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Annonacin and Squamocin Conjugation with Nanodiamond Alters Metastatic Marker Expression in Breast Cancer Cell Line

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ABSTRACT

Breast cancer can perform metastasis to distant organs and cause more than 90% of malignancy-related deaths. The anti-metastasis potency of nanodiamond-conjugated annonacin and squamocin against MCF-7 cells is currently studied. First, IC_{50} determination of both free annonacin and squamocin to evaluate their potency as cytotoxic agents. Upon getting the IC_{50} value, both compounds are conjugated into nanodiamonds. Drug loading efficiencies of nanodiamond-conjugated annonacin and squamocin are 88.9% and 89.1%, respectively. Meanwhile, the ND-annonacin and ND-squamocin complex size is 150-300 nm based on SEM imaging. Subsequently, cell viability assessment of MCF-7 was performed with six cohort designs, namely, K (control cell), AN (annonacin), SQ (squamocin), NDAN (nanodiamond-conjugated annonacin), and NDSQ (nanodiamond-conjugated squamocin). Both IC_{50} and cell viability are assessed by MTT assay after 24 h incubation. All cohorts also underwent gene expression analysis subject to the metastasis markers *CTNND1* (catenin delta 1), *NOTCH4*, and *C-JUN*. Here, the IC_{50} of both free annonacin (4.52 µg/ml) and squamocin (10.03 µg/ml) are more than IC_{50} of potent anticancer (< 4 µg/ml) for pure compounds. However, nanodiamond conjugation to both compounds can decrease cell viability better than free compounds. Compared to K, nanodiamond-conjugated annonacin and squamocin significantly decreases cell viability after 24 h incubation. Bioinformatics analysis confirmed significant pro-metastasis (*C-JUN* and *NOTCH4*) upregulation and anti-metastasis (*CTNND1*) downregulation in tumors compared to normal. Recent findings demonstrated that nanodiamond-conjugated annonacin can significantly upregulate *CTNND1* and significantly downregulate *C-JUN* and *NOTCH4*. Even so, nanodiamond-conjugated squamocin upregulate *CTNND1* but not significantly and significantly downregulate *C-JUN* and *NOTCH4*.

1. Introduction

Breast cancer is a leading cause of death in women, with approximately 2.3 million new cases and 685,000 deaths reported worldwide in 2020 (Lei *et al.* 2021). Indonesia has 19.2% breast cancer cases, which is the most emerging cancer, with the majority (60-70%) cases reported in late stage (stage III and IV) (Gautama 2022). Such a heterogeneous disease that emerged by the association between genetic risk and environmental factors, breast cancer, poses major subtypes, namely luminal, $HER2^+$ and triple-negative (Ataollahi *et al.* 2015; Zhou *et al.* 2020). Breast cancer is known to perform metastasis, allowing malignant cells to spread to other organs through epithelial-to-mesenchymal transition (EMT) (Zhou *et al.* 2020). Metastasis is primarily responsible for treatment failure and causes more than 90% of malignancy-related deaths (Wang *et al.* 2021). Here, several markers such as *NOTCH4, C-JUN/AP-1*, and *CTNND1* (catenin delta 1) have been associated with invasive features of aggressive breast cancer (Zhang *et al.* 2007; Zhou *et al.* 2020; Lin *et al.* 2021). Breast cancer therapy may vary and optimally depends on tumor subtype, anatomic cancer stage, and patient

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preferences (Waks and Winer 2019). Among several endeavours, chemotherapy is preferred as part of primary breast cancer treatment to induce tumor cell death and reduce tumor mass. Meanwhile, the main treatment for cancer metastasis also uses chemoagent-based therapy (ACS 2022). However, chemoresistance has been redundantly reported due to fast drug efflux from the cell (Benson and Amini 2020).

In accordance with the need for effective anticancer agents in resolving chemoresistance, nanodiamonds or NDs (carbon-based nanomaterial) offers potency as a drug carrier due to their ability to control drug efflux that leads to good drug bioavailability in tumor. NDs exhibit tunable surfaces, excellent biocompatibility, and a large surface area for the conjugation of drugs for intra and extracellular delivery. In addition, NDs are very potent as a carrier in drug delivery systems with enhanced drug efficacy and decreased toxicity level, and thus can act as a safer medication (Li *et al.* 2014; Chauhan *et al.* 2019). NDs can be designed with different functional groups, allowing interaction with water molecules or biologically relevant conjugates. This interaction has resolved dose-dependent side effects by releasing the drug without any chemical modification, as reported in ND-conjugated anthracyclines (Benson and Amini 2020). Although NDs have good biocompatibility, they are still a foreign, non-degradable material for biological organisms and may possess side effects when their exposure concentration exceeds a certain limit. Many studies that aimed to evaluate the side effects of NDs concluded that NDs have no or a small toxic side effect on biological systems. Under a different route of administration, the in vivo absorption, distribution, excretion, and metabolism of NDs may be different, which allows us to understand better the risk that NDs may pose to human health and provides an important basis for designing an ND-based drug delivery system (Liu *et al.* 2010; Zhu *et al.* 2012; Li *et al.* 2014; Zhang *et al.* 2015).

Regarding abundant anticancer agents already available, two annonaceous acetogenin compounds originating from *Annona muricata* (annonacin) and *Annona squamosa* (squamocin) hold promising therapeutic as cytotoxic agents. Annonacin is reported to have cytotoxic effects against HeLa and HeLa S3 cervical cancer cell lines, SKOV3 and PA-1 ovarian cancer cell lines, BCC-1 skin cancer cell line, and MCF-7 breast cancer cell line (Wahab *et al.* 2018). In the meanwhile, squamocin shows cytotoxic activity towards several cancer cell lines, including MCF-7 (breast cancer); H460 (lung cancer); BGC 803 (gastric cancer); BEL 7402, HepG2, dan SMCC-7721 (liver cancer) (Miao *et al.* 2016). These annonaceous acetogenins can modulate cell apoptosis via activating apoptosis effector caspase 3 and 8, which can enhance cell death with a tiny dose. Thus, both annonacin and squamocin may overcome the dose-dependent side effects of chemo agents (Jacobo-Herrera *et al.* 2019). Previous in vivo studies reported that conjugation of annonacin and squamocin with NDs increased the anti-cancer activity of the bioactive compounds in rats-induced breast cancer (Dewi *et al.* 2022, 2023). Nevertheless, the potency of NDs as a carrier of annonacin and squamocin to regulate the expression of *NOTCH4*, *C-JUN/AP-1*, and *CTNND1* as metastatic markers in breast cancer cells has not yet been reported.

This study aimed to know the antimetastatic effect of annonacin and squamocin, which are both conjugated to ND towards the breast cancer cell line, Michigan Cancer Foundation-7 (MCF-7). The use of ND as a drug carrier rather than free drug compounds is expected to further reduce cell viability by decreasing the expression of pro-metastatic markers such as *NOTCH4* and *C-JUN/AP-1*, as well as increasing anti-metastatic marker, *CTNND1*.

2. Materials and Methods

2.1. Materials

Annonacin and squamocin (Anhui Minmetals Development LTD, China); nanodiamond (particle size : <10nm) (Carboxyl-modified), TCI America™; NaOH; DSPE-PEG; chloroform; MCF-7 cell line; Roswell Park Memorial Institute (RPMI) medium; fetal bovine serum (FBS); Penicilin-Streptomycin; 3-(4,5'-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reagent; phosphate buffer saline (PBS); dimethyl sulfoxide (DMSO); SV Total RNA Isolation System (Promega); GoScriptTM Reverse Transcription System (Promega), GoTaq® qPCR Master Mix (Promega); primer forward and reverse for *AP-1, NOTCH4*, and *CTNND1* (Integrated DNA Technologies, Inc.); cDNA sample, H_2O, SYBR green (Sigma Aldrich).

2.2. Synthesis of Nanodiamond-Conjugated Annonacin and Squamocin and Characterization

Prior to conjugation, nanodiamond (ND) was first designed to bear the carboxyl groups by performing EDC-NHS coupling. NDs 1 mg/ml in deionized water were sonicated for 5 min. EDC (8.35 µg), and Sulfo-NHS (9.5 µg) were added to NDs, and then the solution was stirred for 30 min. mPEG-amine (200 µg) was added to the NDs solution and stirred overnight at room temperature. After overnight incubation, centrifugation at 14,000 rpm for 2 h was performed with NDs solution. The pellets were subsequently washed using distilled water and dispersed in 2.5 mM NaOH (Dewi *et al.* 2022, 2023).

Annonacin and squamocin in DMSO were added to NDs solution after sonicated for 5 min. The mixture was placed on the shaker and incubated overnight to let conjugation. NDs solution was then performed centrifugation (14,000 rpm, 2 h). The supernatant was measured with its absorbance at 589 nm to assess the concentration of the unconjugated mixture. Finally, the pellets were gain-washed in distilled water and ready for treatment. The binding efficiency of annonacin and squamocin to NDs (E) can be calculated using the formula below (Locharoenrat 2019):

$$E = \frac{\text{Concentration (before conjugation - after conjugation)}}{\text{Concentration before conjugation}} \times 100\%$$

The characterization of nanodiamonds conjugated with annonacin and squamocin was performed by UV-Vis spectrophotometer, scanning transmission microscope (TEM), and scanning electron microscope (SEM) imaging.

2.3. IC_{50} Determination of Annonacin and Squamocin

MCF-7 cells were cultured in triplicate in a 96-wells plate with a density 5 × 10^3 cell/100 µl, then incubated overnight inside an incubator (37°C, 5% CO_2) to let cell adherence. The cells were treated with series concentrations of both annonacin (AN) and squamocin (SQ), 0.2, 0.4, 0.8, 1.6, 3.2, 6.25, 12.5, 25, 50 and 100 µg/ml and then performed incubation (37°C, 5% CO_2) for 24 h. Subsequently, MTT reagent 100 µl was added to every well. The reaction was stopped by adding SDS 10% in 0,1 N HCl after 6 h incubation and then re-incubated overnight without illumination at room temperature. Absorbance was read at 595 nm. Linear regression was obtained as y = a + bx, which was used to determine each sample's IC_{50} (50% inhibitor concentration) value. By entering 50 in variable y, the value of x was obtained as the value of IC_{50} (He *et al.* 2016; Dewi *et al.* 2022).

2.4. Cell Viability Assessment

MCF-7 cells were cultured in 96-wells plates with the density 2.5 × 10^3 cells/ml. The culture was then incubated overnight inside an incubator (37°C, 5% CO_2) to let cell adherence. Subsequently, the cells were exposed to 12.5 µg/ml annonacin (AN); 12.5 µg/ml squamocin (SQ); 12.5 µg/m nanodiamond-conjugated annonacin (NDAN); 12.5 µg/ml nanodiamond-conjugated squamocin (NDSQ). The previous cohort was denoted as the treated group, while the untreated group or control was MCF-7 cells only. Both treated and untreated groups were made in quadruplicate and incubated inside an incubator (37°C, 5% CO_2) for 24 h. As much as 20 µL of MTT reagent was added to every well. Incubation (37°C, 5% CO_2) was performed in 4 h. Solution was replaced by 100 µl DMSO to dissolve the formazan crystals upon incubation. Absorbance was further read at 595 nm (Ghasemi *et al.* 2021).

2.5. Bioinformatics and Data Analysis

Bioinformatics analysis was performed to validate the different expressions of the preferred genes (*CTNND1, C-JUN*, and *NOTCH4*) between normal and breast cancer patients. Here, mRNA expression of preferred genes in breast cancer patients was obtained from GEO datasheet (Accession No. GSE54002, https://www.ncbi.nlm.nih.gov/geo/GSE54002) and (GSE45821, https://www.ncbi.nlm.nih.gov/geo/GSE45821). The correlations between copy number gain and mRNA expression were obtained through the cBio Cancer Genomics Portal (http://www.cbioportal.org/). All bioinformatics data were accessed and analyzed accordingly (Dewi *et al.* 2018).

2.6. RNA Expression Analysis

Isolation of mRNA following Spin protocol (Promega). Design forward and reverse primers for *NOTCH4, C-JUN,* and *CTNND1* (Table 1) were obtained from Genebank (https://www.ncbi.nlm.nih.gov/) and assessed by NetPrimer: Premier Biosoft, Primer3Plus, and PrimerBlast. Here, Actin (ACTB) primer was a control or reference gene. PCR reactions were carried out in a Rotor gene (MyGo Pro) thermocycler. The fold change of mRNA expression was calculated by the pattern below:

Table 1. Forward and reverse primers for target sample and control

Gene	Primer forward (5'→3')	Primer reverse (5'→3')
NOTCH4	CTAGGGGCTCTTCTC GTC CT	CAACTTCTGCCTTTGG CTTC
C-JUN	CCCCAAGATCCTGAA AC AGA	CCGTTGCTGGACTGGA TTAT
CTNND1	TCTGCCATAGCTGAC CTCCT	GGAGTTCT CTGTCCTC CTG
ACTB	CCACACTGTGCCCAT CTA CG	CAACTTCTGCCTTTGG CTTC

$$2^{-}[(Ctg - Cct) - average\ of\ Cct]$$

Description:
Ct = cycle threshold
tg = target gene
ct = control gene

2.7. Statistical Analysis

All data were obtained as mean ± SD. Statistical analysis was performed using IBM SPSS statistics 25 and Graphpad Prism 8. Shapiro -Wilk test (α>0.05) was performed to evaluate data normality. Either parametric or nonparametric analysis was further carried out depending on the normality of the data.

3. Results

3.1. Characterization of Annonacin and Squamocin Conjugation with Nanodiamond

The result of characterization by TEM microscopy showed that the size of ND alone was less than 10 nm, while the size of ND-annonacin and ND-squamocin complex was 150-300 nm accordingly SEM microscopy. The drug loading efficiency of annonacin coating on ND and squamocin coating on ND were 88.9% and 89.1%, respectively, based on the analysis with UV-Vis spectrophotometer (Figure 1).

3.2. Free Annonacin and Squamocin Were Toxic Towards Mcf-7, But Conjugation with Nanodiamond Decreased Cell Viability More Significantly

Both annonacin and squamocin conveyed a trend of decrease in cell viability with increasing concentration after 24 h. Among these two compounds, annonacin (IC_{50} = 4.52 µg/ml) was more toxic to MCF-7 cells than squamocin (IC_{50} = 10.03 µg/ml). Further conjugation of both pure compounds with nanodiamonds was assessed for their cytotoxicity against MCF-7. After 24 h incubation, all cohorts exhibited statistically highly significant (P = 0.0009) based on Brown-Forsythe test. Based on unpaired t-test analysis (P<0.05), AN (P = 0.0008), NDAN (P = 0.0008), and NDSQ (P = 0.0021) significantly decreased cell viability compared to K. Based on descriptive statistics, NDAN (79.78±5.54%) reduced cell viability better than AN (89.23±17.64%). Even so, NDSQ (84.27±5.07%) reduced cell viability better than SQ (96.59±10.23%). This result proved the conjugation of annonacin and squamocin with nanodiamonds could enhance the cytotoxic effect over free annonacin and squamocin (Figure 2).

3.3. The Correlation between Copy Number Gain and mRNA Expression of *Ctnnd1, C-Jun,* and *Notch4* in Breast Cancer Patients

The expression levels of *CTNND1, C-JUN*, and *NOTCH4* were derived from copy-number analysis algorithms and indicate the copy number level per gene. Deep deletion (-2) indicates a deep loss, possibly a homozygous deletion; shallow deletion (-1) indicates a shallow loss, possibly a heterozygous deletion; diploid (0) indicates no deletion/amplification; gain (1) indicates low-level gain; amplification (2) indicates high-level amplification or more copies. The analysis expression of *C-JUN* showed the presence of deep deletion and shallow deletion, while *CTNND1* and *NOTCH4* only exhibit shallow deletion. In addition, the result indicates *CTNND1* and *C-JUN*, but not *NOTCH4*, have a positive correlation between mRNA expression and copy number gain in breast cancer patients. The expression of *CTNND1* and *C-JUN* were high in breast cancer patients, while the expression of *NOTCH4* mRNA did not increase in breast cancer patients (Figure 3).

3.4. The Expression of *CTNND1, C-JUN,* and *NOTCH4* in Normal and Breast Cancer Patients

To know the alteration of *CTNND1, C-JUN*, and *NOTCH4* expression in breast cancer patients, *in silico* analysis of both normal and breast cancer patients was performed. All gene data were obtained from GEO datasheets (https://www.ncbi.nlm.nih.gov/gds). Based on datasheet analysis (Figure 4), the expression of *C-JUN* and *NOTCH4* were significantly

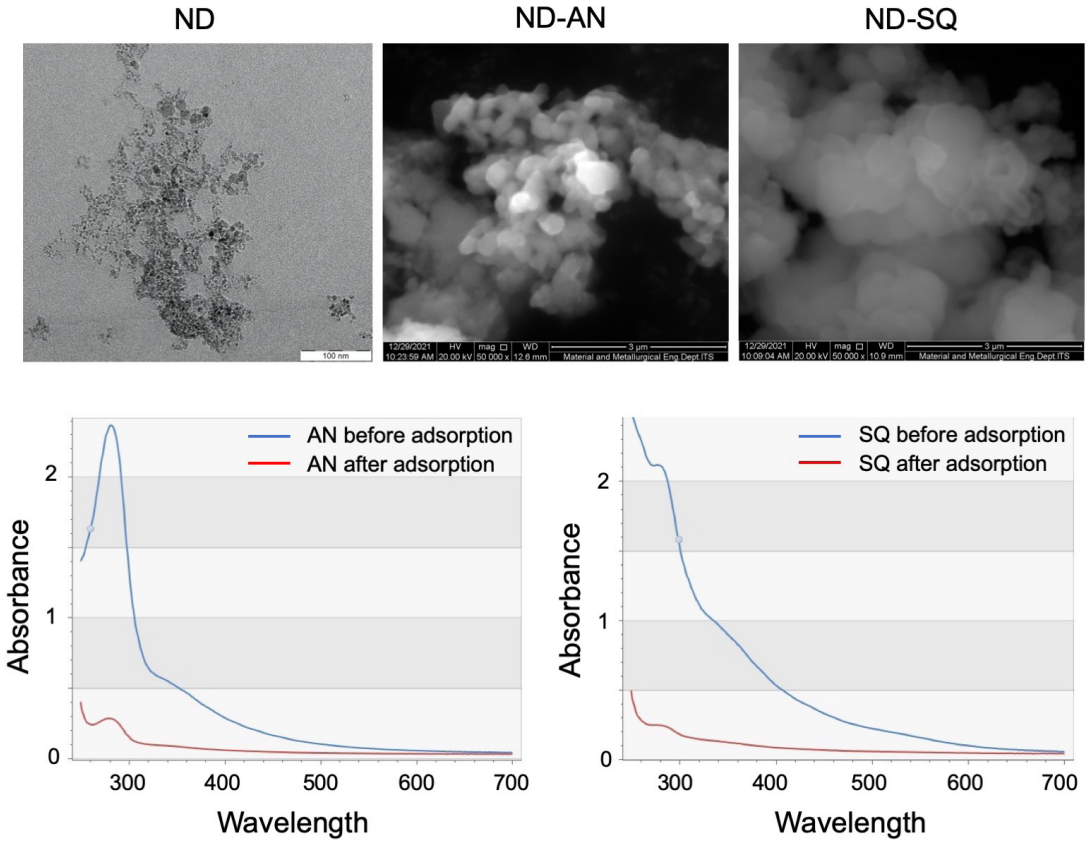

Figure 1. TEM imaging of nanodiamond (ND) and SEM imaging of nanodiamond-conjugated annonacin (ND-AN) and nanodiamond-conjugated squamocin (ND-SQ) (upper); UV-Vis spectra showed absorbance before and after loading of both annonacin (AN) and squamocin (SQ)

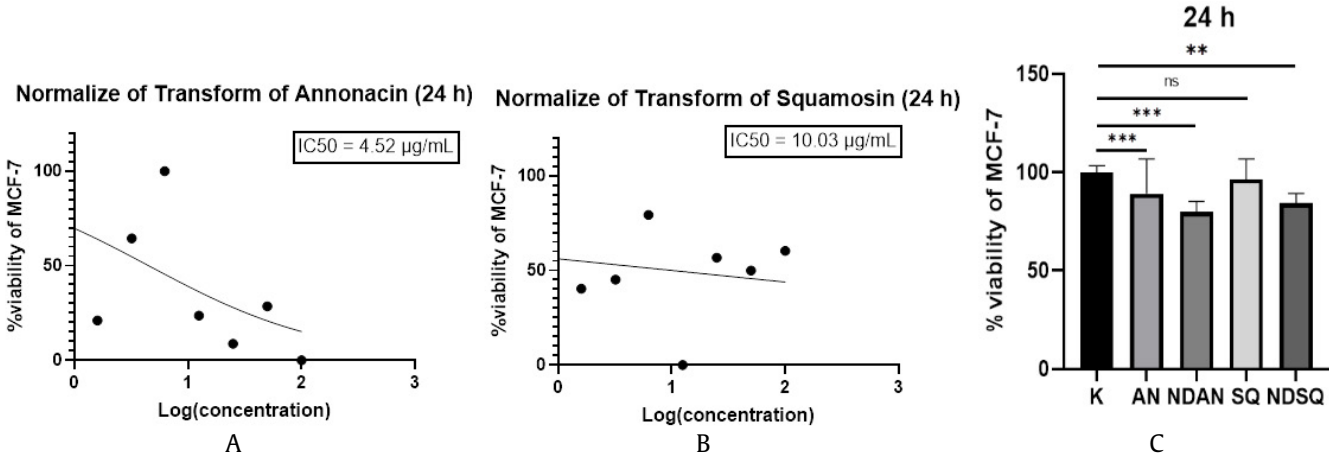

Figure 2. Non-linear regression showing cytotoxic activity and IC_{50} of free annonacin and squamocin; MCF-7 cell viability after being treated by annonacin (AN), nanodiamond-conjugated annonacin (NDAN), squamocin (SQ), and nanodiamond-conjugated squamocin (NDSQ) (*** indicate P value < 0.001, ** indicate P value < 0.01)

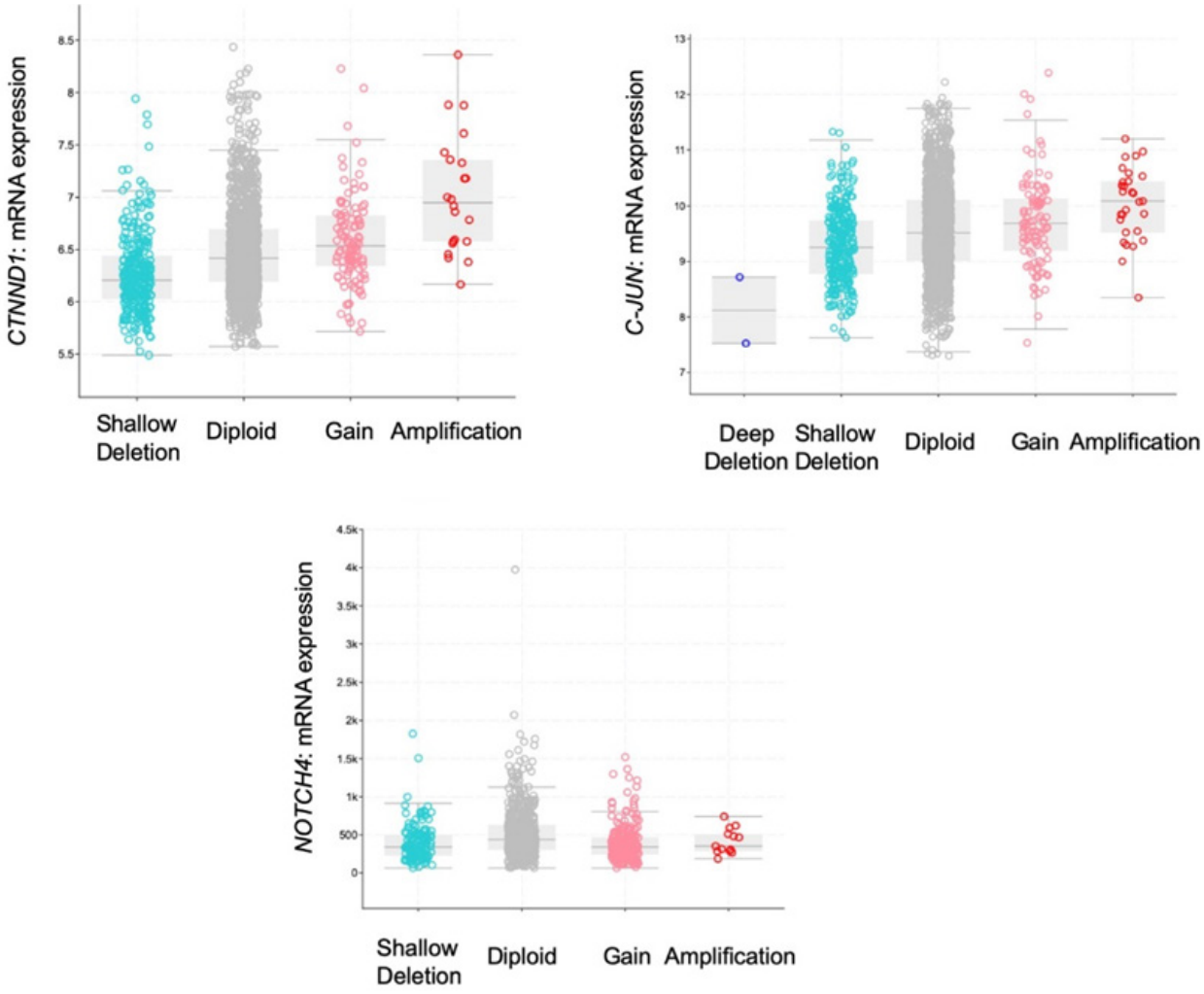

Figure 3. Box plots presenting the correlation between mRNA expression and copy number gain of *CTNND1*, *C-JUN*, and *NOTCH4* in breast cancer patients. The numerous copy number categories (deep deletion; shallow deletion; diploid; gain; amplification) are indicated along the x-axis

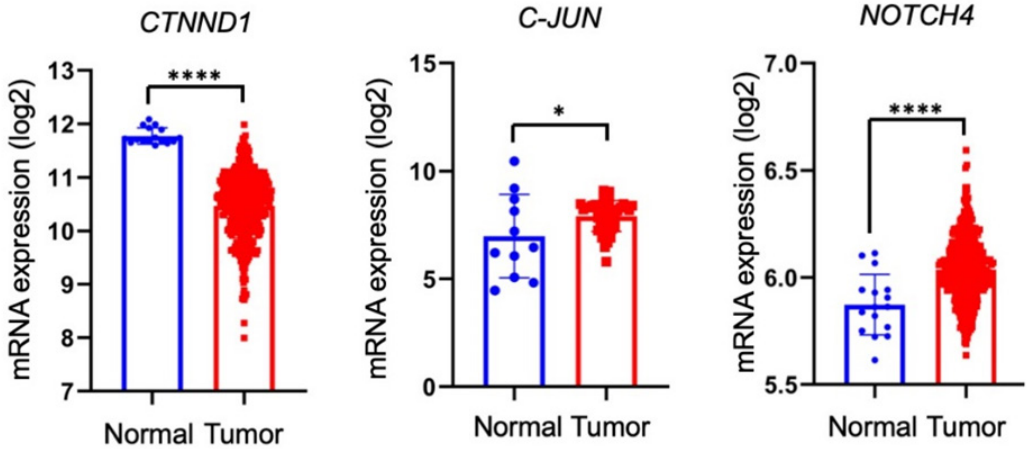

Figure 4. Difference *CTNND1*, *C-JUN* and *NOTCH4* expression among normal and breast cancer patients (**** indicate P value < 0.0001, * indicate P value < 0.05)

upregulated in tumor patients compared to nontumor patients. Conversely, the expression of *CTNND1* was significantly downregulated in tumor patients over nontumor patients.

3.5. Nanodiamond-conjugated Annonacin and Squamocin Influence the Expression of Metastatic Markers in MCF-7 Cells

Expression of *CTNND1* was upregulated in AN (1.03±0.621-fold), NDAN (2.11±0.092-fold), and NDSQ (2.43±0.493-fold) weighed up to K (1.01±0.224-fold). This upregulation was significant in NDAN (P = 0.0237) only. Conversely, downregulation of *CTNND1* expression was reported in SQ (1.00±0.221-fold) but not significantly compared to K (1.01±0.224-fold). All cohorts, AN (0.196±0.029-fold; P = 0.0157), NDAN (0.098±0.005-fold; P = 0.0108), SQ (0.230±0.056-fold; P = 0.0185), and NDSQ (0.074±0.010-fold; P = 0.0099), exhibited significant decrease of *C-JUN* expression over K (1.049±0.365-fold). Meanwhile, the expression of *NOTCH4* was downregulated in all treatment groups. Herein, downregulation of *NOTCH4* in NDAN (0.2673±0.043-fold; P = 0.0086) and NDSQ (0.4241±0.326-fold; P = 0.0469) were significant compared to K (1.180±0.326-fold). However, downregulation of *NOTCH4* in AN (0.4889±0.420-fold; P = 0.0876) and SQ (0.8132±0.157-fold; P = 0.1540) were not significant compared to K (1.180±0.326-fold) (Figure 5).

4. Discussion

Metastasis allows cancer cells to depart from site of origin, spread systemically, and colonize at a distant organ, results in challenging treatments including resistance to cytotoxic agents (Jin and Mu 2015). Metastasis is associated with aberrant gene expression. In this study, metastasis marker gene expression alteration was reported in breast cancer patients (Figure 3 and 4). In particular, pro-metastatic markers *C-JUN* and *NOTCH4* were significantly upregulated in breast cancer patients compared to non-cancer/normal patients. A previous study reported that *C-JUN* was expressed in metastatic lung tumors, but normal lung tissue generally did not express *C-JUN* (Szabo *et al.* 1996). *C-JUN* expression was also reportedly higher in colorectal adenocarcinoma compared to control (Wang *et al.* 2000). *NOTCH4* expression was significantly higher in breast cancer patients compared to normal (Figure 4). Most studies suggested that *NOTCH4* is abnormally overexpressed during cancer development and is involved in the regulation of several tumor-cell behaviours, mainly in stem cell-like self-renewal, epithelial–mesenchymal transition (EMT), radio-/chemo resistance and angiogenesis (Wu *et al.* 2014; Lin *et al.* 2016; Wu *et al.* 2018; Shaik *et al.* 2020; Xiu *et al.* 2021). In contrast to *C-JUN* and *NOTCH4*, the expression of

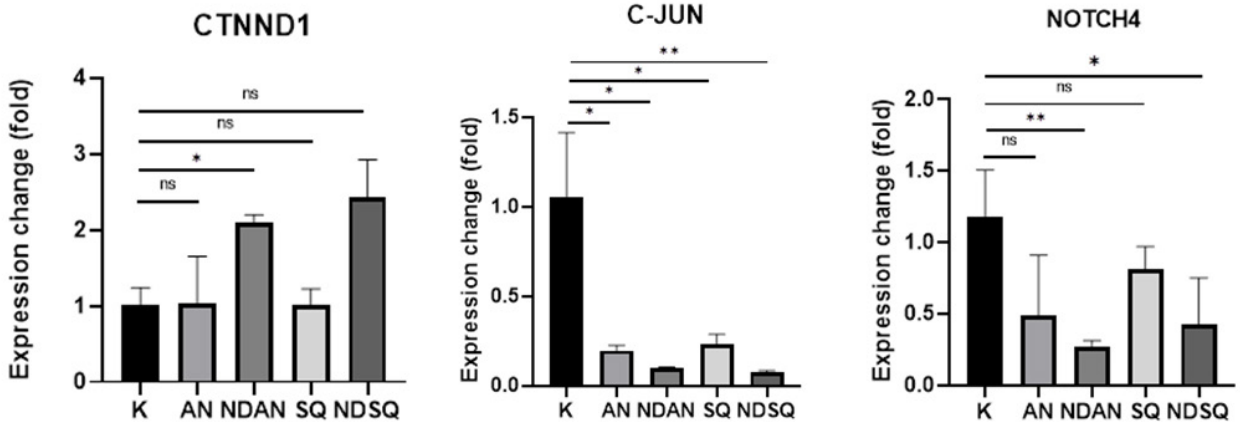

Figure 5. Expression of metastatic marker of MCF-7 cells after being treated by annonacin (AN), nanodiamond-conjugated annonacin (NDAN), squamocin (SQ), and nanodiamond-conjugated squamocin (NDSQ) (** indicate P value < 0.01, * indicate P value < 0.05)

CTNND1 was significantly lower in breast cancer patients (Figure 4). A similar result was reported by Lin *et al.* (2021), showing the reduction of *CTNND1* in bone-metastatic tumors from triple-negative breast cancer (TNBC) patients. The bone metastasis by silencing *CTNND1* was mediated by upregulation of CXCR4/CXCL12 axis and neutrophils infiltration in bone.

In addition to the results of bioinformatics analysis, the *in vitro* study revealed that the expression of *C-JUN* showed an upregulation in the control (K) group, while treatment of the drug formulation was able to reduce *C-JUN* expression (Figure 5). Targeting *C-JUN* in adenocarcinomic human alveolar basal epithelial cells (A549) exhibits antiangiogenic activity *in vitro* and *in vivo* through exosome/miRNA-494-3p/PTEN signalling pathway (Shao *et al.* 2021). Overexpression of *NOTCH4* is reported in cancer development and is involved in EMT, angiogenesis and even radio-/chemo resistance (Xiu *et al.* 2021). In breast cancer, RNA-binding protein (RBP) DCAF13 induces *NOTCH4* overexpression by inhibiting the activation of the E3 ubiquitin-protein ligase DTX3 that prevents *NOTCH4* degradation (Liu *et al.* 2020). *In vitro* study indicates crosstalk between *NOTCH4* and EMT-promoting Stat3-MMP signaling in BC metastasis: N4ICD can physically interact with the EMT-promoting Stat3 protein to activate it in BC cells, promoting upregulation of MMP2 expression (Bui *et al.* 2017). Overexpression of *NOTCH4* in the untreated cohort (K) is also indicated in this study (Figure 5).

On the contrary, silencing *CTNND1* (catenin delta 1) expression may contribute to breast cancer cells' survival in distant organ microenvironment, especially bone. In the TNBC case, *in vivo* studies conveyed the knockdown of *CTNND1* increases both tumor cell metastasis to bone and neutrophil infiltration in bone. Even so, *CTNND1* downregulation can accelerate epithelial–mesenchymal transformation (EMT) of tumor cells and their migration to bone by upregulating CXCR4 via activating the PI3K/AKT/HIF-1α pathway (Lin *et al.* 2021). In our study, the treatment of annonacin and squamocin conjugated onto nanodiamond increased the expression of *CTNND1* compared to the control group (Figure 5).

Nowadays, a cytotoxic agent that harms the tumor as well as eradicate metastasis is vastly available. However, research to discover drugs with better effectiveness is still being carried out. One of them is a drug from pure compounds isolated from plants. Annonacin and squamocin are among the pure compounds that potent to be evaluated as anticancer drugs. As per standard, pure compounds with IC_{50} 4 µg/ml or less in cell culture studies are further being used as chemotherapeutic agents in preclinical studies using animal models (Badisa *et al.* 2009). However, IC_{50} of free annonacin (4.52 µg/ml) and squamocin (10.03 µg/ml) against MCF-7 obtained in this study cannot be categorized as potent (Figure 2). Even though treatment of annonacin in endometrial cancer cell lines (ECC-1 and HEC-1A) exhibited potent antiproliferative effects (IC_{50} = 4 µg/ml) while squamocin caused apoptosis on T24 bladder cancer cells (Yuan *et al.* 2006; Yap *et al.* 2017). The difference in the effectiveness of this cytotoxic activity is due to the different genetic make-up of each cell culture (Mansoori *et al.* 2017). Modification on drug design by performing nanodiamond conjugation is expected to increase drug effectivity in reducing cell viability. Herein, both annonacin and squamocin that are conjugated to nanodiamond can significantly decrease cell viability in comparison to K (control cell) at 24 h exposure. Moreover, cell viability in nanodiamond-conjugated annonacin and squamocin is lower than in free annonacin and squamocin (Figure 2). This is because nanodiamond can maintain drug bioavailability by controlling drug efflux in tumor (Benson and Amini 2020).

In this study, nanodiamond-conjugated annonacin and squamocin suppress the expression of *C-JUN* and *NOTCH4* (Figure 5), commonly overexpressed in cancer cells. Previous studies reported that conjugation of annonacin and squamocin onto nanodiamond increased the anti-cancer activity of annonacin and squamocin in rat-induced breast cancer (Dewi *et al.* 2022, 2023). Conjugation of annonacin with nanodiamond significantly reduced the serum level of CA-153, a metastatic marker in breast cancer, and significantly increased p53 expression (Dewi *et al.* 2022). Acetogenin (a large group of annonacin and squamocin) can downregulate cyclin D1 (*CCND1*), the gene mediated intercellular efflux pumps-mediated chemoresistance, via p53 mediation as reported in T47D breast cancer cells (Kanedi 2016; Agu *et al.* 2018;). Upon silencing cyclin D1, *C-JUN* also inhibited leading to cell cycle arrest at G1/S transition (Jacobo-Herrera *et al.* 2019; Koeppel *et al.* 2011). Acetogenin found in *A. squamosa* inhibit

53.54% of tumor growth in mice bearing H22 cells through reducing inflammatory cytokine, IL-6 (Chen *et al.* 2016). Meanwhile, IL-6 plays role in Notch expression (Guo *et al.* 2011). The reduction of *NOTCH4* expression in this study presumably correlates with this axis. On the contrary, the expression of-catenin delta 1 (*CTNND1*) was upregulated in the group treated by nanodiamond-conjugated annonacin and squamocin (Figure 5). *A. muricata* leaf extract induces apoptosis and inhibits cell proliferation by silencing phosphor-AKT in in MIA PaCa-2 cells (Yiallouris *et al.* 2018). It makes sense as the deactivation of AKT signalling associated with *CTNND1* upregulation (Lin *et al.* 2021).

This study reveals that free annonacin (IC_{50}: 4.52 µg/ml) and squamocin (IC_{50}: 10.03 µg/ml) are toxic to MCF-7 at 24 h incubation but cannot be categorized as potent drugs. The conjugation of both compounds with nanodiamonds enhanced their cytotoxicity by significantly decreasing cell viability at 24 h incubation. At the molecular level, this cytotoxicity is related to antimetastatic potency since these drug formulations downregulate the expression of pro-metastatic markers *C-JUN*, and *NOTCH4* while upregulate anti-metastatic marker *CTNND1*.

Data Availability

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Conflict of Interest

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

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