link Road, Bengaluru - 560 100, India).

2.2. Cell Line Maintenance

PC-3 cell lines were obtained from the National Centre for Cell Science (Pune, India). The cells were grown in T-25 culture flasks in RPMI medium supplemented with 10% FBS with 1% penicillin/streptomycin and 1% amphotericin B. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. On attaining the confluence, the cells were trypsinized and plated[13]

2.3. *Cell Viability Assay*

The cell viability assay was performed by MTT assay, which determines mitochondrial activity in living cells[14]. Cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated for 24 h at 37°C, 5% CO_2 incubator. After attachment, cells were washed with PBS and then incubated with a serum-free medium for 6–12 h. EA was dissolved in DMSO with different concentration emblicanin-A (10, 25, 50, 100, and 150 µM) and added to the cells. After the treatment period, 20 µl medium was removed and 20 µl of MTT was added and incubated for 30 min. Then, 100 µl of DMSO was added to solubilize the crystals and was kept in dark for 10 min. The intensity of color development was measured at 570 nm in ELISA reader. The cell viability was calculated as follows: Cell viability = absorbance of treated cells/absorbance of control cells ×100%.

2.4. *mRNA expression analysis by Quantitative RT-PCR*

mRNA expression levels of Bcl-2, p53, Caspase-3 and caspase-9 were examined using real-time PCR[15]. The total RNA was isolated by using Tri Reagent (Sigma). Total RNA (2 µg) from each sample was reverse transcribed using a commercial Superscript III first strand cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's protocol. The list of primers and the internal control sequence are as follows. Human Bcl-2 FW:5'-ATTGGGAAGTTTCAAATCAGC-3'; Human Bcl-2 RW:5'-TGCATTCTTGGACGAGGG-3'. Human Caspase-3 FW: 5'-GGCATTGAGACAGACAGTGG-3'; Human Caspase-3 RW: 5'-CATGGAATCTGTTTCTTTGC-3'. Human Caspase-9 FW:5'-GAGTCAGGCTCTTCCTTTG-3'; Human Casepase9 RW:5'-CCTCAAACTCTCAAGAGCAC-3. Human p53FW:5' GAGGTTGGCTCTGACTGTACC-3'; Human p53 RW: 5'-TCCGTCCCAGTAGATTACCAC-3'. Human Human GAPDH FW: 5' -TTGGTATCGTGGAAGGACTCA-3'; GAPDH RW: 5' -TGTCATCATATTTGGCAGGTTT-3'. Real time-PCR was carried out in CFX 96 Real Time system software (Bio-Rad). Reaction was performed using MESA Green PCR master mix (It contains all the PCR components along with SYBR green dye).The specificity of the amplification product was determined by melting curve analysis for each primer pair. The data were analyzed by comparative CT method and the fold change is calculated by $2^{-\Delta\Delta CT}$ method using CFX Manager Version 2.1 (Bio Rad, USA).

3. STATISTICAL ANALYSIS

Data were analyzed statistically using one-way analysis of Variance (one-way ANOVA) followed by Duncan's tests for comparison between treatment and control values using the Statistical Package for Student version 17.0 (SPSS Inc.,

Chicago, IL) software. $p< 0.05$ was considered to be statistically significant.

4. RESULTS

4.1. *Effect of emblicanin A on the cell viability in PC-3 cells*

To evaluate the cytotoxic activity of emblicanin-A against human prostate cancer, cells were incubated with different doses (10, 25, 50, 100, and 150 µM /ml) of emblicanin-A. After 24hours of incubation, cell viability was determined by the MTT assay. Emblicanin-A induced cell cytotoxicity in a concentration dependent manner, as illustrated. The results of cytotoxicity assay are presented in (fig.1). It reduced the viability of PC-3 cells in a dose-dependent manner concentration. However, it significantly decreased *(p<0.05)* the viability of PC-3 cells in 24 h with IC_{50} values of 100 and 150 µM/ml, respectively. Hence, 100 and 150 µM concentrations were used for further analysis of mRNA expression of apoptosis signaling cascade.

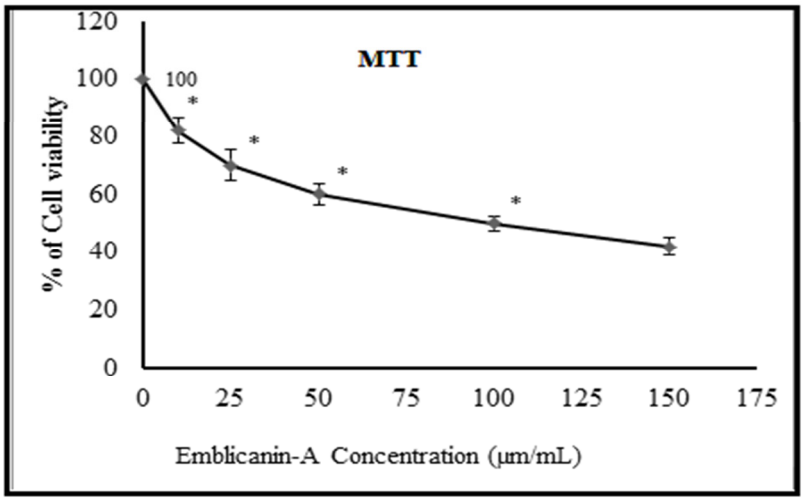


Figure 1.Effect of emblicanin-A on the viability of prostate cancer cells. PC-3 cells were culturedin RPMI medium supplemented with 10% FBS and incubated with indicated concentrations of emblicanin-A for 24 h. For cell viability assay, cells were exposed to different doses (10–150 µM /ml) of emblicanin-A for 24 h. It inhibited growth (as determined by MTT assay) of human prostate cancer cells. Each bar represents the mean±SEM of five independent observations and the statistical significance between control and the treated groups at p<0.05 level.

4.2. *Effect of emblicanin-A on Bcl-2 mRNA expression in PC-3 Cells*

To examine the status of intracellular signaling molecules in the emblicaninA - treated cells, protein expression analysis was performed. Emblicanin-A treatment significantly decreased ($p<0.001$) the Bcl-2 mRNA expression (Fig.2).

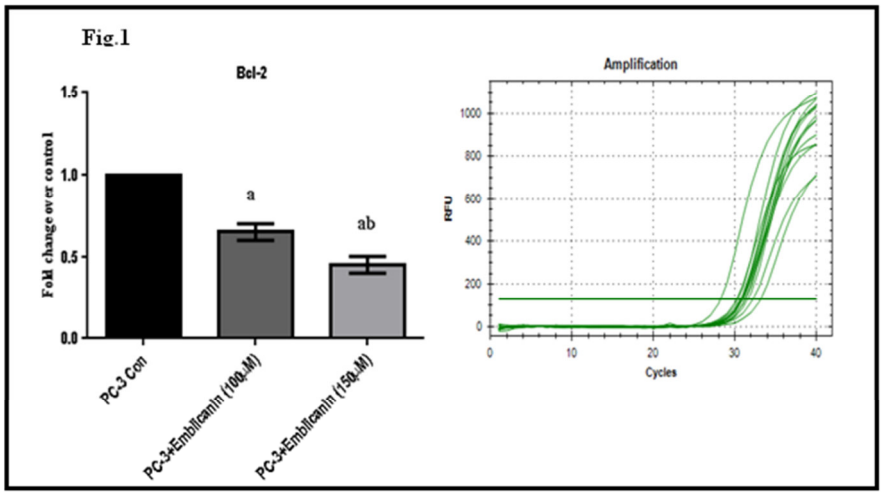


Fig.2. Effect of emblicanin-A on the Bcl-2 mRNA and in PC-3 cells. Cells were treated with 100 and 150µM/ml concentrations of emblicanin-A for 24 h. The mRNA expression of Bcl-2 was analyzed by real-time PCR using SYBR Green dye. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean±SEM of five independent observations and the statistical significance between control and the treated groups at p<0.05 level. a – compared with untreated control cells; b-compared with Emblicanin-A treated (100µM) treated cells

4.3. *Effect of emblicanin-A on p53 mRNA expression in PC-3 cells*

To determine whether tumor suppressor protein is involved in the EA-induced apoptosis, the mRNA expression of p53 was analyzed by Real-Time-PCR. The results showed that emblicanin-A could effectively ($p<0.05$) up regulated tumor suppressor protein (p53) expression in PC-3 cells (Fig.3) suggesting that the emblicanin-A plays an important role on tumor suppressor protein in prostate cancer cells.

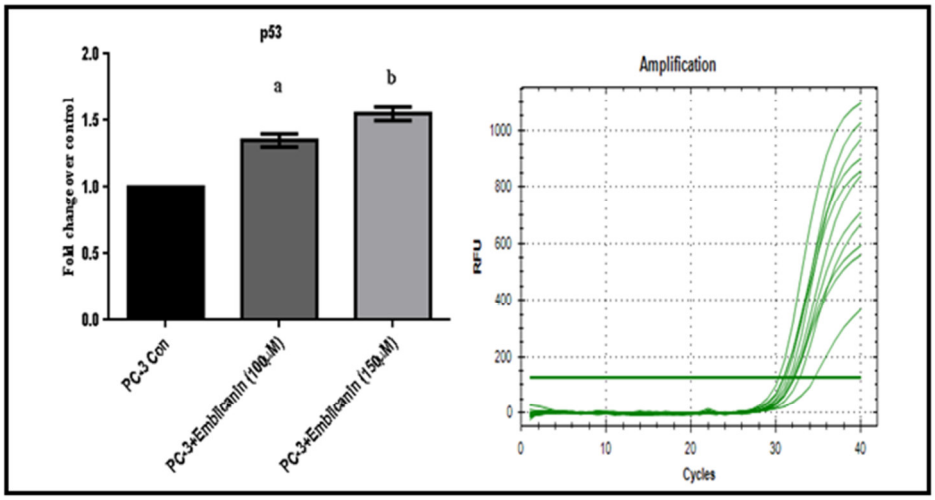
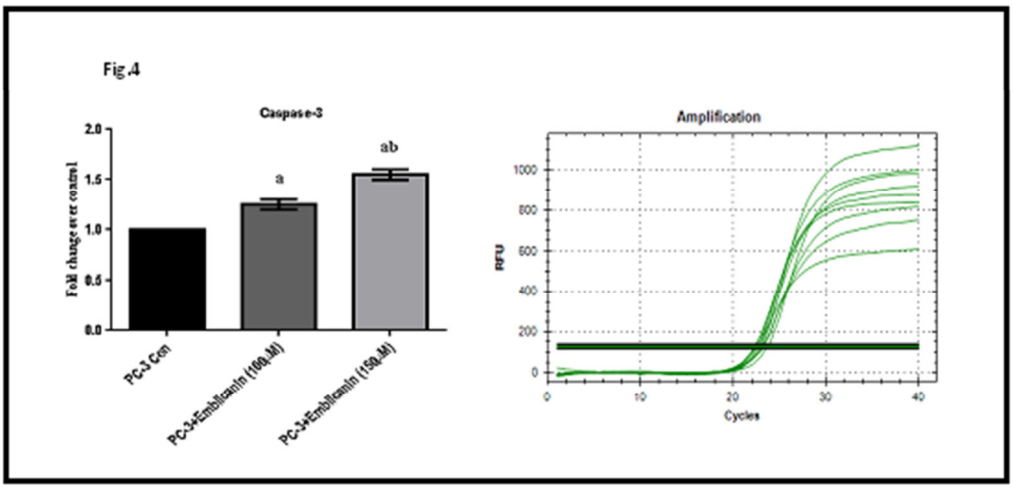


Fig.3. Effect of emblicanin-A on the p53 mRNA and in PC-3 cells. Cells were treated with 100 and 150µM/ml concentrations of emblicanin-A for 24 h. The mRNA expression of p53was analyzed by real-time PCR using SYBR Green dye. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean±SEM of five independent observations and the statistical significance between control and the treated groups at p<0.05 level.a – compared with untreated control cells; b-compared with Emblicanin-A treated (100µM) treated cells

4.4. *Effect of emblicanin-A on caspase-3 and -9 mRNA expressions in PC-3 cells*

In order to check the possible mechanism of action of emblicanin-A on intrinsic apoptotic signaling molecules, caspase-3 and-9 mRNA expression were examined. In the present study, emblicanin-A treatment to PC-3 cells significantly increased ($p<0.05$) the mRNA expression of the caspase-3 and-9 (Fig.4 & 5). This study clearly shows that emblicanin-A mediated cell death in PC-3 cells is mediated through intrinsic apoptotic mechanisms.



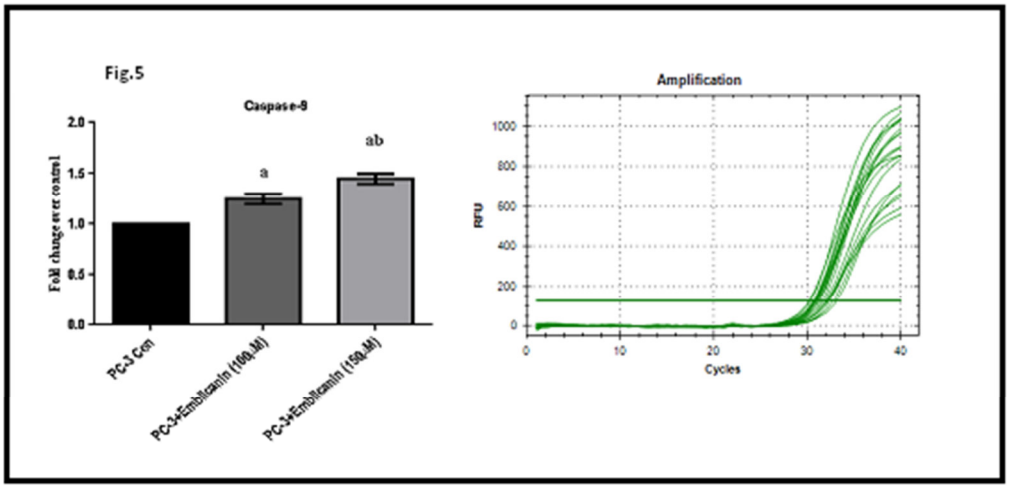


Fig.4 & 5. Effect of emblicanin-A on the caspase-3 and-9 mRNA and in PC-3 cells. Cells were treated with 100 and 150µM/ml concentrations of emblicanin-A for 24 h. The mRNA expression of caspase-3 and-9 weres analyzed by real-time PCR using SYBR Green dye. Target gene expression is normalized to GAPDH mRNA expression and the results are expressed as fold change from control. Each bar represents the mean±SEM of five independent observations and the statistical significance between control and the treated groups at p<0.05 level.

5. DISCUSSION

Apoptosis is a key process in the development of cancer. Evading of apoptosis process is one of the hallmarks of cancer. Treatment for prostate cancer begins with withdrawal of androgen where most of the cells die by apoptotic process. However, in some conditions, the cells derive the resistance to the therapy and leading to the metastatic prostate cancer[13]. Cancer management is being made with the help of several therapeutic strategies, yet there is an increase in the prevalence of the disease with high range of mortality. Plants and plant-based medicines are being used as medicines to cure several diseases including cancer[16]. There are several phytocompounds identified to possess anticancer activity and drugs from medicinal herbs are of great interest for their efficacy and safety[17]. In this study we interested to test effect of emblicanin-A on the advance stage of cancer though targeting intrinsic apoptotic signaling mechanisms. Hence, we selected hormone-dependent cell line PC-3. The results of the present study showed that emblicanin-A inhibits proliferation of human PC-3 cells in a dose-dependent manner with the IC_{50} value of 100µM which clearly indicates that emblicanin-A possesses anti-proliferative effects in PC-3 cells. The present study is first to report that emblicanin-A anticancer activity in PC-3 cells. In this regard, studies have shown that E.officinalis has anti-proliferative potential which is mediated through the activation of apoptotic signaling cascade[18-21]. Tannins compounds such as chebulagic acid, ellagic acid and corilagin isolated from emblicaofficinalis extract showed antiproliferative effect through NF-kappaB inhibitory pathways. Moreover, studies on effect of E.offcinalins on other cancer cell lines such as pancreatic and ovarian cancer cells showed antiproliferative and proapoptotic properties against cancer cells[22-24].Intrinsic pathway of apoptosis is one of the pathways activated by many cytotoxic drugs. Bcl-2 members family play a major role in the intrinsic pathway and this family contains 25 pro and antiapoptotic members which interact to maintain the balance between newly forming and old dying cells[25]. These proteins are classified into three subfamilies. Antiapoptotic subfamily contains the Bcl-2, Bcl- XL, Bcl-w, Mcl-1, Bfl1/A-1 and Bcl-B proteins which suppress apoptosis, some pro-apoptotic proteins like Bax, Bak and Bok, other pro-apoptotic proteins such as Bim Bad and Bid. Theantiapoptotic proteins are overexpressed in prostate cancer. These proteins heterodimerize with pro-apoptotic protein such as Bax/Bak, which will prevent the cell death. Drugs have been used for prostate cancer targeting the antiapoptotic proteins. These drugs induce the activation of tumor suppressor protein p53 thereby increase the expression of pro-apoptotic protein and reducing the expression of antiapoptotic protein[26]. The same mechanism was observed in the emblicanin-A treated the PC-3 cells of the present study. There was an increase in the level of p53 mRNA and decrease in the mRNA levels of antiapoptoticproteins Bcl-2 and this study suggest that emblicanin-A promotes apoptosis in PC-3 through the modulation of intrinsic pathway. In support of the present findings, Malik et al. (2016)[27] reported that the E.officinalisexract significantly decreased the Bcl-2 protein expression in cisplatin-induced nephrotoxicity in rats *in vivo*.Caspases serve as a primary mediator of apoptosis located in the cytosolic space[28]. In the present study, the treatment of emblicanin-A to PC-3 cells increased the caspase-3 and-9 mRNA expression compared to untreated cells which might the mechanism that emblicanin-A might have lead to the release of cytochrome c from mitochondrial space which would have been combined with an adaptor molecule apoptosis protease activating factor 1 and also with an inactive initiator caspase, pro-caspase-9 within a multiprotein complex called the apoptosome[29]. This a popto some would have caused a series of caspase activation which starts from 9 to 3, 6, 7 that ultimately would have caused apoptosis. Hence, it clearly demonstrates that emblicanin-A activates apoptosis by the intrinsic pathway. In support of the present investigation, it has been reported that E.officinalis fruit extract treated rats significantly increased the caspase-3 protein expression[27].

6. CONCLUSION

The current study provides a evidence that emblicanin-A strongly inhibits the growth of prostate cancer cells by modulating the apoptotic pathway. It down-regulated the antiapoptotic protein and up-regulated the p53 pro-apoptotic protein thereby paved the way to cell death. The caspase-3 and caspase-9 activation exhibited that emblicanin-A acts

through intrinsic apoptotic pathway. Hence, emblicanin-A could serve as potent chemotherapeutic candidate for the treatment of prostate cancer. In the present study we have measured only mRNA expression analysis of intrinsic apoptotic signaling mechanisms protein expressions on the same need to be studied. Further studies on experiments through animal model and clinical trial may lead emblicanin-A to be a potent drug in future.

7. AUTHORS CONTRIBUTION STATEMENT

Selvaraj Jayaraman involved in conceptualization and investigation. Ponnulakshmi Rajagopal and Vishnu priya Veeraraghavan analyzed the data and necessary inputs were given towards the designing of the manuscript. Sindhura Myneni and Sai Ravi Teja Kamineni are involved in manuscript writing. Divya Ravikuma and Surapaneni Krishna Mohan were involved in statistical analysis and manuscript corrections. All authors discussed the methodology and results and contributed to the final manuscript.

8. ACKNOWLEDGMENTS

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9. CONFLICT OF INTEREST

Conflict of interest declared none

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