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Phytochemical Investigation and Molecular Profiling by p21 and NF-κB of *Chorisia crispiflora* Hexane Extract in Human Breast Cancer Cells *in Vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SSA performed cytotoxix activity, sample preparation (tissue culture + DNA preparation), statistical analysis and figure presentations. Author AMA performed DNA fragmentation, detection of nuclear factor kappa B levels, detection of p21 levels. Author OAE performed phytochemical investigation and interpretation of compounds. All authors write, read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The current study targets two main aims; 1st aim is the phytochemical investigation of the hexane extract of *Chorisia crispiflora* leaves. The 2nd aim is the evaluation of the *in-vitro* cytotoxic activity of the extract then examination of the molecular mechanisms underlying this cytotoxic effect.
Study Design: Isolation and identification of the compounds, cytotoxic activity investigation on breast cancer cell line and molecular mechanisms underlying the cytotoxic extract of *Chorisia crispiflora* which may interfere with several cell signaling pathways and insert anti-cancer effects through the suppression of NF-κB or activation of p21, on breast cancer cell lines MCF-7.
Place and Duration of Study: Faculty of Pharmacy, Ain Shams University and National

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Cancer Institute, Cairo University, Egypt. The study was completed within 10 months.
Methodology: *n*-hexane extract was tested against breast cancer cell line then investigated for its effect on NF-κB, p21 and DNA fragmentation. The compounds isolated were identified using different spectroscopic techniques.
Results: In this regard, three main compounds were isolated; β-sitosterol 1, β-sitosterol 3-glucoside 2 and stigmasterol 3-glucoside 3. The extract exhibited IC_{50} values of 7 and 4.2 µg/ml following 48 and 72 hrs of treatment; indicating its significant *in-vitro* cytotoxic activity. This cytotoxic activity was proven to be mediated through down regulation of NF-κB.
Conclusion: Our results suggest that *n*-hexane extract has potent cytotoxic effect on MCF7 cells in addition to down regulation of NF-κB. These findings consequently merit further exploration of the extract in subsequent *in-vivo* studies and later in controlled clinical trials.

Keywords: Chorisia crispiflora; phytosterols; MCF7; NF-κB.

1. INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide [1]. Over 1.1 million cases of breast cancer are diagnosed across the world each year, compared with about 500,000 cases in 1975. This represents about 10% of all new cancer cases and 23% of all female cancers [2,3]. An annual prevalence of more than 4.4 million cases of breast cancer is expected worldwide by the year 2012 [4]. About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries [1]. In Egypt, data reported by Salem, 2010 indicated that it is the most common cancer among women, representing 18.9% of total cancer cases (35.1% in women and 2.2% in men) among the Egyptian National Cancer Institute (NCI).

It has been well known that many genes play important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiologic processes [5,6]. Among these genes, NF-κB plays a key role in the tumorigenesis and progression of several inflammation-linked cancers [7,8], in part, by enabling malignant cells to resist apoptosis-based tumor surveillance mechanisms [8]. Consequently, targeting signaling pathways mediated by NF-κB, directly or indirectly, represents a viable strategy to improve therapeutic outcome in patients with cancer.

Furthermore, human cancers arise from an imbalance of cell growth and cell death. Among key proteins that govern this balance are those that mediate the cell cycle. Notably, loss of expression or function of the G1-checkpoint CDK inhibitors-p21 (CDKN1A) has been implicated in the genesis or progression of many human malignancies [9]. In support, several studies have shown that natural herbal products e.g. American ginseng and purified components, ginsenosides, may inhibit cancer cell proliferation by inducing gene and protein expression of the cell cycle regulatory protein p21, thus arresting tumor cell cycle progression [10]. Furthermore, treatment of the immortalized human mammary epithelial cell line MCF10A with the anti-proliferative phytochemical indole-3-carbinol (I3C) stimulated expression of downstream transcriptional target, p21 [11].

Our hypothesis is that *Chorisia crispiflora* extract decreases proliferation of human breast cancer cells via multiple molecular signaling pathways. Targeting these pathways may be applicable for a novel chemotherapeutic regimen for breast and other cancers. Thus, the present study was designed to target two specific aims; first, the phytochemical investigation of the extract and second the evaluation of *in-vitro* cytotoxic activity of extract of *Chorisia crispiflora* and examination of the molecular mechanisms underlying this putative cytotoxic effect.

2. MATERIALS AND METHODS

2.1 Phytochemical Investigation

2.1.1 Plant material

Chorisia leaves were collected from Zoo Garden in Giza, Egypt, 2010 and were authenticated by Prof. Dr Abdel Salam El Noyehy, Prof. of Taxonomy, Faculty of Science, Ain Shams University, Cairo, Egypt. Voucher specimen was deposited in the herbarium of Pharmacognosy Department (voucher specimen number; CCB-73), Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. The leaves were dried in shade and milled to a fine powder.

2.1.2 Extraction and isolation

Powder of air dried leaves of *Chorisia crispiflora* (1kg) was extracted with 70% ethanol on cold. The hydroalcoholic extract was dried using rota vapor and then dissolved in least amount of water to be then partioned with *n*-hexane. Eight grams of *n*-hexane extract was applied on silica gel column (180g) then eluted with hexane and ethyl acetate with increasing polarity. Compound 1 (β-sitosterol) was isolated from fraction 6 (9:1). Compound 2 [25 mg] and 3 (β-sitosterol 3-glucoside [23 mg] and stigmasterol 3-glucoside [21 mg]) were isolated from fraction 18 (4: 6).

2.1.3 Chemicals

Silica gel 60 for column chromatography (E-Merk), particle size (70-230 mesh), precoated silica gel 60 F 254 sheets for TLC (Riedel-De Haem AG, Germany) were also used.

2.1.4 Nuclear magnetic resonance spectroscopic analysis

The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^{1}H-spectra run at 500 MHz and ^{13}C- spectra were run at 75.46 MHz in deutrated dimethylsulphoxide (DMSO-d_6). Chemical shifts are quoted in δ and were related to that of the solvents.

2.2 Cytotoxic Activity

2.2.1 Cell line

Breast cancer cell line (MCF7) were obtained from the National Cancer Institute, Cairo University, maintained in RPMI medium (SIGMA) supplemented with 10% fetal bovine serum

(SIGMA Aldrich Co., USA) in the presence of penicillin (100 units/mL) and streptomycin (100 µg/mL). These cells were cultured in 5% CO_2 incubator in a humidified incubator at 37ºC.

2.2.2 Cell cytotoxicity measurements

Sulforhodamine B assays were conducted to measure the effects of *Chorisia crispiflora* extract on the proliferation of MCF-7 cells as previously described by Skehan et al. [12]. Cells were seeded at a concentration of 2.5×10^4 cells/ml in 96-well plates, and allowed to recover for 24 hr prior to drug addition. The extract was prepared in a stock solution in DMSO, and then serially diluted to multiwell plates to a final concentration of 5, 12.5, 25 or 50 µg/ml. Control wells received diluted vehicle only, corresponding to the amount present in the 50 µg/ml extract well (0.1% DMSO solution). Following 48 and 72 hr of treatment, the 96 well plates was fixed with absolute methanol containing 1% acetic acid (at -10ºC) for 30 minutes, the methanol decanted, and the plate again air-dried. Sulforhodamine B (0.5% in 1% acetic acid) was added to each well, and the plate incubated at 35ºC for 1 hour. Plates were rinsed with 1% acetic acid, air-dried, and the bound dye eluted with 1 ml of 10 mM Tris buffer, pH 10. The absorbance was measured in a spectrophotometer at 540 nm; the amount of dye released is proportional to the number of cells present in the dish, which is a reliable indicator of cell cytotoxicity.

The percentage of cell survival was calculated as follows:

Survival fraction= O.D. (treated cells)/ O.D. (control cells)

The IC_{50} values (the concentrations of the treatments required to produce 50% inhibition of cell growth) were calculated using GraphPad, Prism software incorporated. The percentage of inhibition has been calculated where the inhibitory effect of each agent added to the cultures was calculated as follows:

Percent of inhibition (%) = [1- (SRB staining in treated wells/SRB staining in control wells)] X 100.

2.3 Molecular Biology

2.3.1 DNA fragmentation

MCF-7 cells were collected after incubation with different concentrations of *Chorisia crispiflora* extract and different time intervals. Total DNA was extracted and purified from the treated and control samples with Genomic DNA Purification kit (Gentra, Minnapolis, Minnesota, USA) in accordance with the manufacturer's instructions. The required amount of DNA was separated on 1% agarose gel with 0.5 X TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8.0). Ethidium bromide-stained DNA in the gel was visualized under UV light and photographed.

Preparation of cell extracts and immunoblot analysis MDA-MB-231 cells were detached, washed once in cold phosphate buffered saline (PBS), and suspended in 100 µL RIPA buffer (150mM NaCl, 1% NP-40, 0.5% dexycholic acid, 0.1% SDS, 50 mM Tris-base, pH 7.5). The suspension was put on ice for 30 min and then centrifuged at 12,000 rpm for 20 min at 4ºC. Total protein content was determined using a Bio-Rad protein assay reagent, with bovine serum albumin as the standard; protein extracts were used for detection of NF-κB and p21.

2.3.2 Detection of nuclear factor kappa bp56

Nuclear factor Kappa Bp65 was determined by ELISA using kits supplied from Glory Science Co., Ltd, USA according to Adams, 2009 [13]. This assay employs the quantitative sandwich immunoassay technique. An antibody specific for NF-κBp65 has been coated onto the wells of the microtiter strips provided. Samples, including a standard containing NF-κBp65, unknowns, and NF-κBp65 antibodies labeled with biotin, and combined with streptavidin-HRP are pipetted into these wells. During the incubation NF-κBp65 antibodies labeled with biotin, and combined with streptavidin-HRP react with the NF-κBp65 antigen bound to the immobilized (capture) antibody. After washing, tetra-methylbenzidine (TMB) substrate solution is added, which is acted upon by the bound enzyme to produce blue color which turn into yellow color after addition of stopping reagent. The intensity of this colored product is directly proportional to the concentration of NF-κB p65 present in the original specimen. The colored reaction product is quantified using a spectrophotometer.

2.3.3 Quantitative determination of human p21 in cell lysates

p21 was determined quatitively in cell lysate using enzyme immunometric assay (EIA) [14,15] Kit supplied from Assay designs company (Catalog#900-161).

2.4 Statistical Analysis

Data are presented as mean ± S.E.M of at least three separate experiments. Multiple comparisons were carried out using one way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis for the pair wise comparisons. Statistical significance was acceptable to a level of $P<0.05$. Data analysis was achieved using software program Graphpad Instat (version 2).

3. RESULTS

Phytochemical Investigation of *Chorisia crispiflora* leads to the isolation of three compounds, β-sitosterol 1, β-sitosterol 3-glucoside 2 and stigmasterol 3-glucoside 3. The structures of these compounds (Fig. 1) were unambiguously determined by their chromatographic behaviors as well as spectroscopic analysis via ^{1}H-NMR and ^{13}C–NMR [16,17,18]

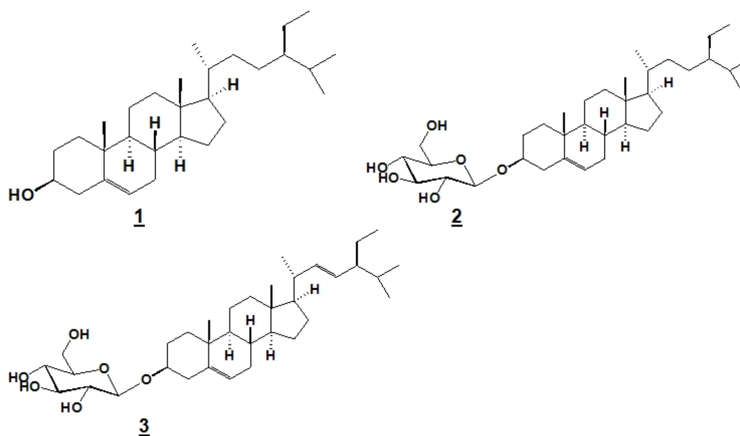

Fig. 1. Compounds isolated from *n*-hexane extract of *Chorisia crispiflora*

3.1 Cell Growth Inhibition by *Chorisia n*-Hexane Extracts Treatment

Cytotoxic effect of the extract was analyzed in human breast carcinoma MCF-7 cells in comparison to doxorubicin. Cell viability was expressed as survival fraction of treated cells compared with untreated control cells following 48 and 72 hr of treatment exposure (Table 1a). Moreover, data were presented as percentage inhibition of cell growth in treated cells (Table 1b). SRB assay revealed that the median inhibitory concentration IC_{50} values for cells treated with extract and doxorubicin following 48 hr were 7 and 3.3 µg/mL, Fig. 2a. Furthermore, the IC_{50} values following 72 hr were 4.2 and 4 µg/mL for extract and doxorubicin, Fig. 2b. The extract cytotoxicity was significantly time and concentration dependent at concentration range varying from 5 to 50 µg/mL.

Table 1a. Cytotoxic effect of *Chorisia crispiflora n*-hexane in MCF-7 cell line following 48 hr of treatment in comparison to doxorubicin

Cell line		Doxorubicin	Extract
	Concentration (µg/mL)	Survival fraction	Survival fraction
48 hr	0	1.00±0.00	0.99±0.01
	5	0.19[a]±0.01	0.69[a]±0.03
	12.5	0.17[a]±0.01	0.27[a]±0.01
	25	0.19[a]±0.00	0.23[a]±0.01
	50	0.20[a]±0.01	0.28[a]±0.02
72 hr	0	1.00 ±0.00	1.00±0.00
	5	0.21[a]±0.01	0.29[a]±0.01
	12.5	0.17[a]±0.01	0.27[a]±0.01
	25	0.14[a]±0.01	0.23[a]±0.00
	50	0.11[a]±0.01	0.20[a]±0.01

Each point is the mean ± SEM of 3 separate experiments performed in triplicate.
[a]: Statistical significance as compared to the control value using one way analysis of variance (ANOVA) followed by Tukey test for post-hoc analysis where a has a P-value (P<0.05).

Table 1b. Percentage inhibition of cell growth by *Chorisia crispiflora n*-hexane in MCF-7 cell line following 48 hr of treatment in comparison to doxorubicin

Cell line		Doxorubicin	Extract
	Concentration (µg/mL)	% Inhibition	% Inhibition
48 hr	0	0±0	0±0
	5	81[a]±1	31[a]±3
	12.5	83[a]±1	73[a]±1
	25	81[a]±0	77[a]±1
	50	80[a]±1	72[a]±2
72 hr	0	0±0	0±0
	5	79[a]±1	71[a]±1
	12.5	83[a]±1	73[a]±1
	25	86[a]±1	77[a]±0
	50	89[a]±1	80[a]±1

Each point is the mean ± SEM of 3 separate experiments performed in triplicate.
[a]: Statistical significance as compared to the control value using one way analysis of variance (ANOVA) followed by Tukey test for post-hoc analysis where a has a P-value (P<0.05).

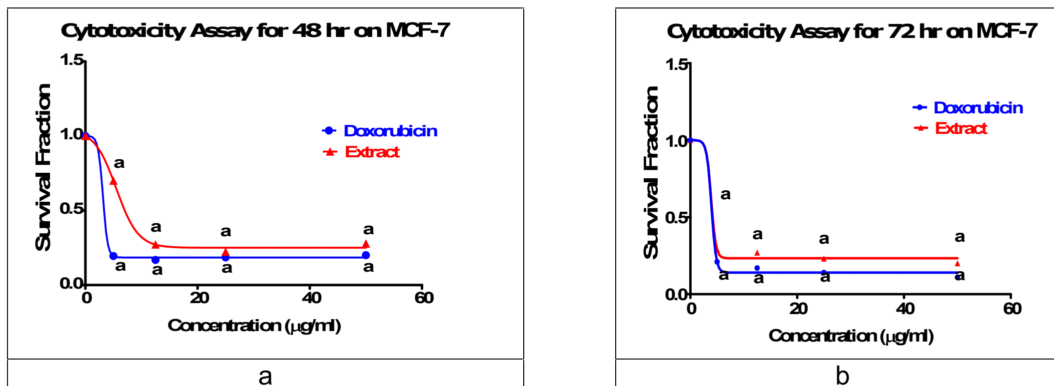

Fig. 2a and 2b. Cytotoxic effect of *Chorisia crispiflora n*-hexane in MCF-7 cell line following 48 & 72 hrs of treatment in comparison to doxorubicin

[a]: Statistical significance as compared to the control value using one way analysis of variance (ANOVA) followed by Tukey test for post-hoc analysis where a has a P-value (P<0.05).

Inhibition of cell proliferation observed by SRB could be due to altered regulation of several gene expressions by chorisia extract treatment. Hence, we further investigated the p21 & NF-κB gene expression profile of MCF-7 breast cancer cells treated with IC_{50} concentration of chorisia extract.

3.2 Effect on DNA Fragmentation and Apoptosis

Fig. 3b showed that incubation of *Chorisia crispiflora* extract with MCF-7 cells at concentration (7 µg/mL and 4.2 µg/mL) at both time intervals (48 and 72 hrs) respectively, there was a significant DNA fragmentation in the treated cells compared to control untreated group (Fig. 3a).

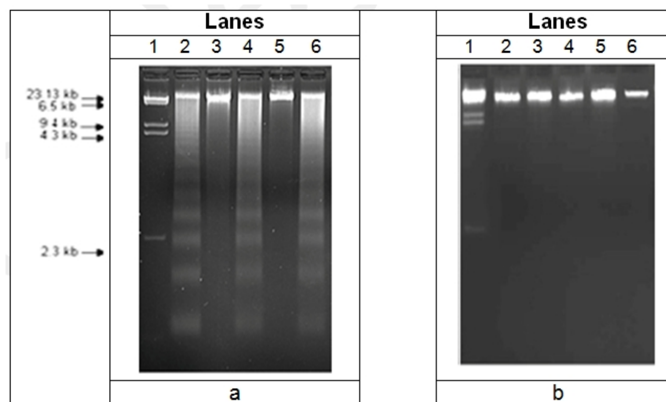

Fig. 3. *Chorisia crispiflora n*-hexane extract induced DNA fragmentation in MCF-7 cells

(a) lane 1:DNA marker

The extracted DNA from control group (lanes 3, 5)

The extracted DNA from treated MCF-7cells with standard doxorubicin (lane 2),

The extracted DNA from treated MCF-7cells with chorisia crispiflora n-hexane extract (5.2 µg/ml and 4.2 µg/ml) at both time intervals (48 and 72 hrs) (lanes 4 and 6) respectively.

(b) Lane 1: DNA marker

Lanes 2 to 6: The extracted DNA from untreated MCF-7cells (control group)

3.3 Detection of NF-κB and p21

Chorisia crispiflora diminished the level of NF-κB in MCF-7 cells at dose (7 µg/mL and 4.2 µg/mL) at both time intervals (48 and 72 hrs) respectively. Significant decrease in NF-κB level was detected compared to control (untreated MCF-7 cells) and standard samples treated with (doxorubicin) at dose (3.3 µg/mL and 4µg/mL) after 48hr incubation (*P<0.05*), (Fig. 4a) and a significant difference was observed between treated group and the control only (P<0.05), with no difference with the standard group, after 72hr incubation (Fig. 4b). However no significant difference was observed in p21 level among the different studied groups (Fig. 5a and 5b).

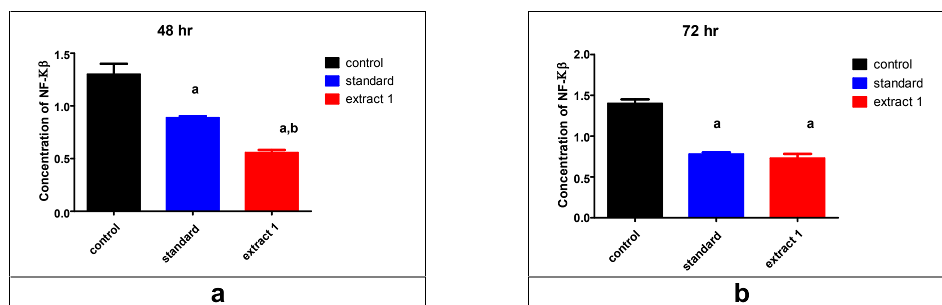

Fig. 4a and 4b. Detection of NF-κB level by ELISA method in MCF-7 cell line treated with *Chorisia crispiflora n*-hexane extract following 48 & 72 hrs

Each point is the mean ± SEM of 3 separate experiments.

[a]: Statistical significance as compared to the control value using one way analysis of variance (ANOVA) followed by Tukey test for post-hoc analysis where a has a P-value (P<0.05).

[b]: Statistical significance as compared to the standard (doxorubicin) value using one way analysis of variance (ANOVA) followed by Tukey test for post-hoc analysis where a has a P-value (P<0.05).

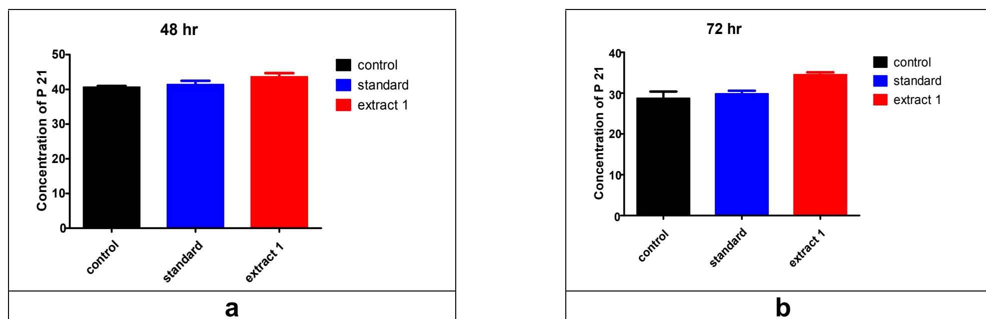

Fig. 5a and 5b. Detection of p21 level by ELISA method in MCF-7 cell line treated with *Chorisia crispiflora n*-hexane extract following 48 & 72 hrs

Each point is the mean ± SEM of 3 separate experiments.

4. DISCUSSION

There has been a recent surge in interest in phytochemicals as medicinal anticancer agents, due to the favorable efficacy and toxicity profiles of these agents. Thus, investigation into the mechanisms of action of plant-derived compounds remains an important approach in the

search for new and more effective anti-cancer agents [19]. Our aim was to examine the cytotoxic activity as well as the underlying molecular mechanisms of the *n*-hexane extract. In our previous published data, *n*-hexane extract was the second effective extract of *Chorisia crispiflora* leaves against MCF-7 breast cancer cells [20]. As the crude extract with an IC_{50} less than 30 μg/mL is considered to be cytotoxic according to the American National Cancer Institute (NCI) [21] so, the *n*-hexane extract of *Chorisia crispiflora* with IC_{50} value of 7 and 4.2 μg/mL following 48 and 72 hrs; possesses a significant cytotoxic activity in the examined human breast carcinoma cell line.

Phytochemical investigation of the extract was the following step which led to the isolation of three phytosterols; β-sitosterol β-sitosterol 3-glucoside and stigmasterol 3-glucoside.

Phytosterols are structurally similar to cholesterol but with some modifications. These modifications involve the side chain and include the addition of a double bond and/or methyl or ethyl group [22]. The most common dietary phytosterols are β-sitosterol (SITO), campesterol and stigmasterol. In recent studies, phytosterols reduced the absorption of dietary cholesterol [23], and cancer. Epidemiological evidence especially showed that SITO inhibited tumor growth of human colon cancer HT-29 [24] and human prostate cancer LNCaP [25]. In addition, the effect of SITO and campesterol on the growth and apoptosis of human breast cancer MDAMB- 231 has been investigated [26]. Although SITO and campesterol were detected in blood, SITO inhibited the growth of tumor cells, but campesterol did not [27]. These epidemiological results supported that SITO has a protective role in cancer development.

In the present study, we showed that chorisia *n*-hexane extract elicits a significant effect on growth inhibition and induction of apoptotic processes in MCF-7 breast cancer cells. Such cytotoxic effect could be mediated by alterations in the gene expression of cell cycle and apoptosis regulatory genes. To provide proof for this premise, we examined the expression of NF-κB and p-21 in the treated cells.

Because of the central role of NF-κB in cell survival and proliferation, we explored this transcription factor as a target for chorisia extract. Several potential mechanisms could explain why NF-κB down-regulation by chorisia extract abrogates the survival of malignant cells. Nuclear factor kappa B (NF-κB) transcription factors regulate several important physiological processes, including inflammation and immune responses, cell growth, and apoptosis. Therefore, the NF-κB signaling pathway has also provided a focus for pharmacological intervention, primarily in situations of chronic inflammation or in cancer, where the pathway is often constitutively active and plays a key role in the disease. Now that many of the molecular details of the NF-κB pathway are known, it is clear that modulators of this pathway can act at several levels. variety of natural and synthetic molecules that include antioxidants, peptides, small RNA/DNA, microbial and viral proteins, small molecules, and engineered dominant-negative or constitutively active polypeptides act as general inhibitors of NF-κB induction, Moreover, the therapeutic and preventative effects of many natural products may, at least in part, be due to their ability to inhibit NF-κB [28].

This study showed a significant decrease in NF-κB level in samples treated with the *Chorisia crispiflora* extract compared to standard and control which may indicate that extract of *Chorisia crispiflora* has an inhibitory effect on NF-κB activity that is time- and concentration-dependent and this inhibitory effect may play an important role in the degradation of the DNA of the cancer cells which confirmed by DNA fragmentation indicating that this extract may exhibit antitumor activity as well.

It seems likely that the most promising near term ses for NF-κB inhibitors will be in cases where such inhibitors can be applied topically (e.g., cylindromatosis or other skin inflammatory diseases), locally (e.g., airway inflammation), or in a highly directed fashion (e.g., to a specific cell or tumor type). Systemic application of potent NF-κB inhibitors will likely have unwarranted side effects. Nevertheless, it is certainly possible that the long-term systemic ingestion of low-dose NF-κB inhibitors may have general beneficial effects in reducing inflammation and cancer. At least in part, chronic dampening of NF-κB activity may explain the promoted anti-inflammatory, antiaging, and anticancer effects [28].

On the other hand, many studies showed that p21 is activated through NF-κB and is an important mediator of the cell growth arrest response [29]. But our data showed no significant effect of *Chorisia crispiflora* extract on p21 level indicating that many molecular components in overlapping signaling pathways makes it a challenge to find molecules that block specific pathways leading to NF-kB activation without interfering with other signaling cascades. A future goal will be to discover molecules.

5. CONCLUSION

In conclusion, our results show that the hexane extract of *Chorisia crispiflora* potently inhibits breast cancer cells by targeting several signaling pathways that regulate cancer cell survival and progression.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no competing financial or personal interest or any kind of conflict of interest relevant to this study.

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