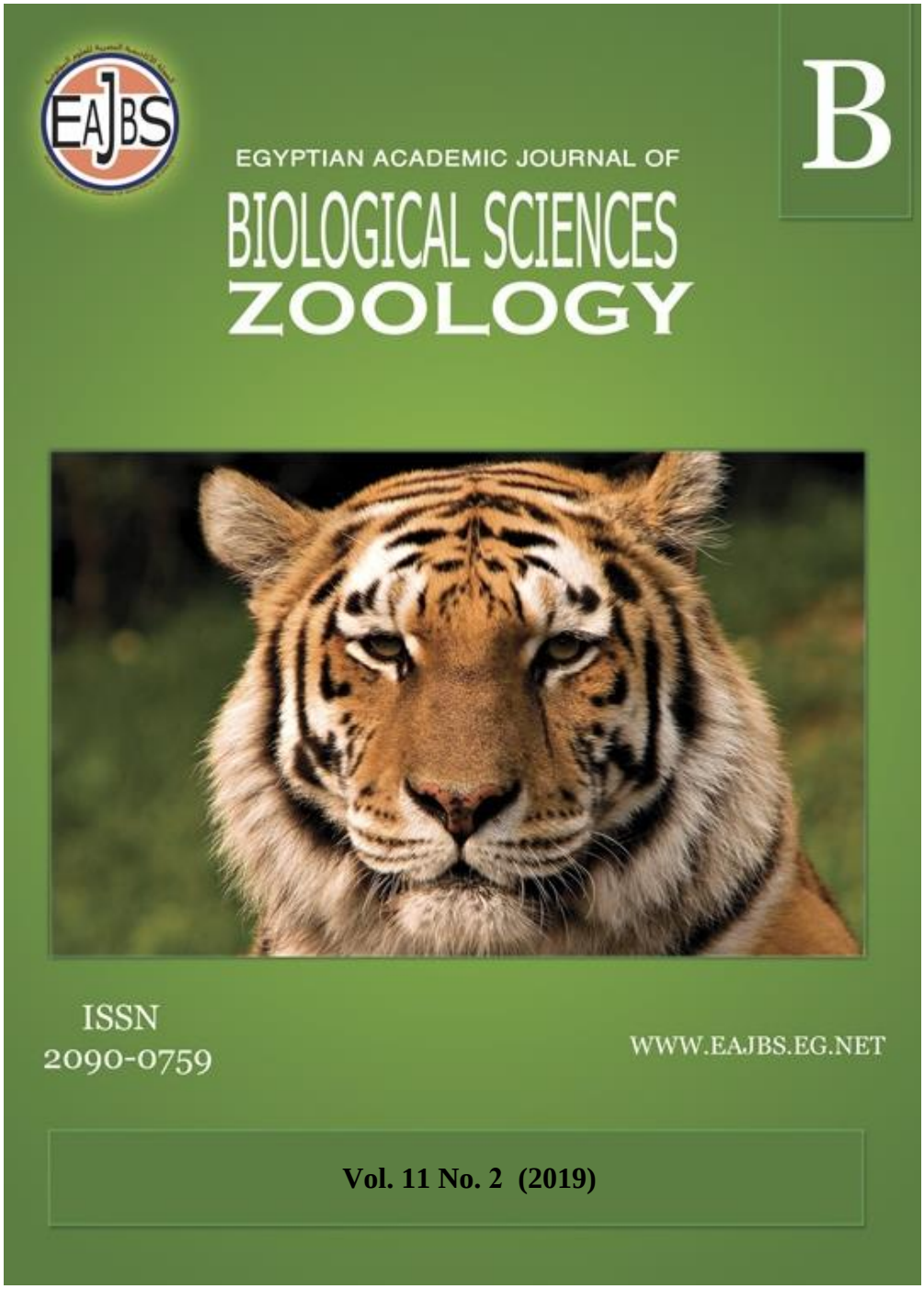

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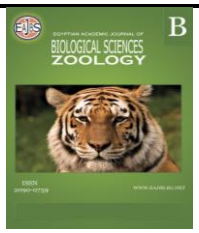

Anti-Tumor Effect of *Cicer Arietinum* against the Triple-Negative Breast Cancer Cells

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ABSTRACT

Triple negative breast cancer (TNBC) is the most aggressive and deadly subtype of breast cancer. The only treatment option available for TNBC is conventional therapy, which associated with debilitating-side effects and drug-resistance. Therefore, using natural chemo-preventive agents to suppress cancer progression or reverse its initiation is an effective and safe way in cancer therapy. The aim of this study was to investigate the antitumor effect of *Cicer arietinum* **(**CAE) *in vitro* employing TNBC cell lines MDA-MB-231 and 4T1. Cell viability, Colony formation as readout for single cell ability to form a colony**,** gelatin-cell adhesion, and immunofluorescence staining were used to assess that effect**.** Relative to control cells, our results indicate that CAE treatment significantly decreased cell viability, colony formation, and cell adhesion. Intriguingly, immunofluorescence microscopy unveiled that TNBC cells exhibited an attenuation of the constitutive and EGF-induced p44/42 MAPK signaling pathway implicated in regulating cell survival. Moreover, a morphological transition of TNBC cells from a fibroblast-like spindle-shaped into epithelial-like cells was observed. In conclusion, CAE may emerge as a promising antitumor agent against TNBC possibly via attenuation of EGF-induced p44/42 MAPK signaling.

INTRODUCTION

Breast cancer, the second leading cause of cancer-related mortality, and the most commonly diagnosed cancer among all women (constitutes 25%) with more than 1.5 million cases every year worldwide (Falah *et al.*, 2017; Ferlay *et al.*, 2015; Torre *et al.*, 2017). According to the expression profile of different receptors, breast cancer can be classified into several molecular subtypes. One of the most aggressive subtypes is triple negative breast cancer (TNBC), that is characterized by loss of expression of estrogen, progesterone, and Her2/neu receptors (Bosch *et al.*, 2010; Elbaz *et al.*, 2015). Therefore, TNBC cannot be targeted by receptors-based antagonist/antibodies therapies**.** Although the conventional therapies are still the main treatment strategies for TNBC namely, chemotherapy and radiotherapy, they are not safe and have toxic side effects (Falah et al., 2017; Salem *et al.*, 2016). Therefore, the development of

natural therapeutic alternatives with lower side effects is highly warranted for cancer inhibition or treatment.

Many phytochemicals such as flavonoids, terpenoids, and steroids have antitumor activities (Ahmed *et al.,* 2011). Since, it has been shown an inverse relation between phenolic compounds intake and breast cancer onset (Pan *et al.,* 2015; Takemura *et al.,* 2013). Genistein and daidzein are widely studied phytochemicals that exhibit biphasic effects on breast cancer cell lines, as they can promote tumor cells proliferation at low concentrations and inhibit the tumor growth at higher concentrations (Poschner *et al.,* 2017). Mahmoud *et al.,* (2014) (Mahmoud *et al.,* 2014) clarified that genistein reverses breast cancer via an apoptotic pathway through inhibition of tyrosine kinase. Similarly, biochanin A is a unique multiple signaling pathway inhibitors as it can down regulate growth, survival, and invasion in HER-2+ breast cancer cells (Sehdev *et al.,* 2009). Furthermore, another isoflavonoid compound the so-called formononetin has anticancer activity against breast cancer (Zhou *et al.,* 2014). The crude methanolic extract of chickpea *Cicer arietinum* (CAE) is composed of multi-biochemical agents that may be more effective than their individual constituents in preventing cancer through both additive and synergetic effects as previously demonstrated (Somasagara *et al.,* 2012). Thus, the potential antitumor effects of CAE are may attributed to its compositions we have previously shown (Fahmy et al., 2015; Sayed *et al.,* 2018) that includes: (1) genistein, daidzein, biochanin A, formononetin, (2) flavonoids, terpenoids, and steroids, and (3) antioxidant and free radical scavenging abilities (Sayed and Elfiky, 2018). Moreover, CAE is considered a safe extract as we have shown that it does not cause any abnormal clinical signs at the dose of 5000 mg/kg rat body weight. Therefore, it has no/low side effects compared to the chemo or radio-therapy (Fahmy et al., 2015). Therefore, in the present study, we investigated for the first time the effect of the crude CAE extract against TNBC behavior using preclinical in vitro models MDA-MB-231 and 4T1 cells.

MATERIALS AND METHODS

Preparation of *Cicer arietinum* Extract (CAE):

CAE was prepared as we have recently reported (Sayed and Elfiky, 2018). Briefly, about one hundred grams of *Cicer arietinum* seeds powder was extracted in 400x (w/v) methanol and heated at 60°C in a water bath for 1 h with constant shaking. Afterward, methanolic extract was subjected to centrifugation at 10,000 rpm for 20 min at 4°C, filtered, concentrated in rotavap apparatus, and finally dried via freezing lyophilization (LABCONCO lyophilizer, shell, freeze system, England, UK) at -40 °C. The final dry yield extract is 15.833%, which re-suspended in distilled water prior to use.

Cell Culture:

The human MDA-MB-231 and mouse 4T1 breast cancer cell lines (a kind gift from Dr. Martin Götte, Münster University Hospital, Münster, Germany) were maintained in DMEM containing 10% FCS, 1% glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Cell Viability Assay:

5×10^3 4T1 cells were cultured into a 96-well plate in complete growth medium and incubated at 37°C for 72 h. Subsequently, cells were incubated with 10 µl/ well of MTT dye solution for 4 h at 37°C. The number of viable cells was directly proportional to the production of formazan, which was dissolved in 100 µl/well DMSO, and measured spectrophotometrically at 570/650 nm using microplate reader (Tecan, Männedorf, Zürich, 165 Switzerland).

Colony Formation Assay:

A total of 5000 4T1 cells were seeded into 6-well plates and incubated at 37˚C in complete growth medium for 24 h. On the next day, cells were treated with different concentrations of CAE (500, 2000 µg/mL) or vehicle (DMSO, control) and maintained in 5% CO_2 in a humidified atmosphere for 4 and 7 days. Cells were then washed twice with PBS, fixed in methanol for 20 min and stained with 0.05% crystal violet for 15 min. Excess stain was removed by water and the plate was left to dry. Colonies contained 50-150 cells were counted and the survival fraction was calculated as a percentage compared with control (Hassan et al., 2013). The stain was then dissolved in 1 mL 10% glacial acetic acid and the color released was spectrophotometrically measured at 570 nm as we previously described (Ibrahim *et al.*, 2017).

Cell Adhesion Assay:

96-well plates were coated with 0.2% gelatin or 10 µg/ml BSA and kept at 4°C overnight, then washed twice with 0.1% BSA in DMEM without FCS. The plate was then incubated for 45 min with blocking buffer (0.5% BSA in DMEM) and washed once. 4T1 cells were released with 2 mM EDTA in PBS, washed twice and resuspended in blocking buffer. 2x 10^4 cells in 100µl were pre-incubated with DMSO, 500µg/ml CAE and 2000µg/ml then added to the coated wells and allowed to attach for 1 h at 37°C, 5% CO2. The cells were washed 3 times with PBS and subsequently fixed with 3.7% PBS buffered formaldehyde for 30 min. Attached cells were stained with 0.2% crystal violet for 30 min. Following 4 times washing with tap water, the cells were lysed in glacial acetic acid and the released stain was quantified by spectrophotometry at 570 nm in a microplate reader (Tecan, Männedorf, Zürich, and 165 Switzerland).

Immunofluorescence:

A total of 50,000 MDA-MB-231 cells were plated on coverslips for attachment overnight in a complete growth medium. On the next day, cells were washed and serum starved overnight. Afterward, cells were preincubated with 500 µg/mL CAE before stimulation with 50 ng/mL EGF for 30 min. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100**,** and stained with anti-phospho-p44/42 antibody (Cell Signalling Technology, Inc., Beverly, MA) at 1:200 dilution followed by incubation with Alexa Fluor 555-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA) at 1:200. Images were captured using fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis:

Results were expressed as the mean ± SEM or SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the Statistical Package for the Social Sciences (SPSS version 20.0, SPSS, Chicago, IL, USA). A P-value <0.05 was considered statistically significant.

RESULTS

Antitumor Effect of CAE against Breast Cancer 4T1 Cells:

A previous study showed that isoflavones extracted from chickpea sprouts have an antitumor effect against human breast cancer cells via mitochondrial-dependent apoptosis (Chen *et al.*, 2015a). Therefore, we assessed the therapeutic effect of different concentrations of CAE extract on colony-forming capability and viability of breast cancer cells, employing colony formation assay and MTT assay, respectively (Fig. 1). To study the effect of CAE on colony formation capacity from a single cell of triple breast cancer, 4T1 cells were seeded as single cells for 4 and 7 days and

examined for differences in colony formation (Fig. 1A). We found that CAE treated cells developed significantly much fewer colonies by at least 30% relative to untreated cells in a concentration-dependent manner (Fig. 1B). Our data revealed that at concentrations of 250 and 500 µg/mL, CAE reduced 4T1 cell viability significantly ($P<0.01$, $P<0.05$, respectively) by 26% and 19% respectively (Fig. 1C), while higher concentrations did not induce cytotoxicity.

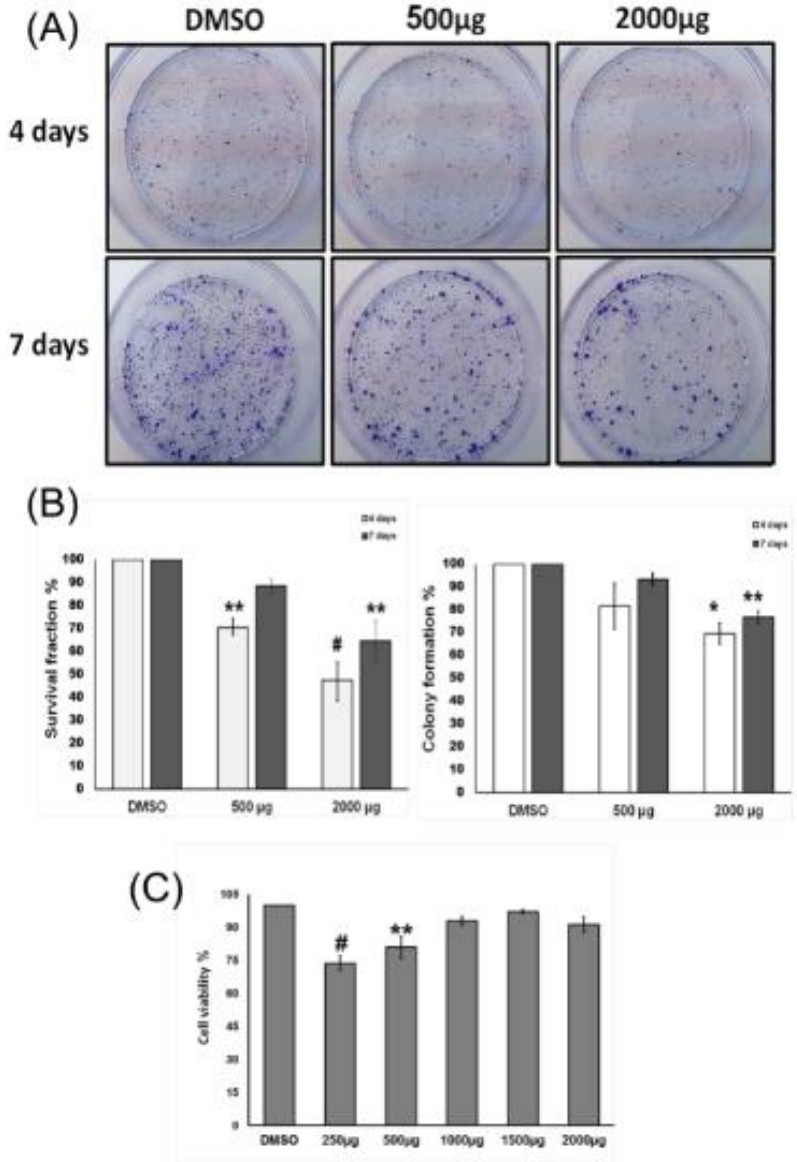

Fig. 1: CAE suppresses the clonogenicity and viability of 4T1 breast cancer cells.
(A) Representative images of single-cell clone proliferation, stained with crystal violet. Equal total numbers of cells were plated into petri dishes to form colonies in presence of DMSO, 500, or 2000 µg/mL CAE for 4 and 7 days. (B) Quantification of the colonies treated with the indicated concentrations of CAE. Values are relative to those of DMSO-treated cells. Left panel represents the quantification of the number of colonies and survival fraction and right panel represents colony formation assay. (C) 4T1 cells were treated with DMSO or CAE with different concentrations for 72 h and subjected to MTT assay. Data represent fold change of cell viability after 72 h relative to DMSO. Data represent mean ± SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparison test.

CAE Modulates Cell Adhesion and Reverses the Mesenchymal Phenotype of Triple Negative Breast Cancer Cells:

Cancer cell adhesion is the first crucial step in tumor metastasis and progression (Bendas and Borsig, 2012). Since cell migration depends on dynamic coordination between assembly and disassembly of cell adhesion to the extracellular matrix that regulated by the turning-over of adhesion molecules (Webb et al., 2002), we assessed the influence of CAE on 4T1 cell adhesion to gelatin-coated wells. The adhesion activity of 4T1 cells was reduced significantly by 30% and 40%, upon treatment with 500µg and 2000µg concentrations, respectively compared with control (Fig. 2A). Epithelial-mesenchymal transition (EMT), is also considered as a key player in cancer invasiveness and resistance to cancer treatment through which epithelial cells lose their cell-cell adhesion and acquired more motile and invasive properties (Nistico *et al.*, 2012). MDA-MB-231 cells are highly invasive breast cancer cells that exhibit a fibroblast-like morphology. Intriguingly, microscopic analysis showed that MDA-MB-231 cells acquired epithelial-like morphology 5h post-treatment with CAE (Fig. 2B), suggesting that CAE induced a phenotypic switch from mesenchymal to epithelial phenotype (mesenchymal-epithelial transition) of MDA-MB-231 cells.

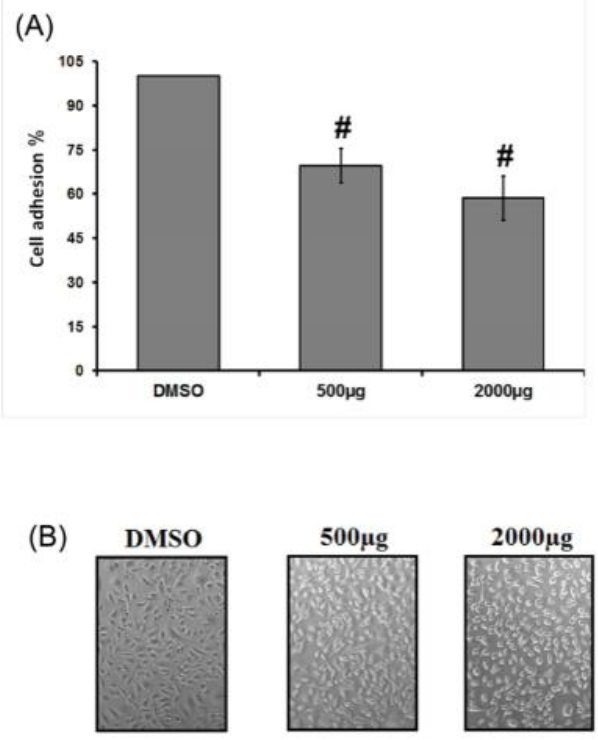

Fig. 2: CAE impairs cell adhesion and induce morphological changes of TNBC in concentration-dependent manner.

(A) Histogram graph panel of cell adhesion is representative of relative adhesion of CAE treated cells (500µg and 2000µg/ml) as compared with DMSO. 4T1 cells were plated on gelatin coated wells and allowed to attach for 1 h at 37°C, 5% CO2. Then, the cells were washed, fixed and stained with crystal violet. Finally, the stain was released to be quantified using spectrophotometry. Data represent mean ± SEM, $n = 3$. * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparison test. (B) Representative images of morphological alteration in MDA-MB-231 cells from fibroblast-like morphology to epithelial one after treatment with CAE for 5h.

CAE Attenuates Activation of EGF-induced p44/42 MAPK in MDA-MB-231 Cells:

A previous study demonstrated that p44/42 MAPK, the downstream signaling of EGF pathway, is implicated in human breast cancer cell survival and apoptotic resistance (Peng *et al.*, 2006). Furthermore, it has been reported that EGF/EGFR signaling plays a pivotal role in enhancing focal adhesion formation (Elbaz *et al.*, 2015) and remodeling of cell adhesion in different types of cancer (Vial and McKeown-Longo, 2012). To decipher whether CAE treatment had an influence on EGF-induced p44/42 MAPK signaling, MDA-MB-231 cells were pretreated with 500 µg/mL CAE before EGF stimulation for 30 min. Immunofluorescence microscopy revealed that CAE pretreatment attenuated activation of p44/42 MAPK upon stimulation with EGF (Fig. 3), substantiating the inhibitory effect of CAE on the downstream signaling of EGF stimulation.

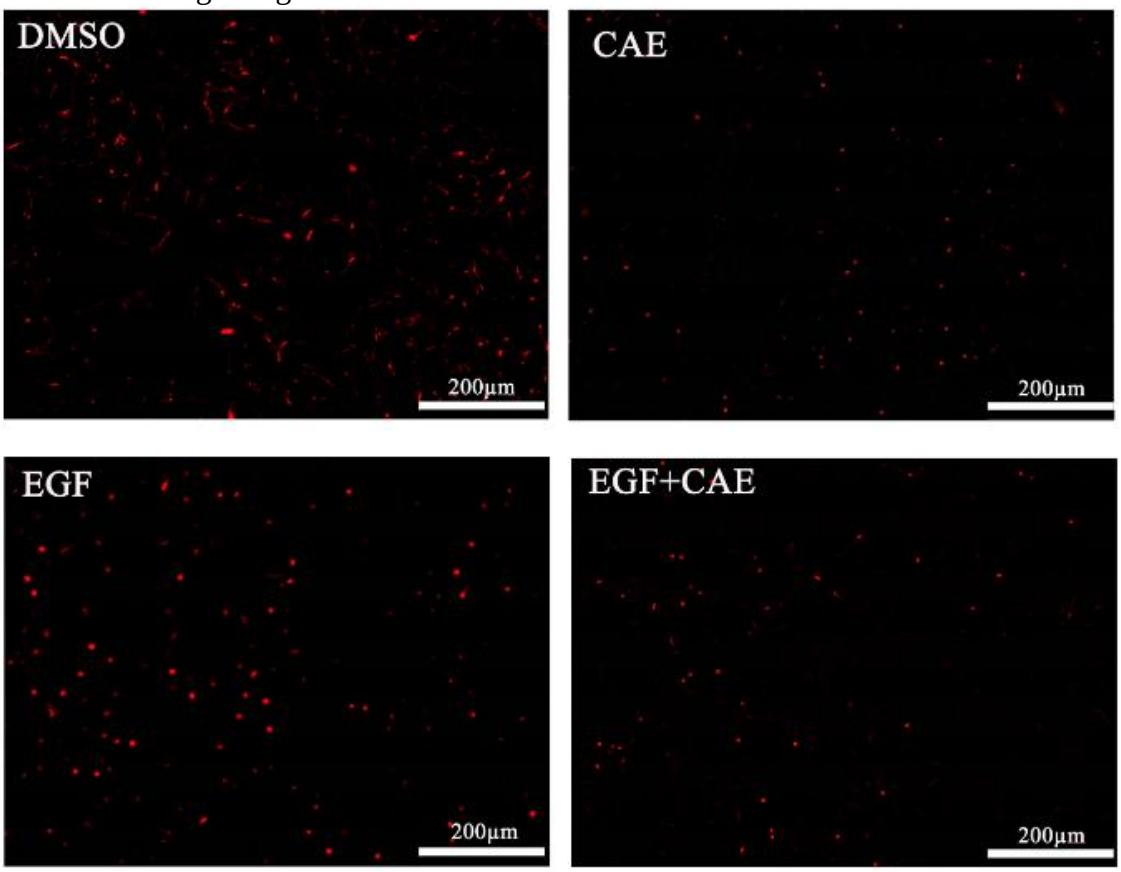

Fig. 3: CAE attenuates the activation of EGF-induced p44/42 MAPK signaling. Immunofluorescence microscopy revealed a reduced ph-p44/42 MAPK in MDA-MB-231 cells were treated with or without 50 ng/mL EGF for 30 min after 500 µg/mL CEA pretreatment. The cells were fixed with 4% PFA, permeabilized, and incubated with rabbit anti-ph-p44/42 antibody followed by anti-rabbit Alexa Flour 555 secondary antibody.

DISCUSSION

Several studies have been conducted to investigate the impact of natural phytochemical extracts on cancer regression (Wang *et al.*, 2012). CAE is considered as a source of a wide variety of phytochemical compounds; such as isoflavones that act as a natural chemoprotective agent for breast cancer (Chen et al., 2015a; Chen *et al.*, 2015b). Previous studies on CAE were performed to isolate a specific substance and to

analyze its effect on cancer development, whereas the protective/therapeutic effects of the whole CAE with its all-bioactive compounds on breast cancer initiation/progression have not been fully investigated. This is a study aimed to evaluate the synergistic antitumor effect of the combined bioactive compounds from dietary CAE against cancer using *in vitro* models.

Our finding indicates that CAE treatment reduced colony formation, cell adhesion and reversed a mesenchymal phenotype of triple breast cancer cells to an epithelial phenotype at the morphological level in a concentration-dependent manner. We suggest that the antitumor activity of CAE possibly mediated by EGFR signaling inhibition.

A large body of evidence indicates that overexpression of EGFR and the constitutive activation of its downstream signaling pathways ERK1/2 MAPK and PI3K are strongly related to tumor aggressiveness, specifically in the triple negative breast cancer (Ai *et al.,* 2006; Palanivel e*t al.,* 2014; Peddi et al., 2012; Peng *et al.,* 2006). Indeed, our results suggest that the bioactive constituents of CAE did not only reduce the basal constitutive activity of ERK1/2, but also inhibited ERK1/2 MAPK signaling upon EGF stimulation. This may be attributed to its genistein content, which has the ability to inhibit EGFR signaling (Rudrabhatla and Rajasekharan, 2004).

One of our most interesting findings was that CAE reversed the fibroblast-like morphology of the triple negative breast cancer MDA-MB-231 cells to an epithelial-like morphology, suggesting a phenotypic switch that might be Erk-dependent. In agreement with this finding, it has been found that Erk inhibition reverses the mesenchymal phenotype of inflammatory breast cancer and prostate cancer (Bhat *et al.,* 2014; Zhang *et al.,* 2009). Cell adhesion and proliferative ability of cancer cells are additional clues implicated in cancer progression in EGFR signaling dependent-manner (Elbaz *et al.,* 2015; Xie et al., 1998; Zhang *et al.,* 2009). Moreover, it has been recently shown that a triple combination of siRNA targeting growth factor receptors, EGFR/Her2/ER induces cell survival inhibition and tumor regression via ERK1/2 MAPK and PI3K pathways in breast cancer (Kamaruzman *et al.,* 2018). In this context, our data revealed that CAE treatment exerted antiproliferative and antiadhesive activities in triple negative breast cancer cells. this could be reasoned to flavonoids content of CAE that have a chemo-preventive property through its ability to inhibit cell proliferation, adhesion and induce a mesenchymal-epithelial phenotypic switch via blocking of EGFR/ERK signaling pathway [30](Bhat *et al.,* 2014; Firdous *et al.,* 2014; Lee *et al.,* 2016). Additionally, a glycoprotein termed lectin is one of CAE components, has an antiproliferative and apoptosis-inducing roles (Gupta *et al.,* 2018).

Conclusion

Overall, this study underpinning the relevance of CAE as a promising antitumor agent that mediates its effect via suppression of cell proliferation, cell adhesion and morphological changes possibly via attenuation of EGF-induced p44/42 MAPK signaling. Our findings suggest the possible therapeutic implication of CAE for the triple-negative breast cancer.

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ARABIC SUMMARY

" النشاط المضاد للورم للحمص فى خلايا سرطان الثدي السلبية الثلاثية "

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سرطان الثدي السلبي الثلاثي (TNBC) هو النوع الفرعي الأكثر عدوانيه وفتكا من سرطان الثدي. العلاج الوحيد المتاح لهذا النوع من سرطان الثدى هو العلاج الكيماوي و/أو ا الاشعاعي، والذي يترتب عليه الآثار الجانبية ومقاومه العلاج ولذلك، فان استخدام العوامل الكيماوية الوقائية الطبيعية لقمع تقدم السرطان أو بدئه هو وسيله فعاله وأمنه في علاج السرطان. الهدف من هذه الدراسة فحص تاثيرمستخلص الحمص كمضاد للورم فى المختبر بإستخدام خطوط الخلايا MDA-MB-231 and 4T1. تم إختبار قدرة االخلايا علي البقاء، تكوين مستعمره، التصاق الخلايا للجيلاتين،استخدام تقنية الفلورة المناعية لتقييم هذا التاثير. مقارنة بالخلايا الضابضة تشير النتائج إلى انخفاض قدرة الخلايا علي البقاء، تكوين مستعمره، التصاقها. كما كشف المجهر الفلوري ضعف مسار الاشارة p44/42 MAPK الاساسى والمستحث بـ EGF المتحكم في بقاء الخلية. وعلاوة علي ذلك، لوحظ التحول الظاهرى للخلايا من الشكل المغزلى إلى الشكل الكروى. في الختام تشير هذه الدراسة الي ان مستخلص الحمص الطبيعي يعمل كمضاد واعد لسرطان الثدي السلبي الثلاثي ربما من خلال تتضعيف مسار الاشارة p44/42 MAPK الاساسى والمستحث بـ EGF.