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# COMPARING EFFECT OF ANNONACEAE AND EMETINE NANOPARTICLES ON NFκB p65 PATHWAY IN TONGUE SQUAMOUS CELL CARCINOMA (scc-25)

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## *ABSTRACT*

**Objective**: The transformed cells in oral cancer secrete inflammatory mediators which generate an inflammatory microenvironment result in malignant transformation. Nuclear factor-ϰB (NF-ϰB) regulates a large number of genes which play a critical roles of the immune, inflammatory responses, cell proliferation, differentiation, and survival. Nanoparticles are 100 to 10,000 times smaller than human cells, could be designed to be highly selective for tumors and allow a slow release of active anticancer agents. We performed this study to investigate the effect of annonaceae nanoparticles and Emetine/Chitosan nanoparticles against scc-25 cell lines human tongue squamous cell carcinoma by studying their effect on NF-ϰB p65 expression.

**Methods**: Cell Line cells were obtained from American Type Culture Collection, cells were cultured using DMEM (Invitrogen/Life Technologies). The effect of Annonaceae nanoparticles and EMETIN drug were investigated by Enzyme-Linked Immunosorbent Assay. The Signal is generated proportionally to the amount of bound NFκB p65 and the intensity is measured by ROBONIK P2000 ELISA READER at 450 nm.

**Results**: Treatment of tongue scc-25 cells with both annonacea NP and Emetine/Chitosan NP revealed a significant increase in expression of NFκB p65 in cancer cells by (227 & 261 pg/ml) respectively at incubation period 48 hours . While the control untreated cancer cells scc-25 revealed lower expression of NFκB p65 at (147 pg/ml at incubation period 48 hour.

**Conclusion**: Our findings demonstrate that annonaceae and Emetine nanoparticle might have regulatory effect on canonical NF-kb pathway by upregulation of NFκB p65 expression signals.

**KEYWORDS:** Annonaceae, Emetine drug, chitosan nanoparticles, NFkB65.

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## INTRODUCTION

The transformed cells in oral cancer secrete inflammatory mediators which generate an inflammatory microenvironment result in activation of several transcription factors such as NF-ϰB, STAT-3, and HIF-1 that lead to the accumulation of pro-inflammatory molecules like chemokines, cytokines, and PGHS-2 into the tumor microenvironment results in marked initiation of inflammatory condition which as well activate tumor growth and invasiveness [1].

Nanomedicine was developed to improve the anticancer effect of synthetic and natural therapeutic products against numerous cancer types[2]. Yingchoncharoen p. et al, **2016** reported that nanoparticles are 100 to 10,000 times smaller than human cells and can interrelate with biological factors intra and extracellularly. They reported that nanoparticles can be designed to be highly selective for tumors and allow a steady release of active anticancer agents, to lessen the possible systemic toxicity and improve the distribution and circulation time of these agents in the body [3].

So applying the nanotechnology on natural compounds like Annona Cherimola which revealed cytotoxic properties in treatment of different types of cancer cell lines like breast (MCF-7) and colon (HT-29), and liver cancer cell lines could be encouraging anticancer strategy [4]. It was revealed that ethanolic extract of Annona Cherimola seeds, have shown a cytotoxic effect on AML cell lines, by promoting apoptotic signals through a p53-dependent action through stimulating the intrinsic and extrinsic apoptotic pathways and decreasing the ROS levels [5].

Annona muricata is a lowland tropical, tree laden with fruit of the family Annonaceae found in the South America, South East Asia and rainforests of Africa A. muricata, includes numerous bioactive compounds like Alkaloids, terpenoids and acetogenins [6,7]. Certain types of alkaloids, as jerantinine B liriodenine and vinoreline , reveal the ability to induce apoptosis and block the cell cycle in the G1 phase [8].

The major bioactive elements that have been released from A. muricata's parts are known as annonaceous acetogenins (AGEs). These are imitatives of long-chain (C32 or C34) fatty acids derived from the polyketide. They are shown to be selectively toxic to cancer cells, stimulating multidrug-resistant cancer cell lines [9]. Annonaceous acetogenins promotes cytotoxicity by blocking mitochondrial complex I, which is involved in oxidative phosphorylation and ATP synthesis, since, cancer cells have a higher request for ATP than the normal cells, mitochondrial complex I blockers have possibility in cancer therapy [10].

Moreover, the ethanolic extract of A. muricata leaves stimulates apoptosis in COLO-205 colon cancer cells by upregulation of proapoptotic caspase-3 action. Also, in HT-29 colorectal cancer cells, annomuricin E pulled out from leaves of A. muricata stimulate apoptosis through activation of caspases 3/7 and 9, increase of BAX, and decrease of BCL-2 at the mRNA and protein levels [11] An aqueous leaf extract revealed encouraging anti-proliferative action by blocking the cell cycle in the G2M phase in SCC-25 squamous cell carcinoma [12]

Emetine dihydrochloride hydrate (emetine) was recognized as one of the mini molecule blockers of the Wnt/β-catenin pathway with a long history of application for the treatment of amoebiasis, it is one of the natural active agents withdrawn from the plant Psychotria ipecacuanha [13]. Emetine has been shown to apply antitumor results by promoting apoptosis in leukemia cell lines, down regulating Bcl-XL in ovarian carcinoma cells, inducing apoptosis and autophagy in bladder cancer cells, and regulating the ERK and p38 pathways in human NSCLC [14, 15]

Nuclear factor-ϰB (NF-ϰB) is considered a family of transcription factors that modulates a large number of genes which plays a major roles of the immune, inflammatory responses, cell proliferation, differentiation, and survival [16]. This family is made of five structurally linked members, includ-

ing NF-κB1 (as well named p50), NF-κB2 (as well named p52), RelA (as well named p65), RelB and c-Rel, which promotes transcription of target genes by binding to a particular DNA element, κB enhancer, as various hetero- or homo-dimers. The most copious member in many cell types is the p65/p50 complex. In unstimulated cells, NF-κB is inactive in the cytoplasm and bound by I-κB proteins [17].

NF-κB activation happen in two pathways canonical and non-canonical pathways and relies on phosphorylation-induced ubiquitination of IκB proteins. NF-κB member which is turned on by the canonical pathway is the p65 and p50 heterodimers. The canonical NF-κB pathway is stimulated within minutes of contact to pro-inflammatory signals such as pathogens, cytokines and danger-present molecules [18].

Numerous studies introduced different results about the role of NF-kb pathway in cancer microenvironment. Many studied reported the oncogenic role of NF-kb through stimulation of cell cycle mediators (cyclin D1, c-Myc), anti-apoptotic (c-FLIP, survivin, Bcl-$X_L$) and adhesion molecules (ICAM-1, ELAM-1, VCAM-17) [19], proteolytic enzymes (e.g., MMP, uPA), and pro-inflammatory factors (PGHS-2, cytokines) that encourage an invasive phenotype[20], while other studies revealed that NF-KB mediates induction of FADD and caspase 8 to the death-inducing signaling complex to elevate tumor cell sensitivity to Fas-mediated apoptosis in tumor cells [21].

So the aim of the present study was to investigate the effect of treatment of annonaceae nanoparticles and Emetine/chitosan nanoparticles on NF-kb/p65 expression in tongue scc-25 cell line since many studies proved the anti-proliferative and apoptotic effect of these two compounds [22, 23].

## MATERIALS AND METHODS

We used in the present study Scc-25 Cell Line (human tongue squamous cell carcinoma). Cells were acquired from American Type Culture Collection. Cells were cultured using DMEM (Invitrogen/Life Technologies) augmented with 10% FBS (Hyclone), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin. All chemicals and reactants were from Sigma, or Invitrogen.

Cell culture protocol was done according to conventional procedures at confirmatory diagnostic unit, VACSERA-Egypt. . Plate cells (cells density 1.2 – 1.8 × 10,000 cells/well) in a volume of 100$\mu$l complete growth medium + 100 ul of the tested compound per well in a 96-well plate for 24 hours before the Enzyme-Linked Immunosorbent Assay.

The cultures were incubated at 37°C for 24 hrs. After treatment of SCC-25 cells with the serial concentrations of the nano- annonaceae and Emetine drug (100, 25, 6.25, 1.56 and 0.39 up/ml). Incubation is carried out for 48 h at 37ºC, then the plates are to be examined under the inverted microscope and proceed for the enzyme immunoassay, ELIZA for NF-κB 65 expression (the standard protocol derived from www.abcam.com).

### Preparation of Nanoparticles Containing Annona Muricata Leaves Extract Using Gelation Ionic Method

The specimen was in the form plant leaves powder of Annona. Muricata code E536, Origin Peru. Chemicals used in the study involve 2, 2-diphenyl-1-picrylhydrazil (DPPH) (Sigma Aldrich), poly (acrylic acid) (PAA) (Sigma-Aldrich), calcium chloride, ascorbic acid, dimethyl sulfoxide (DMSO), propylenglycol.

### Nanoparticles preparation

Annona Muricata and Emetine nanoparticles were prepared at National research institution, Egypt. The plant extract was used to develop nano-suspension with poly acrylic acid (PAA) and calcium chloride. PAA solution was used at a concentration of 0.05% (in water) with NaOH to pH 8 and 0.1% calcium chloride solution in water. [24].

Evaluation of nanoparticle included particles size using a Malvern particle sizer and examination of the zeta potential using a Malvern zeta potential measuring device. Moreover, the nanoparticles plant suspension was dried at inlet temperature of 190˚C and outlet temperature of 90˚C [25].

**EMETINE Encapsulated Chitosan Nanoparticles preparation**

Chitosan obtained from crab shell was purchased from Sigma-Aldrich (Medium Molecular Weight, Catalogue no. 448877). The degree of deacetylation and molecular weight for the medium-molecular-weight chitosan (MWM chitosan) is 75–85% and 190–310 kDa according on viscosity, respectively. [25].

Sodium tripolyphosphate (TPP) (purity: 85%), EMETIN (purity: 99%), and phosphate-buffered saline (PBS) tablets (pH 7.4), were maintain chitosan concentration at 0.75 (mg/mL). the formed chitosan solutions were stirred with EMETINE solutions (EMETINE dissolved in water), and 1.0mg/mL EMETIN containing chitosan solutions were maintained. Tween 80 (Sigma, Germany) (0.5% (v/v) was added to chitosan solutions, and pH was set as 4.6–4.8. The formed EMETIN-containing chitosan solutions were blended with 0.5mg/mL TPP solutions with a volume ratio of (2: 1) (v/v) (chitosan: TPP). The nanoparticle solution was agitated for 20 min at room temperature to permit excess EMETINE adsorption on the nanoparticles to achieve isothermal equilibrium. EMETIN encapsulated chitosan nanoparticles were centrifuged at 12000 g for 30 min, dispersed in water and freeze-dried used for future use. Prepared EMETIN encapsulated chitosan nanoparticles were examined by Zetasizer Nano S (Malvern, UK) in order to determine mean average particle size distributions.[26]. nanoparticle solutions prepared for the current study were sonicated prior to application at confirmatory diagnostic unit, VACSERA-Egypt. Nanoparticles were sanitized with UV exposure preceding to the application.

**Abcam's NFκB p65 in vitro Simple Step ELISA™ (Enzyme-Linked Immunosorbent Assay).**

The kit was used for semi-quantitative measurement of NFκB p65 protein in squamous cell carcinoma cell lines (SSC-25).The Simple Step ELISA™ employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immune-capture the sample analyst in solution (enzyme-assay kit: ab176648–Fib p65 Total Simple Step ELISA™ Kit). This entire complex (capture antibody/analyst/detector antibody) is in turn immobilized via immune-affinity of an anti-tag antibody coating the well. To perform the assay, samples are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB substrate is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyst and the intensity is measured by ROBONIK P2000 ELISA READER at 450 nm.

**Statistical analysis**

Data were tested for the normal assumption using Shapiro Wilk test and proved normally distributed. Therefore, we described our variables in terms of mean ± standard deviation (± SD) and compared using one way analysis of variance (ANOVA) test. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows.

## RESULTS

**NFκB p65 expression protein level**

In the present study, after treatment of tongue scc-25 cells with both annonaceae NP and Emetine/CH NP, there was a significant increase in expression of NFκB p65 in cancer cells by (227.2 & 261.6 pg/ml) respectively at incubation period 48 hours.

While the control untreated cancer cells scc-25 revealed lower expression of NFκB p65 at (147.8 pg/ml) table (1). We also noticed that of NFκB p65 was expressed significantly more with cancer cells treated with Emetine/CH NP than in cancer cells treated with anonaceae NP fig (1).

TABLE (1). Showing highly significant difference expression of NFκB p65 between controls untreated cancer cells and the treated cancer cells with either Annonaceae NP or Emetine/Chitosan NP ANOVA test.

| Compounds | | SCC25 | |
|---|---|---|---|
| | | NFκB p65 (Total) | |
| | | pg/ml (mean ± SD) | FLD |
| 1 | Annonaceae NP | 227.2 ± 3.62 | 1.537212 |
| 2 | Emetine/CH NP | 261.6± 3.54 | 1.769959 |
| 3 | Control | 147.8± 1.5 | 1 |
| | p- value | 0.000 | |

***NP: Nanoparticles.***

***Emetine/CH NP: Emetine loaded chitosan nanoparticles.***

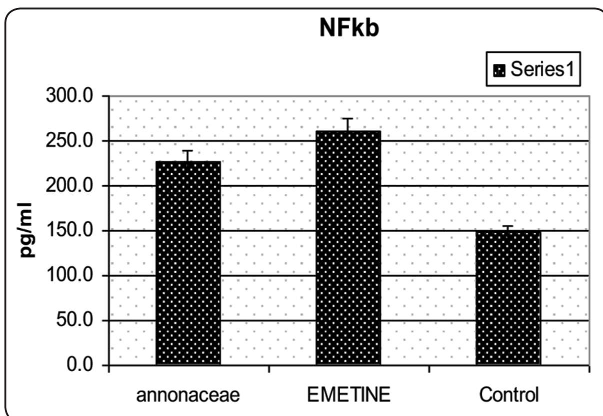


Fig. (1) Bar chart showing comparison of NFκB p65 protein expression values between groups of study. Notice the significant higher expression of NFκB p65 in announce NP/scc25 & Emetine NP/SCC25 in comparison to the untreated control SCC-25.

Moreover, it was noticed that NFκB p65 expression increased by increasing the concentration of the tested materials (annonaceae nanoparticles and Emetine/Chitosan nanoparticle which denotes a positive linear relation between the tested materials and NFκB p65 fig (2).

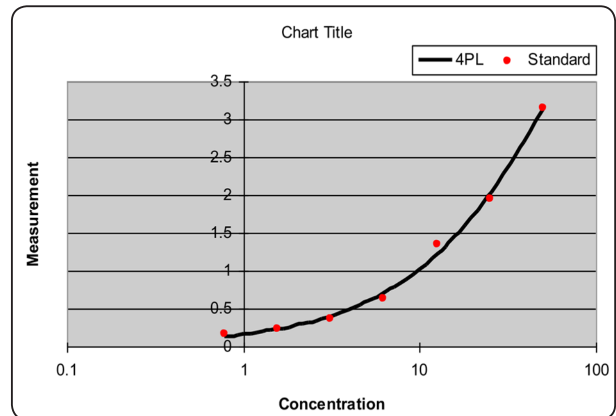


Fig. (2) Graph demonstrates a positive linear relation between the serial concentrations of tested materials and NFκB p65 expression in cancer cells (SCC-25) during the first 48 hours incubation period.

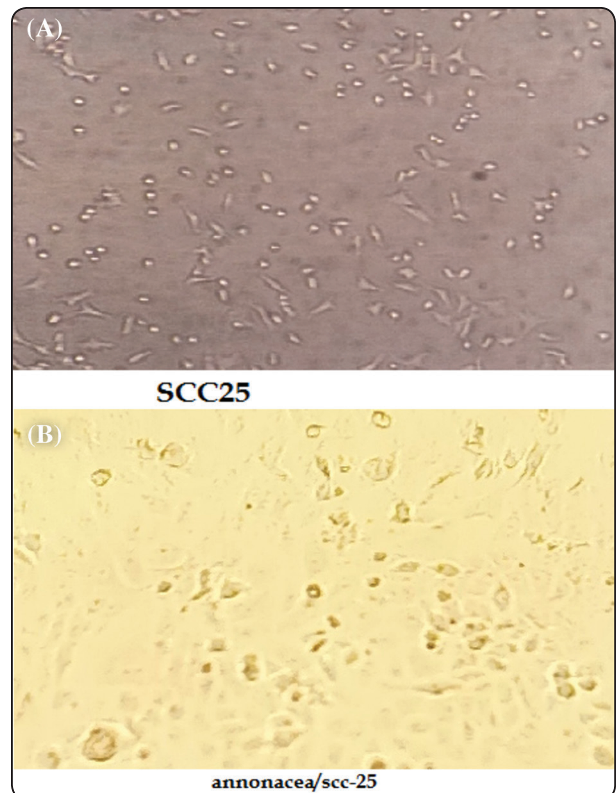


Fig. (3) Cell culture images by inverted microscope. A. SCC-25 cell line before treatment. B. SCC-25 when treatment with **annonacea** nanoparticles after 48hs incubation.

## DISCUSSION

The classic therapeutic strategies to treat caners relay on attacking the differentiated cancer cells, leaving some cancer cells that has capability of self –renewal and eventually relapse of the cancer itself [27]. Interestingly, More than 47% of current anticancer drugs on the market are natural products and more than 25,000 identified phytochemicals have been shown to possess potent anticancer activities [28]. Continuous researches are conducted to develop therapeutic medication of natural plants with implementation of nanotechnology for better cytotoxic effect of anticancer therapy on tumors cells, since the documented prominent antitumor activity of Emetine and annonacea we conducted the present research for further identification of their role in cancer therapy [12, 15, 29].

Previous studies revealed that annonaceae nanoparticles have significant ($p<0.005$) cytotoxicity at IC50 7.64 $\mu$g/ml on SSC-25 cancer cell lines when contrast to the standard untreated cancer cells at time period 48 hours, as well as growth arrest of large number of cancer cells at G1 phase 54.15% when treated by annonaceae NP. Furthermore, apoptosis of 17% of cancer cells at pre G1 Phase, while, the non-treated tongue cancer cells Scc-25 showed minimum apoptosis of 1.89 % of cancer cells [30].

In addition, it was found that Murita annonacea might have powerful anti-proliferative effect through G0/G1 cell cycle arrest and stimulation of apoptosis by interruption of membrane mitochondrial potential resulting in release of apoptotic factors leading to cell cycle arrest at G0/G1 phase and inhibiting cell proliferation on Human promyelocytic leukemia (HL-60 cells) [31].

Interestingly in the present study, after treatment of tongue scc-25 cells with both annonaceae NP and Emetine/CH nanoparticles, there was a significant increase in expression of NFκB p65 in cancer cells by (227 & 261 pg/ml) respectively at incubation period 48 hours. While the control untreated cancer cells scc-25 revealed lower expression of NFκB p65 at (147 pg/ml) table (1). In addition, we noticed that of NFκB p65 was expressed significantly higher in cancer cells treated with Emetine/CH NP than in cancer cells treated with annonaceae NP, which might reflect the stronger regulatory effect of Emetine/CH NP than annonaceae NP on NFκB p65 member of NFkB family.

These finding raises an important question, although the documented anticancer effect of annonaceae and Emetine drug [32, 33], why NF-kbP65, the member of NFkB family was highly expressed in the treated cancer cells?

Numerous researches have recorded that NF-kB act as a tumor-promoting transcription factor[34]. Many studies revealed that NF-ϰB pathway is switched on in all types of cancers including oral cancer where it commands the expression of genes regulating the transformation, survival, proliferation, apoptosis, invasion, angiogenesis, and metastasis of tumor cells [35, 36]. So there was a growing concept about being NF-ϰB signaling pathway is a target for anticancer therapy [37].

But other studies have started to introduce new function of NF-kB as a promoter of apoptosis and deterioration [38-39]. It was found that canonical NFκB stimulates enrolling of FADD and caspase 8 to the death-inducing signaling complex to increase tumor cell sensitivity to Fas mediated apoptosis in tumor cells thus promoting the cytotoxic effect of chemotherapy, which might show the tumor-suppressor function of NF-kb [40].

An interesting study by Liu F et al 2012, they reported that canonical pathway of NF-κB functions in an opposing way in stimulating FasL-induced apoptosis, they found that the canonical NF-κB is a Fas transcription activator which upregulates Fas-mediated apoptosis [41]. Therefore, a regulatory procedure might take place between pro survival signals derived from other oncogenes and canonical NFκB activity which may promote apoptosis.

Therefore, canonical NF-kB signaling might enhances tumor cell sensitivity to Fas-mediated apoptosis thereupon exerting a tumor-suppressor role. On the contrary, they found that, alternate NF-kB (non-canonical pathway) is a Fas transcription repressor in the two human colon carcinoma cells and MEF cells [42].

Bister, P et al 2010 explained that alternate NF-κB is frequently consecutively activated after the canonical NF-B, so, it is conceivable that alternate NF-κB might function as a Fast transcription repressor to shut off canonical NF-B-activated Fast transcription to stop sustained Fast transcription activation [43].

Also another study by Yang HJ et al, 2015 applied on neuroblastoma cells SH-EP1, they revealed the pro-apoptotic effect of NF-ϰB stimulation in the course of Fas-induced apoptosis, where NF-ϰB p65 was transfered to nucleus where NF-ϰB activity was obvious through the apoptotic process by enhancing the expression of caspase-4. The previous results might verify that NF-ϰB plays a role in Fas killing through caspase-4 protease and high light that caspase-4 is a new mediator of NF-ϰB pro-apoptotic pathway in neuroblastoma cells [44].

Another study by Saegusa et.al, 2007. They investigated the alterations in NF-ϰB and β-catenin signaling pathways linking them to E-cadherin, vimentin, GSK-3β, Snail mRNA, TNF-α, and estrogen and progesterone receptors (ER and PR) expression. The results of their study revealed significant rise of NF-kbp65 levels in endometrial glandular cells in the secretory phase with reference to increase of vimentin and E-cadherin expression. They explained that the increase of NF-ϰB/p65 signals are mandatory for reconstruction and repair of the epithelial cells secretory properties [45].

The inhibition of NF- ϰB could be useful in treating inflammatory disease, but the NF- ϰB role has important need to maintain the cell survival and immune response of the body against inflammation, so the inhibition of NF- ϰB could cause undesirable side effect. It was found that the chemotherapeutic approach of some agents can hinder NF- ϰB signaling, which could develop resistance to Fas-mediated apoptosis, thus quell the Fas-mediated apoptosis of the host cancer immune system [46].

From the previous studies, a growing concept about the dual role of NF-κB either by acting through the canonical pathway (NF-ϰB/p65 signals) exerting tumor-suppressor function through Fas-mediated apoptosis and the alternate NF-κB which might function as a oncogenesis transcription factor providing a strong target for anticancer strategies. So it is highly recommended that therapeutic strategy to inhibit NF-κB signaling should consider the dual action of NF-kb pathways to avoid tumor cell resistance to apoptosis and host immune surveillance system due to total inhibition of NF-κB canonical pathway.

The recent study provided by Haykal T et al, 2019 and Ammoury C et al, 2019 proved the apoptotic effect of annonaceae extracts. They reported that Annona cherimola Seeds Extract (ASEE) promotes upregulation of pro-apoptotic proteins like p53, as well as cleaved poly (ADP-ribose) polymerase (PARP)-1 to induce apoptosis and inhibition of cell proliferation, by increase of cytochrome-c release from mitochondria leading to upregulation of cleaved caspase-8 and cleaved caspase-9 [47]. Moreover, Annona cherimola ethanolic leaf extract displayed evident pro-apoptotic effect on Acute Myeloid Leukemia cell lines in vitro, through the upregulation of Bax, downregulation of Bcl2, and cleavage of PARP [48].

In reference to the chemotherapeutic procedure of Emetine (EMTDTC-55). it was shown to depend on promoting PMEPA1, which is involved in down-regulation of the androgen receptor, and increased ubiquitination and proteosomal degradation of the androgen receptor, alongside with an increased expression of CDKN1A, NFKB1 and TP53,

its activity was dependent on initiation of apoptosis and repression the viability ,migration, and invasion in breast cancer cells in addition to suppression of Wnt/β-catenin signaling cascade in metastatic androgen-independent PC3[49,50].

From the previous studies and our observation in the present study, we can suggest that annonaceae plant extract nanoparticles and Emetine nanoparticles might have anti-cancer sequel and may regulate the NF-κB pathway in the direction of apoptotic cascade and cell cycle arrest by upregulation the NF-kB/65 which is considered a key player in the NF-κB canonical pathway.

## CONCLUSION

The annonaceae and Emetine nanoparticle might have direct regulatory effect against SCC-25 cell lines by upregulation of NF-kb/p65 signals. Emetine_CH/NP revealed significant higher upregulation of NF-kb/p65 expression in comparison to Annonaceae NP which might reflect the stronger regulatory effect of Emetine/CH nanoparticles.

## RECOMMENDATION

Further studies should be performed to analyze the annonaceae extracts and their effect with other factors present in cell signal cascade controlling NF-κB canonical and alternate pathways. In addition, great attention should apply to therapeutic strategies which implement inhibitory mechanism on NF-κB pathway.

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## Compliance with ethical standards

Conflicts of interest. The authors declare that they have no conflict of interest.

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