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RESEARCH

Lantana camara Induces Apoptosis by Bcl-2 Family and Caspases Activation

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Abstract Breast cancer is one of the most common cancers worldwide, and the second most fatal cancer in women after lung cancer. Because there are instances of cancer resistance to existing therapies, studies focused on the identification of novel therapeutic drugs are very important. In this study, we identified a natural anticancer agent from *Lantana camara*, a flowering plant species of the genus *Verbena*. The extract obtained from the *L. camara* exhibited cell death properties in the human breast cancer cell line, MCF-7. We found that the apoptosis induced by treatment with the *L. camara* extract was regulated by the Bcl-2 family. Bid and Bax was increased and Bcl-2 was decreased by *L. camara* extract. *L. camara* extract modulated cleavage of caspase-8, and caspase-9, as well as poly (ADP-ribose) polymerase (PARP). Our results support the potential use of the *L. camara* extract as an anti-breast cancer drug.

Keywords Apoptosis · Bcl-2 family · Breast cancer · Caspase · *Lantana camara*

Introduction

Lantana camara is a flowering plant species of the genus *Verbena*, family Verbenaceae, which is a native of the American tropics. This plant has been introduced into other parts of the world as an ornamental plant, and has been considered an invasive species in many tropical and sub-tropical areas.

L. camara has a broad distribution worldwide and has many folk medicinal properties. A substantial number of phytochemical studies have been conducted using *L. camara*, and the initial discovery of its anti-microbial activity by Deena and Thoppil [7] has motivated many subsequent studies to focus on the plant's anti-bacterial and anti-fungal activities [17, 14]. *L. camara* is currently used in herbal medicines for the treatment of skin itches, and its wound healing efficacy has been demonstrated in vivo [18]. Recently, it was revealed that lantadene, the most abundant pentacyclic triterpenoid in *L. camara*, has anti-tumor effects in vitro in human promyelocytic leukemia cells, cervical, colon and lung cancer cell lines and in vivo [26, 25]. Therefore, considering the absence of study about breast cancer cell line, we hypothesized that it may have anti-breast cancer effects.

Breast cancer is one of the most commonly diagnosed cancers in women, and is associated with a high fatality rate. Breast cancer therapies, including chemotherapy, monoclonal antibody therapy and radiation, are associated with tolerance and side effects [23, 24, 31, 29]. Breast cancers without hormone receptors, cancers that have spread to the lymph nodes in the armpits, or those associated with specific genetic characteristics, pose a higher risk than other cancers and are therefore treated more aggressively [8, 3].

There are three main cell death pathways: apoptosis, autophagy, and necrosis. In case of necrosis, engendered by extracellular irritation, the cell membrane is rupture and inflammatory process is performed. Apoptosis and autophagy have been considered to be "programmed cell death". In much tumor, proliferation is abnormal because the programmed cell death system collapses. And apoptosis have been known as clear type of death because of its characteristics included cell shrinkage with maintain of membrane, chromatin condensation, formation of apoptotic bodies and phagocytosis by

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macrophages without inflammation while autophagy have been studied. Targeting apoptosis pathway, therefore, is suitable method in cancer therapy [15, 28, 21, 2].

Previous studies have shown the anti-cancer effects of *L. camara*; however, its mechanism of action is still unclear. Therefore, in this study, we evaluated the chemotherapeutic potential of *L. camara* extract in breast cancer cell line and elucidated its mechanism.

Materials and Methods

Materials and Reagents

An ethanol extract (95 %) of *L. camara* was obtained from Korean Plant Extract Bank. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, and antibiotic-antimycotic were purchased from Gibco (Life Technologies, Carlsbad, CA, USA). DMSO was purchased from Sigma (St. Louis, MO, USA). RIPA buffer was purchased from Biosesang Inc (Seongnam, Korea). Protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). Antibodies against Bid and poly (ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology (Danvers, MA, USA) and antibodies against Bax, Bcl-2, caspase-8 and caspase-9 Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

Cell Culture

The human breast cancer cell line Michigan Cancer Foundation (MCF)-7 was purchased from the Korean Cell Line Bank. MCF-7 cells were cultured in DMEM with 10 % (v/v) fetal bovine serum and 1 % (v/v) antibiotic-antimycotic. The cells were incubated in a humidified incubator in 5 % CO_2 at 37 °C.

Cell Proliferation Assay

Cell proliferation was evaluated by performing the MTT assay. The cells were seeded into a 96-well plate and incubated for 24 h. The cells were exposed to different concentrations of *L. camara* (0, 10, 20, 40, and 80 μg/mL). After incubation for 6, 12, and 24 h at 37 °C, 1 mg/mL MTT containing medium without serum was added to each well, and incubation was performed for 2–4 h at 37 °C; thereafter, the MTT medium was removed from the wells. Next, DMSO was added to the plate, and the plate was placed on a shaker for 5 min. Absorbance was read at 540 nm by using a microplate spectrophotometer (spectra max 190, USA), and the viability rates were calculated as follows: $(OD_{treated}/OD_{control}) \times 100$ %.

Cell Cycle Analysis

After the cells were exposed to different concentrations of *L. camara* for 6 h, changes in cell morphology were assessed using a phase-contrast microscope (CKX41, Olympus. Japan). The cells were then collected, washed twice with PBS, and fixed with 70 % ethanol solution at 4 °C for at least 2 h. After centrifugation, the cell pellets were stained using 50 μg/mL propidiumiodide (PI) solution and treated with 1 μg/mL RNase A at 4 °C in the dark for 30 min. The samples were then analyzed using FACScan flow cytometer (Becton Dickinson USA) and Cell Quest software. The cell cycle distribution was analyzed using ModFit LT software.

Flow Cytometry Analysis of Cell Apoptosis

To determine the effect of *L. camara* on apoptosis, the cells were analyzed by flow cytometry using annexin V and propidium iodide staining. Briefly, the cells were trypsinized, washed in cold PBS, and resuspended in assay buffer. Annexin V and PI solution were added to the cell preparations and incubated for 15 min at room temperature. A binding buffer was then added to each tube, and the samples were analyzed using a FACScan flow cytometer (Becton Dickinson USA) to sort the annexin V-FITC-positive apoptotic cells. The corresponding negative controls were used to determine accurate thresholds for data analysis.

Protein Extraction and Western Blotting

After treatment with various concentrations of *L. camara*, the MCF-7 cells were washed twice with PBS, and lysed by RIPA buffer on ice to get the protein. The protein concentration of the lysate was determined using a bicinchoninic acid (BCA) assay. Next, 20–50 μg of cell proteins were subjected to electrophoresis on 10–15 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Following electrophoresis, the proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5 % skimmed milk in phosphate-buffered saline with 0.1 % Tween 20 (PBST). Then, the proteins were probed using antibodies against the following: Bid, Bax, Bcl-2, caspase-8, caspase-9, and PARP. The immunoblots were developed and visualized using an enhanced chemiluminescence(ECL) detection system (Amersham Biosciences, Piscataway, NJ, USA). The western blot was imaged using the Chemi Documentation Imaging System and quantified using ImageJ. β-Actin was used as an internal control.

Statistical Analysis

All results obtained from at least 3 independent experiments were combined and analyzed using the Student's-*t* test. All

values were expressed as the mean±SD. The acceptable level of significance was established at $P<0.05$

Results

L. Camara Extract Inhibited Growth of Breast Cancer Cells

To study the effect of *L. camara* on breast cancer cell growth, MCF-7 cells were treated with different concentrations of the extract (0, 10, 20, 40, and 80 μg/mL), and a cell viability assay was performed after 6, 12, and 24 h. Figure 1 shows the concentration-dependent inhibition of cell growth by the *L. camara* extract. Compared to no treatment, *L. camara* extract showed significant decrease in cell viability. The estimated median effective concentration (IC_{50}) of *L. camara* after incubation for 6 h was 46.62 μg/mL. These results indicated that *L. camara* might have an anti-cancer potential.

Induction of Apoptotic Cell Death in Breast Cancer Cells

To determine more clearly its effect on the cancer cell death, we introduced annexin V/PI staining following treatment of *L. camara* in the MCF-7 cells. The number of annexin V-positive cells (upper right and lower right quadrants representing late and early apoptosis, respectively) was represented as the percentage of apoptotic cells over the total number of treated cells (upper left and lower left quadrants representing necrosis and live cells, respectively) (Fig. 2a). 5-Fluorouracil (5FU, 10 μg/mL), a known inducer of apoptosis, was used as a positive control [32]. Dose–response studies revealed that *L. camara* dose-dependently increased cell death, which was augmented early apoptosis in 30 μg/mL followed by late apoptosis and necrosis in 60, 90 μg/mL treatment. In comparison, the results indicated that there were most PI-negative and annexin-V-negative cells in the control cultures. Positive control, 5-Fluorouracil-treated cells indicated 30.62 % labeling for annexin-V-positive cells. And in lowest concentration (30 μg/mL) of *L. camara* treated cells, the value was similar to positive control as 30.58 %. To evaluate and quantify the extent of apoptosis induced by treatment with the *L. camara* extract, we performed cell cycle analysis as well [6]. We observed a dose-dependent increase in the number of cells containing sub-G_1 DNA which is indicative of apoptosis (Fig. 2b).

L. Camara Regulates Bcl-2 Family Signaling in Breast Cancer MCF-7 Cells

To evaluate molecular mechanism of the cell death induced by the *L. camara*, we focused on the changes in Bcl-2 family members. 5FU (10 μg/mL) was used as a positive control [20]. The protein levels of Bid, Bax, and Bcl-2 were performed in MCF-7 cells 6 h after *L. camara* treatment. Bcl-2 family is important mediators of the mitochondrial apoptotic pathway. Upon activation of Bid and Bax, the pro-apoptotic proteins of the Bcl-2 family, many apoptogenic factors such as cytochrome C are released from the mitochondria into the cytosol. However, Bcl-2, an anti-apoptotic protein of the Bcl-2 family, blocks this signaling pathway. We found that *L. camara* treatment increased Bid expression levels in a dose-dependent manner and regulated Bax/Bcl-2 signaling which were increase Bax and decreased Bcl-2 expression (Fig. 3a and b). In case of Bax and Bcl-2, moreover, the effects were greater than positive control. These results suggest that the Bcl-2 pathway play a significant role in the apoptosis induced by *L. camara*.

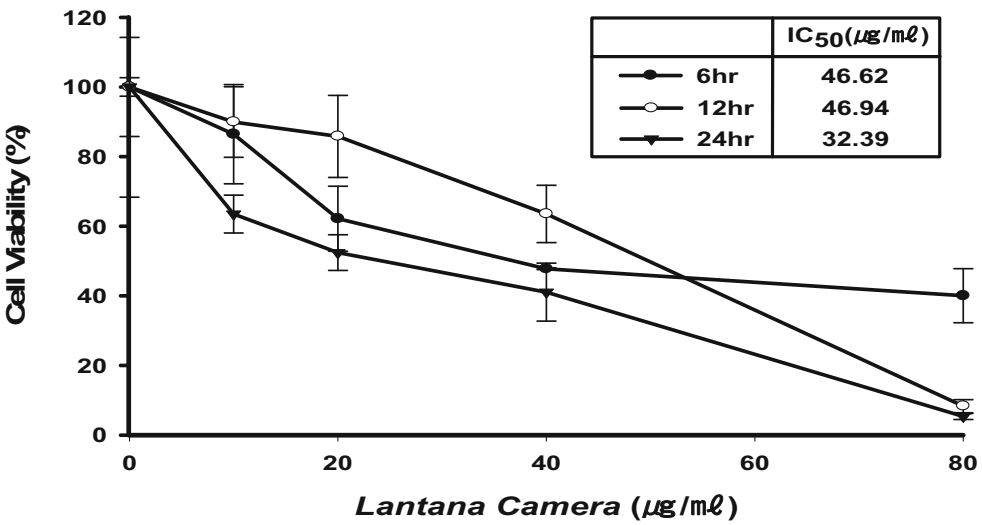

Fig. 1 Examination of the effect of an extract of *L. camara* on breast cancer cell (MCF-7) proliferation by using the MTT assay after extract treatment for 6, 12, and 24 h. Compared to the control cells, the MCF-7cells treated with *L. camara* extract showed a decrease in tumor cell proliferation in a dose- and time-dependent manner

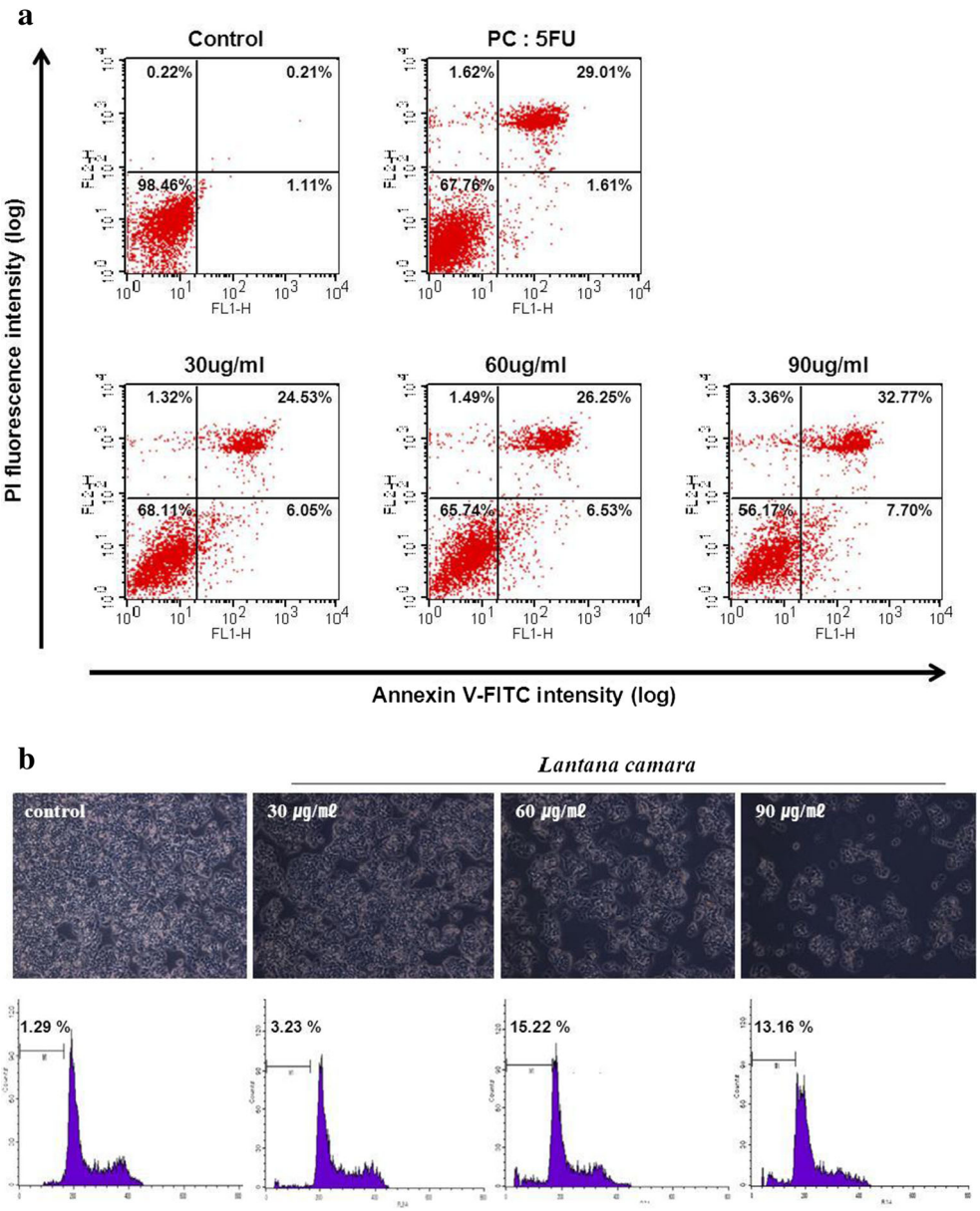

Fig. 2 **a** The MCF-7 cells were treated with the *L. camara* extract (30, 60, and 90 μg/mL) and then stained with Annexin V and propidium iodide (PI) and analyzed by flow cytometry. The number of differentially stained cells was expressed as a percentage of the total cell number. **b** Cells were exposed to various concentrations of *L. camara* extract for 6 h, and changes in cell morphology were assessed using a phase-contrast microscope. Next, the cell cycle was analyzed using flow cytometry. The cell morphologies (400×) and DNA histograms of the MCF-7 cells are shown

Analysis of the Effect of *L. Camara* Treatment on the Modulation of Caspases and PARP Activation in MCF-7 Cells

Next, we evaluated the expression of caspases and PARP using western blotting analysis. A positive control was 5FU (10 μg/mL) [32]. It is well known that cleavage of caspase-8 and caspase-9 induces caspase-3 activation. Moreover, activated caspase-3 mediates the cleavage of apoptotic-proteins such as PARP, which are important in degradation and chromatin condensation in apoptotic cells [4]. *L. camara* treatment (10, 30 and 60 μg/mL) for 6 h resulted in the up-regulation of cleaved-caspase-8 and caspase-9. Pro-PARP, inactivated form of PARP before cleavage, expression levels also significantly decreased in a dose-dependent manner (Fig. 3c and d), and were most likely due to the caspase cleavage induced by *L. camara*.

Discussion

L. camara has a broad distribution worldwide and is used in many folk medicines [11]. To date, *L. camara* has demonstrated numerous medicinal activities, including anti-cancer effects, which have been previously studied in vitro and in vivo [7, 17, 14, 18, 26, 25]. However, the exact mechanisms have not yet been completely elucidated, especially, in the breast cancer. To determine the underlying mechanism, we studied the effects and signaling pathway of an ethanol extract

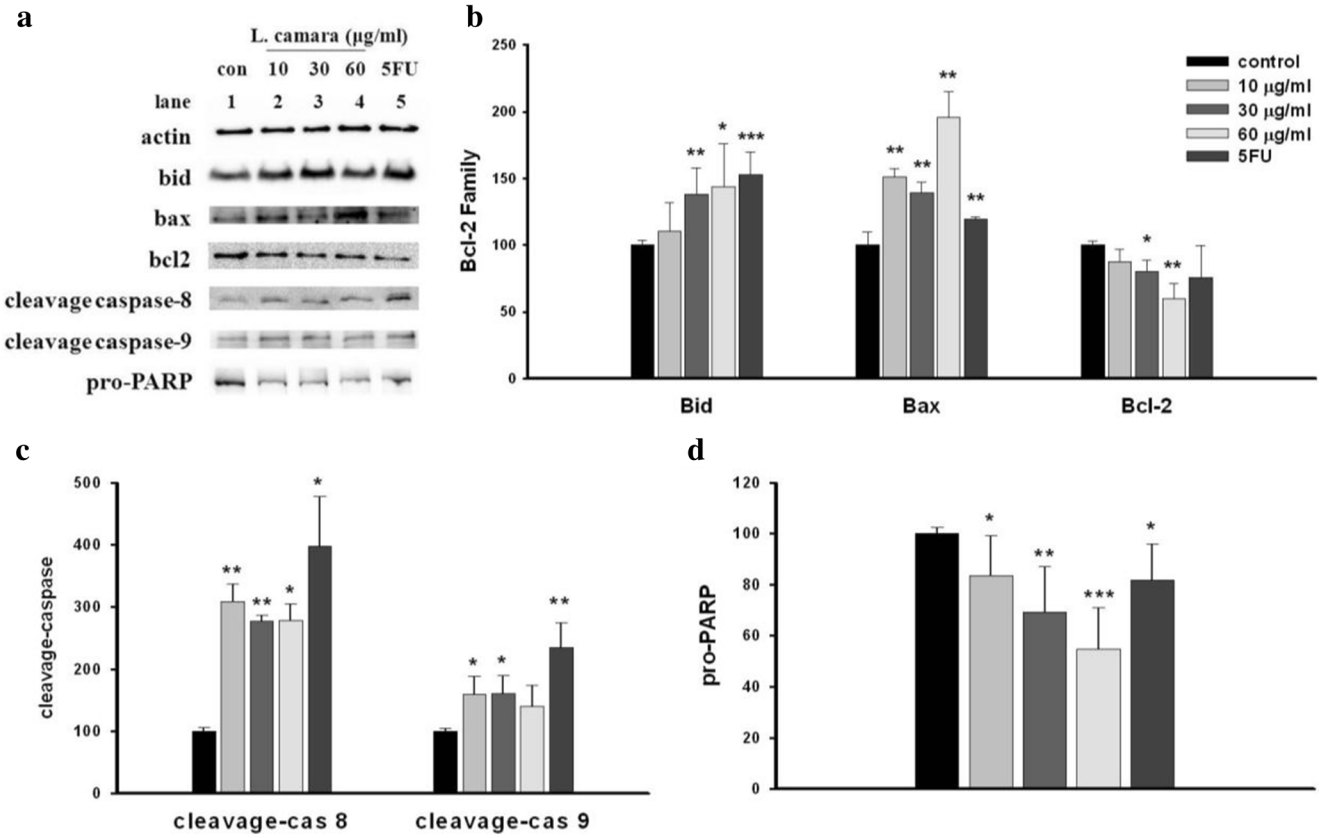

Fig. 3 After treatment with the *L. camara* extract at 10, 30 and 60 μg/mL for 6 h, the cells were harvested and lysed. Proteins obtained from the cell lysate were identified using western blotting. Actin was used as an internal control for protein normalization. The levels were indicated in terms of percentage relative to the control. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ vs. control

from *L. camara* in MCF-7 cells. In this study, we showed that *L. camara* inhibited the proliferation of MCF-7 cells and the IC_{50} value was 46.62 μg/mL at 6 h after treatment (Fig. 1). Breast cancer is associated with one of the highest mortality rates among all cancers that develop in women [27]. Although there are numerous types of cytotoxic chemotherapy, the clinical use of these drugs has been limited by the frequent occurrence of drug resistance. For this reason, natural products which are showing anti-cancer capacity are catching special attention in the development of novel drug. The main signaling pathway underlying the cytotoxic effects of anti-cancer drugs is apoptosis, which is a physiological cell death pathway, but which may be dysfunctional because of some oncogenic mutations [12, 10]. In this study, we found that apoptosis was the major inhibitory pathway underlying the anti-cancer effects of *L. camara* (Fig. 2).

Our mechanistic studies demonstrated that the *L. camara*-induced apoptotic pathway is mediated by the Bcl-2 family, caspases, and PARP. When the MCF-7 cells were treated with the *L. camara* extract at various concentrations, the expression

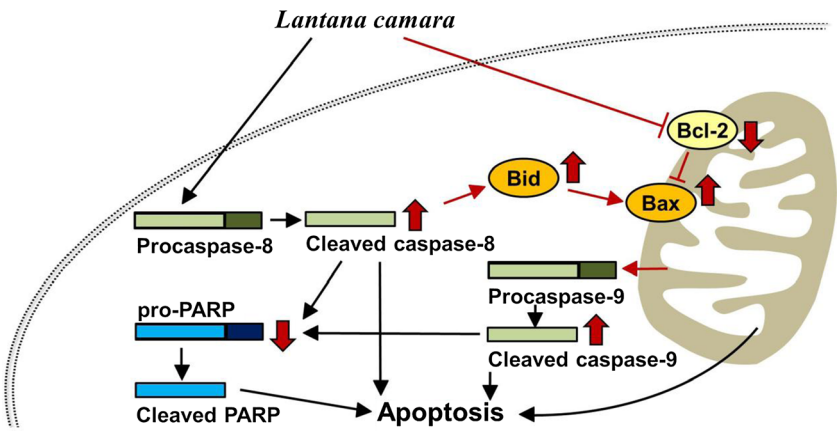

Fig. 4 Effects of *L. camara* on the Bcl-2 family and caspases dependent anticancer mechanism suggested by western blot

levels of the pro-apoptotic protein Bid were increased significantly (Fig. 3b). We also found that treatment with the *L. camara* extract induced the expression of the pro-apoptotic protein Bax and suppressed the expression of the anti-apoptotic protein Bcl-2 (Fig. 3b). These results showed that *L. camara* modulated the expression levels of the Bcl-2 family proteins. The Bcl-2 family regulates apoptosis by inhibiting mitochondria outer membranes permeabilization [30]. Therefore, substances that target Bcl-2 family signals are good drugs for cancer therapies [5, 12, 1]. Caspase-8 and caspase-9 levels were also regulated by the *L. camara* extract. Treatment with the extract for 6 h activated caspase-8 and caspase-9 (Fig. 3c). There are many caspases that stimulating apoptosis and this family is activated two pathway in large. One is the mitochondrial-, and other is non-mitochondrial mediated pathway. The first one is involved in capase-9 activation and caspase-8 is a typical non-mitochondrial mediated protein [9, 30, 5]. The fact that treatment with the extract stimulated not only caspase-9 but also caspase-8 suggests the existence of complex pathway by which *L. camara*-induced apoptosis is mediated. PARP, one of the downstream substrates of caspase, involves with cell death by binding damaged DNA and repairing it [13]. Cleavage of PARP is observed in apoptosis process, similar to caspases [22, 16]. There are many drugs for cancer therapy that induce PARP-mediated apoptosis [19]. In this study, we found that pro-form of PARP levels significantly decreased in a dose-dependent manner (Fig. 3d), which suggest it is converted to active form by cleavage-caspase. In this study, it could be inferred that the PARP is inactivated by cleavage-caspases. Taken together, the molecular and biological mechanisms underlying the induction of apoptosis may be mediated via a complex signaling pathway (Fig. 4) and, thus, further research is warranted to better understand its anti-cancer effect.

In summary, the extract obtained from *L. camara* showed anti-cancer effects through the induction of apoptosis in MCF-7. Furthermore, we revealed the regulatory effect on Bcl-2 family proteins including Bid, Bax, Bcl-2-and caspase-8, caspase-9, as well as PARP in the underlying molecular mechanisms. Finally, we suggest the potential role of *L. camara* to serve as a natural therapeutic agent against breast cancer.

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