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Luffa cylindrica flower extract induces apoptosis and autophagy in breast cancer cells

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Abstract: **Background**: *Luffa cylindrica* flower has been used to treat haemorrhoids and breast hyperplasia, but the mechanism is unclear. **Objectives**: In this study, the antiproliferation activity of *L. cylindrica* flower extract (LCFE) was tested in breast cancer cells. **Methods**: Following LCFE treatment, the CCK-8 assay, Hoechst staining and wound healing assay were used to evaluate cell proliferation, apoptotic cell morphology, and cell migration, respectively. qRT-PCR and western blot were used to analyze the expression of genes and proteins involved in the apoptosis and autophagy pathways. LC-MS was performed to characterize chemical constituents of LCFE. **Results**: CCK-8 and wound healing assays revealed that LCFE suppressed the proliferation and migration of MCF-7 and MDA-MB-231 cells. In these two breast cancer cells, the extract treatment induced apoptotic morphological changes. LCFE treatment induced apoptosis by upregulating ZFP36 and BNIP3 expression, while downregulating Bcl-2 expression in MCF-7 cells. LCFE increases ZFP36 expression while decreasing BMP4 expression in MDA-MB-231 cells, promoting apoptosis. Meanwhile, LCFE treatment induced autophagy by increasing VMP1 expression and activating LC3 in MCF-7 cells. It also triggered autophagy by decreasing TBC1D14 expression, increasing ATG5 and VAMP8 expression, and activating LC3 in MDA-MB-231 cells. **Conclusion**: LCFE exerts an anti-tumor effect by activating apoptosis and autophagy processes in breast cancer cells, while having low cytotoxicity for normal breast cells, highlighting the potential of LCFE as a natural agent for cancer treatment.

Keywords: Apoptosis; Autophagy; Breast cancer cell; *Luffa cylindrica*

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INTRODUCTION

Breast cancer has a high incidence and mortality rate in women (Jemal *et al.,* 2011). Based on the histological characteristics, breast cancer is typically classified into three major groups. They are the estrogen receptor$^+$ (ER^+) or progesterone receptor$^+$ (PR^+) group, the human epidermal receptor 2^+ ($HER2^+$) group, and the triple negative breast cancer (TNBC, (ER^-, PR^-, $HER2^-$)) group (Liedtke *et al.,* 2023). The most common approach to treating breast cancer is surgical resection. However, tumor heterogeneity, metastasis and poor prognosis all have a significant impact on therapeutic efficacy. Other clinical treatments, such as radiotherapy, chemotherapy and targeted endocrine/molecular strategies, are also used to eliminate tumor cells or inhibit tumor growth (Geay, 2013). Chemotherapy is an effective treatment for breast cancer patients, improving their survival and prognosis (Chew, 2001). However, reagent treatment was always accompanied by toxicity and resistance to long-term use, which restricted their application. Therefore, exploring novel bioactive natural products may lead to the discovery of a new agent for breast cancer treatment.

Plant extracts and phytochemicals derived from them are an important source of anti-cancer drugs. These phytochemicals are mainly derived from plant secondary metabolites, which contain a variety of bioactive natural products (DeSantis *et al.,* 2017). Many phytochemicals with anti-cancer and chemoprotection properties have been identified in vegetables, fruits, traditional Chinese medicines, or natural herbal medicines (Gezici and Şekeroğlu, 2019). Up to now, phytochemicals such as capsaicin, catechins, paclitaxel, curcumin, resveratrol, silibinin, benzyl isothiocyanate, genistein, kaempferol, thymoquinone, and quercetin have previously been used in breast cancer treatment (Younas *et al.,* 2018). However, breast cancer heterogeneity and drug resistance make chemotherapy more difficult, especially in TNBC treatment. TNBC patients always have a poor prognosis due to their insensitivity to hormonotherapy, with a higher risk of tumor recurrence and metastasis (Li *et al.,* 2022). Some studies have found that plant extracts or bioactive compounds from traditional Chinese medicines have unique anti-tumor activity in TNBC cells by targeting multiple signaling pathways (Yang *et al.,* 2021). However, for a better treatment of TNBC, new plant extracts with low side effects and novel molecular targets are needed to be discovered.

L. cylindrica, a climbing herb of the Cucurbitaceae family, is widely distributed in Asia and Africa. This plant holds great promise with its long-standing use in traditional medicine for asthma, skin diseases and splenic enlargement (Abdel-Salam *et al.,* 2019). The fruit, also known as Si Gua Luo, has been a part of cancer treatments in China, often in combination with other medicinal materials (Abdel-Salam *et al.,* 2019). Its antioxidative components, including apigenin, luteolin, and diosmetin, demonstrate notable

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potential in combating cancer (Du *et al.,* 2006). Similarly, the leaves of *L. cylindrica* have demonstrated anti-cancer properties in breast cancer MCF-7, BT-474 and MDA-MB-231 cells, inducing caspase 8 and inhibiting the cell cycle (Abdel-Salam *et al.,* 2018). Excitingly, further research has revealed the ability of *L. cylindrica* leaves to suppress the proliferation of breast cancer stem cell $CD44^+$ /24^- (Abdel-Salam *et al.,* 2019). Aside from the fruit and leaves, *L. cylindrica* flower has traditionally been used in China to treat hemorrhoids and breast hyperplasia. However, the active ingredient and the antiproliferation mechanism still need to be studied further.

Breast cancer contains different subtypes, each with its own prognosis and treatment response (Mason *et al.,* 2024). Therefore, the therapeutic strategy differs between breast cancer subtypes. MCF-7 is a hormone-sensitive cell line with ER^+, $PR^{+/-}$, and $HER2^-$ expression profiles that often respond to chemotherapy (Jurzak *et al.,* 2024). MDA-MB-231 (TNBC cell) is a hormone non-sensitive cell line with ER^-, PR^-, and $HER2^-$ expression profiles that are more aggressive, more difficult to treat, and have a poor prognosis characteristic (Jurzak *et al.,* 2024). Understanding these differences is crucial for developing effective treatments for each subtype (Umar *et al.,* 2023). In this study, the effects of LCFE were investigated in breast cancer cells to evaluate its potential value. Furthermore, the related pathways and chemical composition were analyzed for a better understanding of the antiproliferation mechanism in these cells. Our study could lead to the development of a new anti-cancer agent with chemopreventive or chemotherapeutic properties in breast cancer treatment.

MATERIALS AND METHODS

LCFE preparation

L. cylindrica (L.) M. Roem., also known by the Chinese name Si Gua or the English name Luffa, is a climbing herb widely distributed in Asia. The plant was identified by the corresponding authors, and the plant name was also confirmed on http://www.theplantlist.org. *L. cylindrica* flowers were collected at Quanshan Forest Park (N34◦21′, E117◦17’), Xuzhou, Jiangsu province, China. The flowers were dried at 50◦C for 24h. Then, the sample (5g) was powdered and extracted with 75% ethanol (4×40mL, each 24h) and later filtered and evaporated at 50°C. Dried extract was dissolved in DMSO and filtered through a 0.22μm filter membrane until used.

Cell culture

Mammary epithelial cells (MCF-10A) were cultured in MCF-10A special culture medium (Procell, China). MCF-7 cells were grown in DMEM medium and MDA-MB-231 cells were cultured with DMEM/ Ham’s F-12K (1:1) medium (Gibco, USA). Both mediums were supplemented with 10% FBS, 100U/mL penicillin, and 100μg/mL streptomycin. Cells were incubated in 37◦C, 5% CO_2, and 95% humidity conditions.

CCK-8 assay

Cells were seeded in 96-well plates (2×10^4 cells/well) for 24h. Then, LCFE (2.5, 5, 7.5, and 10μg/mL) or 5-Fu (Beyotime, China) (0.5, 1, 2, 3, 4, 5 and 6μg/mL) were added in the medium. After incubated 48h, the medium was removed and CCK-8 (10μL) was added for 4h. The absorbance was measured by Su Per Max 3100 (Shanghai Flash Spectrum, China) at 450 nm. The inhibition ratio was calculated as: $(OD_C - OD_T)/ OD_C \times 100\%$. OD_C and OD_T indicated the absorbance in the control group and the treatment group, respectively.

Hochest stain

Cells were incubated with LCFE (5 and 10μg/mL) for 48 h, and fixed with 4% paraformaldehyde and stained by Hoechst 33342 (1μg/mL, MCE, USA) for 15 min. After removing supernatant, sterilized PBS was added for washing. The cells were observed using an Olympus IX71 microscope (Olympus, Japan).

Quantitative Real-time PCR (qRT-PCR) and western blot

MCF-7 and MDA-MB-231 cells were incubated with LCFE (8μg/mL). After treatment 24 and 48 h, total RNA was acquired using the RNA Isolation Kit (Beyotime, China). The first strand cDNA was synthesized using the TIAN Script II Synthesis Kit (TIANGEN, China). The primers related to cell apoptosis and autophagy genes were designed and listed in Table S1. qRT-PCR was carried out with SYBR Green Mix (MCE, USA) and detected in Roche Lightcycler 96 (Roche, USA). The genes’ relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, and all experiments were repeated three times.

The above cells were collected after LCFE treatment, and lysed in RIPA lysis buffer (Beyotime, China). The supernatants were acquired by centrifuging the cell homogenates at 12,000 × g for 10 min. The proteins’ concentration was measured using the BCA Protein Assay Kit (TransGen, China). Protein samples were separated on 12–15% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride membrane by electrophorese. Then, the membrane was blocked with 5% nonfat milk in PBST (PBS with 0.1% Tween 20) for 2h. The membrane was washed by PBST and incubated with primary antibody for 2h. The membrane was washed and incubated with secondary antibody for 2h, then was pictured using chemiluminescence detection system (Tanon, China). β-Tubulin was used as an internal control. ImageJ software was used to quantify the protein level (Sun *et al.,* 2017).

The antibodies against β-Tubulin, Bcl-2, zinc finger protein 36 (ZFP36), caspase 3, Bcl-2 interacting protein 3 (BNIP3)

and vacuole membrane protein 1 (VMP1) were purchased from Sangon (Sangon, China). Light chain 3 (LC3) and ATG5 antibodies were obtained from Cell Signaling Technology (CST, USA). The antibodies against TBC1 domain family member 14 (TBC1D14) and vesicle associated membrane protein 8 (VAMP8) were obtained from CUSABIO (CUSABIO, China) and ABclonal (ABclonal, China), respectively.

Cell wound healing

Each cell was seeded in 6-well plates (2×10^6 cells/well) previously labelled with traced lines. Atfer culturing for 24 h, the cells were scratched by a pipette tip to form a cell-free zone. After removing the medium, cells were washed with PBS and later added new medium with LCFE and were observed by microscope (Olympus, Japan). The relative migration rate was calculated as we described previously (Ding *et al.,* 2022). Cells were treated with 5-Fu as a positive control.

Liquid chromatography-mass spectrometry (LC-MS)

The composition and relative content of LCFE were determined by LC-MS. UHPLC system (Thermo, USA) with a C18 column (1.8μm*2.1 × 100mm) (Agilent, USA) was used to separate the chemicals in the extract. MS identification was carried out using the Q Exactive HF MS system (Thermo, USA). The detailed procedure and conditions were described previously (Ding *et al.,* 2022). The component identification and classification were performed by referencing Thermo mzCloud and Thermo mz Valut databases.

Statistical analysis

The data are represented as the mean ± SD. One-way ANOVA analysis of variance was performed to test the difference. P-value < 0.05 was considered significant. All analyses were performed using SPSS 18.0 software.

RESULTS

LCFE inhibits breast cancer cells proliferation

Both normal mammary epithelial cells (MCF-10A) and breast cancer cells (MCF-7 and MDA-MB-231) were tested to determine their viability. Following treatment with LCFE, both breast cancer cells displayed a notable decrease in OD_{450} value (fig. 1a). But, the OD_{450} value did not obviously change after the extract treatment in MCF-10A cells. Except MCF-10A cells, these two breast cancer cells demonstrated strong inhibition ratios after treatment with LCFE (fig. 1b). As the positive control group, 5-Fu treatment appeared to suppress breast cancer cells proliferation (fig. 1c). The IC_{50} values of LCFE was 5.5± 0.078μg/mL in MDA-MB-231 cells and 14.1±2.5μg/mL in MCF-7 cells, respectively (fig. 1d). Thus, LCFE inhibits breast cancer cells proliferation, but has no effect on normal breast cells.

LCFE induces cell apoptosis in breast cancer cells

After LCFE treatment, the cell numbers of two breast cancer cells were both reduced (fig. S1). However, the cell number in MCF-10A cells did not change obviously. Hoechst stain results showed that the blue fluorescence intensity and refraction were increased after the extract treatment in two breast cancer cells (fig. 2a and b). However, the blue fluorescence intensity in MCF-10A cells did not change noticeably (fig. 2c). In breast cancer cells, chromatin condensation and an increase in the number of nuclear body fragments were observed after LCFE treatment (fig. 2d and e). Unlike the results in breast cancer cells, MCF-10A cells had uniformly stained cell nuclei after treatment (fig. 2f). These findings implied that LCFE induces cell apoptosis with morphological changes in breast cancer cells, but has no effect on mammary epithelial cell.

LCFE inhibits cell migration in breast cancer cells

LCFE (8 and 12μg/mL) clearly suppressed the closure of wounds after being treated for 24 and 48 h in MDA-MB-231 cells (fig. 3a). The relative migration ratios in the 24 and 48h treatment groups were also significantly less than the control group after treatment (fig. 3b). In the positive control groups, 5-Fu treatment also inhibited MDA-MB-231 cells migration that is consistent with the extract treatment results (fig. 3c and d). Similarly, LCFE treatment groups had larger wound areas and smaller relative migration ratio in MCF-7 cells (fig. 4a and b). 5-Fu treatment also suppressed MCF-7 cells migration (fig. 4c and d). However, with regard to the cell migration effect in MCF-10A cells, the results were not the same. The wound areas and relative migration ratios did not clearly change whether LCFE was applied for 24h or 48h (fig. S2). These results show that LCFE exhibits a strong suppressive ability in the migration of breast cancer cells, but not in normal MCF-10A cells.

LCFE induces gene expression related to apoptosis and autophagy pathway in MCF-7 cells

RNA-seq was carried out to test gene expression after LCFE treatment in MCF-7 cells. Genes involved in the apoptosis and autophagy pathways were selected and analyzed according to RNA-seq results (data not shown). The results confirmed that ZFP36 mRNA expression level was significantly upregulated after 24 and 48h treatment (fig. 5a). ZFP36 protein level increased obviously after 24 h treatment, then decreased after 48h treatment (fig. 5a). BNIP3 mRNA expression was increased after 48h treatment, and protein level was increased after 24h treatment (fig. 5b). Caspase 3 and Bcl-2, critical members of mitochondria-mediated apoptosis, were also analyzed. Caspase 3 protein level was obviously increased after 48 h treatment, and the cleaved caspase 3 was also enhanced (black box) (fig. 5c). Bcl-2, the key anti-apoptotic member, was clearly downregulated after 24 and 48h of LCFE treatment (fig. 5c). VMP1 is a gene that is involved in the

autophagy process. Its mRNA level was upregulated significantly after the extract treatment for 24 and 48 h, and its protein level increased significantly after 48 h treatment (fig. 5d). LC3 (an essential protein in autophagosome) protein level also increased significantly after treatment with LCFE (fig. 5e). The relative LC3-II protein level was strongly upregulated after 24h treatment (fig. 5f). According to these findings, LCFE may induce caspase-dependent apoptosis and VMP1 related autophagy in MCF-7 cells.

LCFE induces gene expression related to apoptosis and autophagy pathway in MDA-MB-231 cells

Based on RNA-seq results, genes related to apoptosis and autophagy pathways were selected and analyzed in MDA-MB-231 cells. BMP4 mRNA expression level was reduced after LCFE treatment 48h, as was protein level after 24 and 48h treatment (fig. 6a). ZFP36 was significantly upregulated after treatment in mRNA level, and the protein level was also clearly increased after 24 h treatment (fig. 6b). The protein level of caspase 3 did not change obviously after treatment, but the level of cleaved caspase 3 increased (fig. 6c). Bcl-2 protein level did not change obviously after treatment (fig. 6c). TBC1D14, an autophagy suppressor, had its mRNA and protein levels down regulated (black box) after treatment for 48 h (fig. 6d). VAMP8 was significantly upregulated after treatment for 48 h in mRNA level, as did protein levels in both the 24 and 48 h treatment groups (fig. 6e). Furthermore, ATG5 and LC3 protein expression were both enhanced after the extract treatment (fig. 6f). The relative protein level of LC3-II was also significantly raised (fig. 6g). The results suggested that LCFE may induce BMP4 and ZFP36 related apoptosis as well as TBC1D14 and VAMP8-related autophagy in MDA-MB-231 cells.

The bioactive chemicals in LCFE

The bioactive compounds in LCFE were identified by LC-MS. In positive ion mode, 155 phytochemicals were detected and listed in Table S2. Among these chemicals, flavonoids, isoflavonoids, coumarins and derivatives have been shown with cancer-suppressive properties. In LCFE with positive ion mode, 16 flavonoids, 2 isoflavonoids, and 5 coumarins and derivatives were identified. Besides, two flavonoids (Nictoflorin and Kaempferol) and one isoflavonoids (Genistein 4'-O-glucuronide) were notably abundant in the extract (fig. 7a). In negative ion mode, 126 chemical compounds were identified which contained 16 flavonoids, 2 isoflavonoids and 1 coumarins and derivatives (Table S3). In LCFE with negative ion mode, two flavonoids (Apigenin-7-O-glucuronide and Afzelin) and one isoflavonoid (Genistein) were abundantly detected (fig. 7b). In addition, two undefined flavonoids (here named compound 1 and compound 2) were found in high concentrations in the extract (fig. 7b, Table S3). These abundant bioactive chemicals may play vital roles in breast cancer cells suppression.

DISCUSSION

Flowers contain a variety of biologically active phytochemicals, some of which have been traditionally used to cure diseases, including cancer. *L. cylindrica* is a traditional Chinese medicinal and edible plant (Wu *et al.,* 2020). In the present study, the antiproliferation effect of LCFE was investigated in breast cancer cells. The findings indicate that LCFE can suppress breast cancer cells proliferation, most notably in generally resistant TNBC cells (MDA-MB-231) (fig. 1). Interestingly, the antiproliferation activity of the extract was limited and minor in normal mammary epithelial MCF-10A cells in all tested concentrations (fig. 1b). Therefore, LCFE appears to contain a selective suppression for breast cancer cells, with very low cytotoxicity for non-cancerous breast cells. Hibiscus *(Hibiscus rosa-sinesis)* flower extract also has this selective cell cytotoxicity in breast cancer, which can be used as an adjuvant agent to reduce the toxicity in chemotherapy (Nguyen *et al.,* 2019). Aside from cell viability, LCFE inhibited breast cancer cells migration (fig. 3 and 4). Similarly, the extract did not inhibit MCF-10A cells migration (fig. S2). These findings further suggest the possibility of supplementing therapeutic regimens with LCFE in cancer treatment. Phytochemicals mainly induce cell apoptosis to kill cancerous cells in chemotherapy (Rajput and Mandal, 2012). Apoptosis has typical morphological features including cell blebbing, cytoplasm condensation, DNA fragmentation, and nuclear condensation (Huschtscha *et al.,* 1996). In our results, except for MCF-10A cells, the tested breast cancer cells displayed apoptotic morphological characteristics like cell shrinkage and condensed chromatin after LCFE treatment. The increased fluorescence intensity and nuclear body fragments were also observed after the extract treatment (fig. 2). These characteristics were also not observed in normal MCF-10A cells. These results strongly indicated that LCFE may induce apoptosis in breast cancer cells, with a minor effect on normal breast cells. Aside from morphological changes, apoptotic cells also exhibit a difference in genes expression (Carneiro and El-Deiry, 2020). LCFE treatment significantly increased ZFP36 and BNIP3 expression in MCF-7 cells (fig. 5a and b). In addition, caspase 3, an apoptotic effector, was cleaved and activated, while Bcl-2, a key anti-apoptotic member, was reduced following treatment (fig. 5c). ZFP36 (also known as TTP), a novel zinc finger protein and a negative regulator, has been proofed to decay the key genes expression in cancer initiation and progression (Park *et al.,* 2018). Overexpression of ZFP36 suppressed cell proliferation and induced lung cancer cells apoptotic death (Dong *et al.,* 2018). BNIP3 has been shown to have the pro-apoptotic effect by interacting with E1B or Bcl-2 proteins (Singh *et al.,* 2018). Therefore, LCFE may induce caspase-dependent apoptosis by the ZFP36/Bcl-2 axis in MCF-7

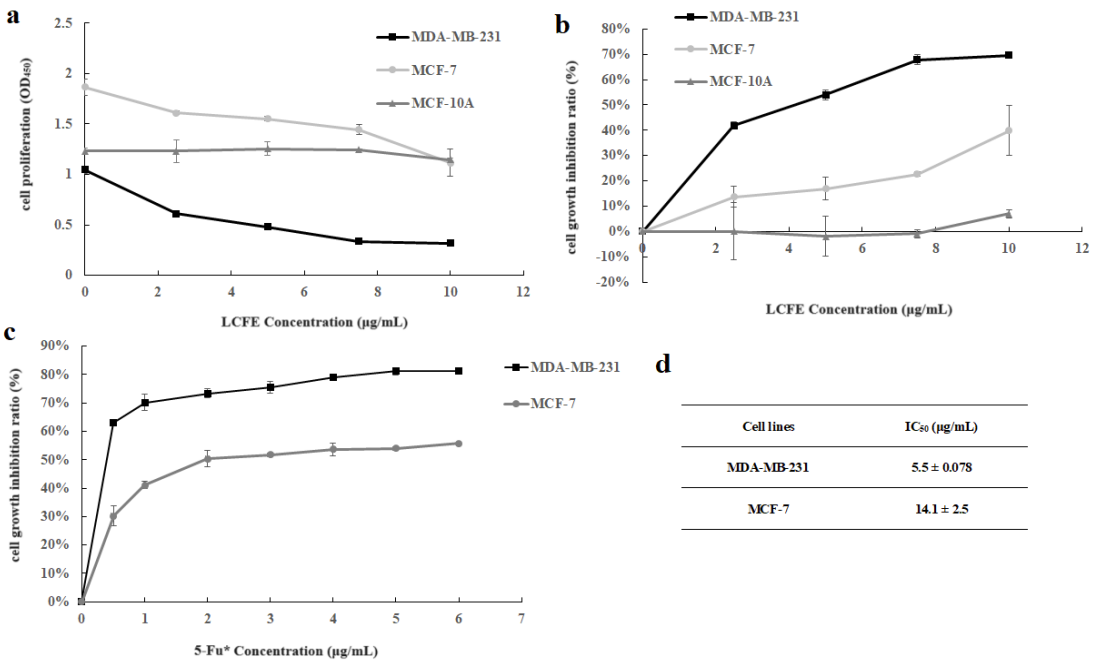

Fig. 1: Anti-proliferative activity of LCFE. (a) CCK-8 assay of MCF-7, MDA-MB-231 and MCF-10A cells after LCFE treatment for 48h. (b) The growth inhibition ratio of each cell after LCFE treatment. (c) The growth inhibition ratio of MCF-7 and MDA-MB-231 cells after 5-Fu treatment for 48h. (d) IC_{50} values of LCFE in MCF-7 and MDA-MB-231 cells.

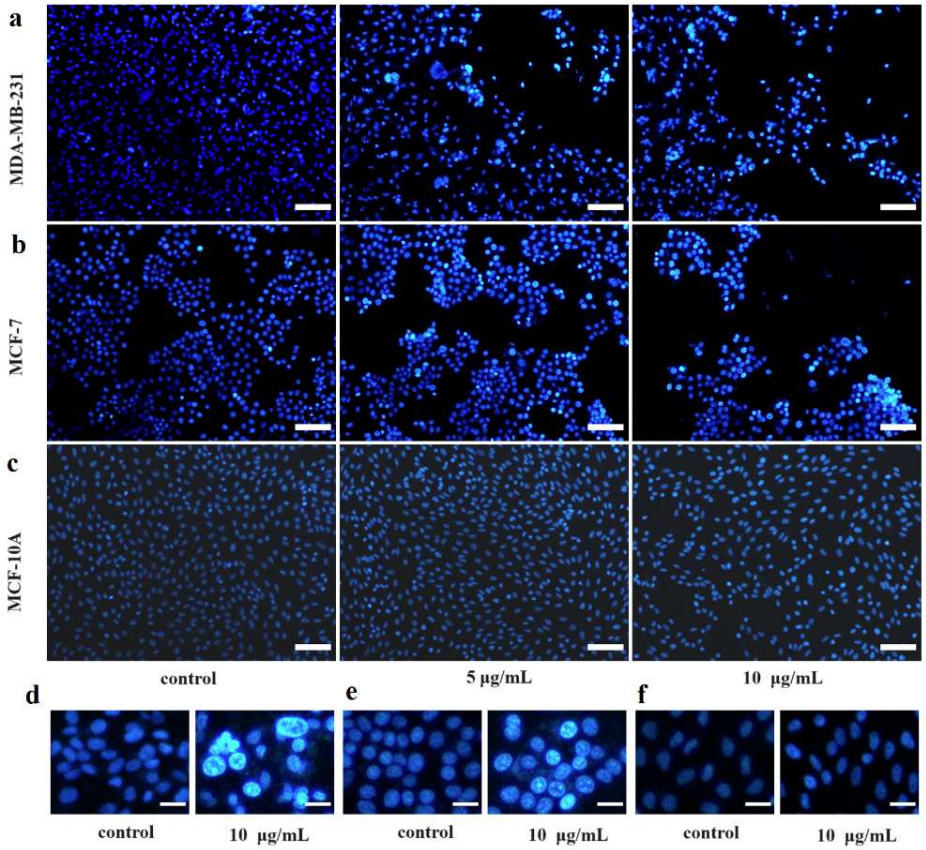

Fig. 2: LCFE increased fluorescence intensity and the number of nuclear body fragments in breast cancer cells. (a) MDA-MB-231, (b) MCF-7 and (c) MCF-10A cells were treated with LCFE for 48h, respectively. Scale bar 100μm. Hoechst staining of 10μg/mL LCFE treated (d) MDA-MB-231, (e) MCF-7 and (f) MCF-10A cells. Scale bar 25μm.

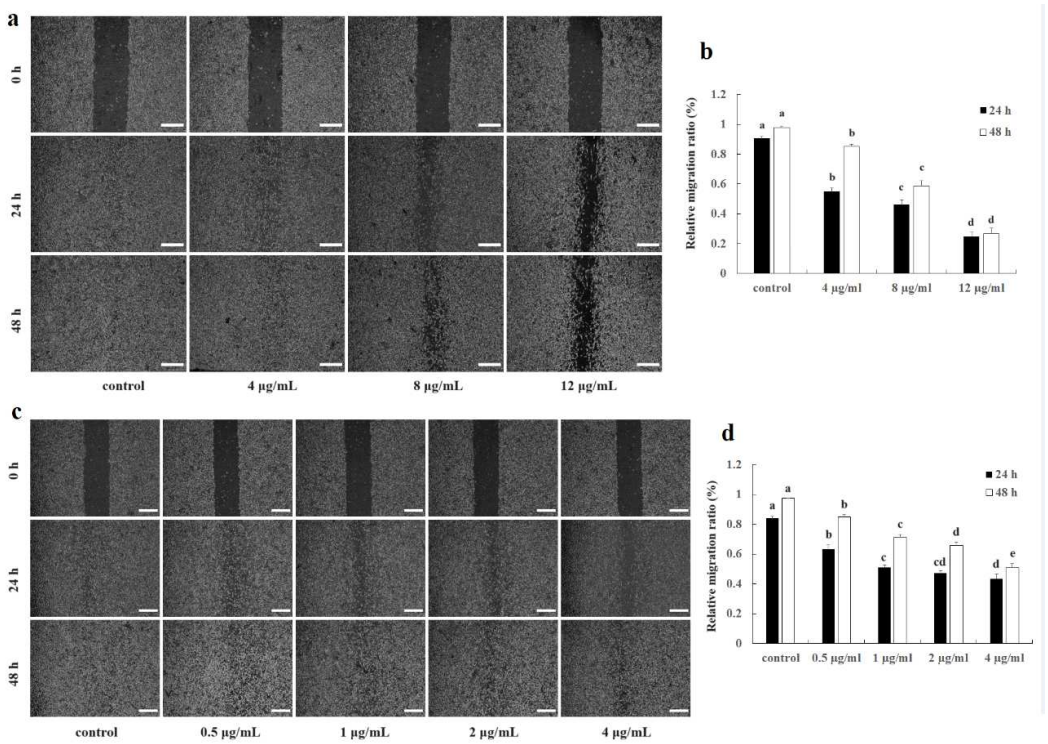

Fig. 3: LCFE inhibited cell migration in MDA-MB-231 cells. (a) and (b) represent the wound healing and the relative migration ratio of MDA-MB-231 cells after LCFE treatment, respectively. (c) and (d) represent the wound healing and the relative migration ratio of MDA-MB-231 cells after 5-Fu treatment, respectively. Scale bar is 500 μm for the cell images. Bars represent mean ± SD (n = 3), and the different letters above the bars are significantly different ($P < 0.05$) at each time point.

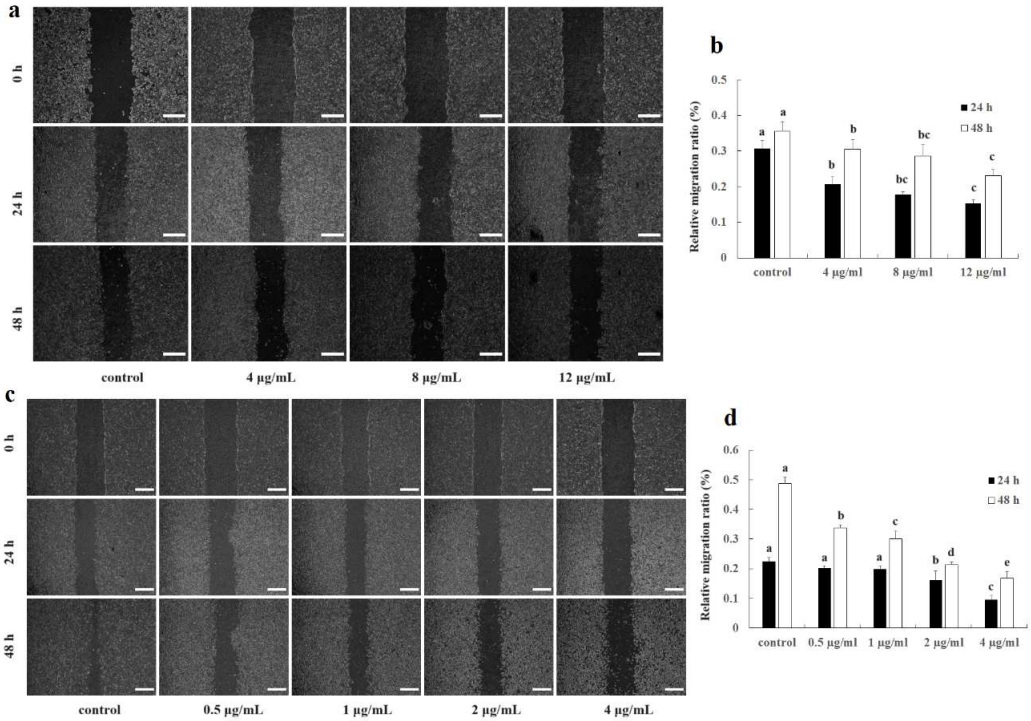

Fig. 4: LCFE inhibited cell migration in MCF-7 cells. (a) and (b) represent the wound healing and the relative migration ratio of MCF-7 cells after LCFE treatment, respectively. (c) and (d) represent the wound healing and the relative migration ratio of MCF-7 cells after 5-Fu treatment, respectively. Scale bar is 500μm for the cell images. Bars represent mean ± SD (n = 3), and the different letters above the bars are significantly different ($P<0.05$) at each time point.

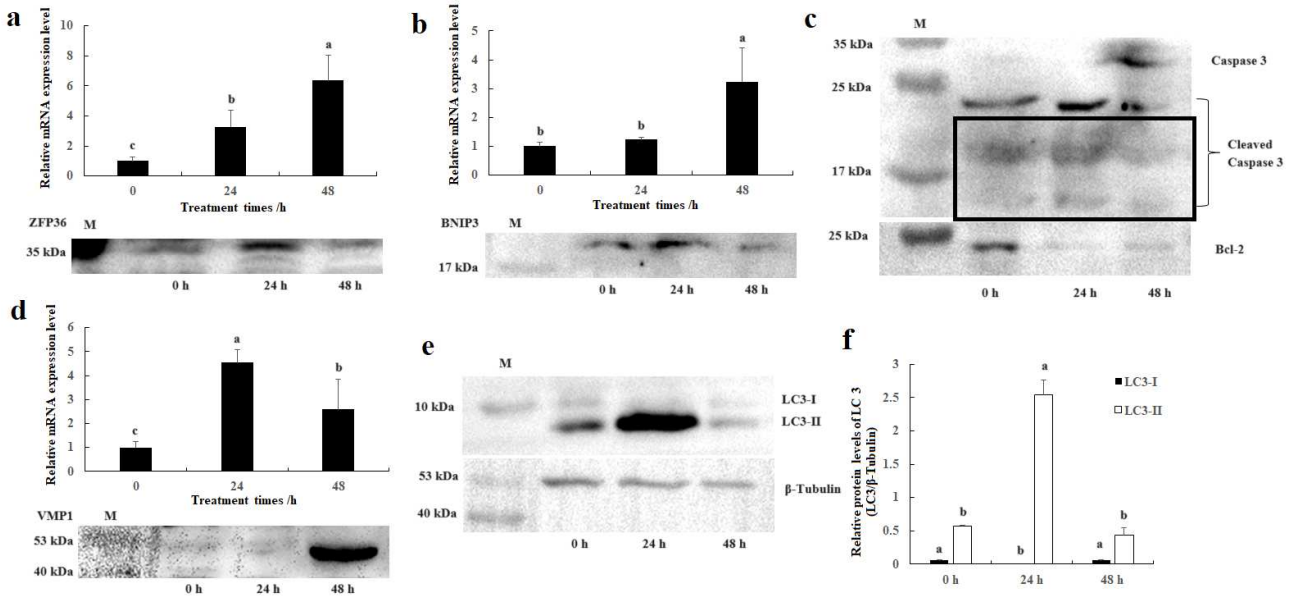

Fig. 5: LCFE affected several genes expressions related to apoptosis and autophagy in MCF-7 cells. ZFP36 (a) and BNIP3 (b) expression after LCFE treatment for 24 and 48h. (c) Caspase 3 and Bcl-2 protein expression after the extract treatment. (d) VMP1 mRNA and protein expression levels after the extract treated. (e) LC3 protein expression after the extract treatment. (f) The relative expression levels of LC3 were calculated by Image-J. Bars represent mean ± SD (n = 3), and the different letters above the bars are significantly different ($P<0.05$) in all histogram. β-Tubulin was used as an internal control in all western blots.

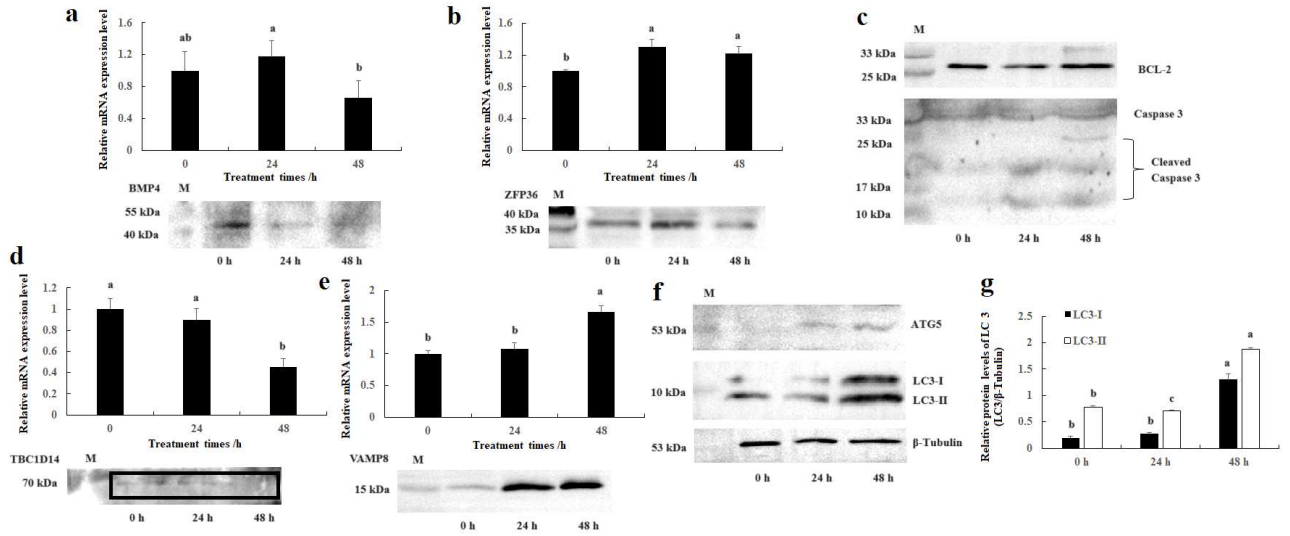

Fig. 6: LCFE affected several gene expressions related to apoptosis and autophagy in MDA-MB-231 cells. BMP4 (a) and ZFP36 (b) mRNA and protein expression after LCFE treatment for 24 and 48h. (c) Bcl-2 and caspase 3 protein expression after the extract treatment. TBC1D14 (d) and VAMP8 (e) mRNA and protein expression after the extract treatment. (f) ATG5 and LC3 protein expression after the extract treatment. (g) The relative expression levels of LC3 were calculated by Image-J. Bars represent mean ± SD (n =3) and the different letters above the bars are significantly different ($P < 0.05$) in all histogram. β-Tubulin was used as an internal control in all western blots.

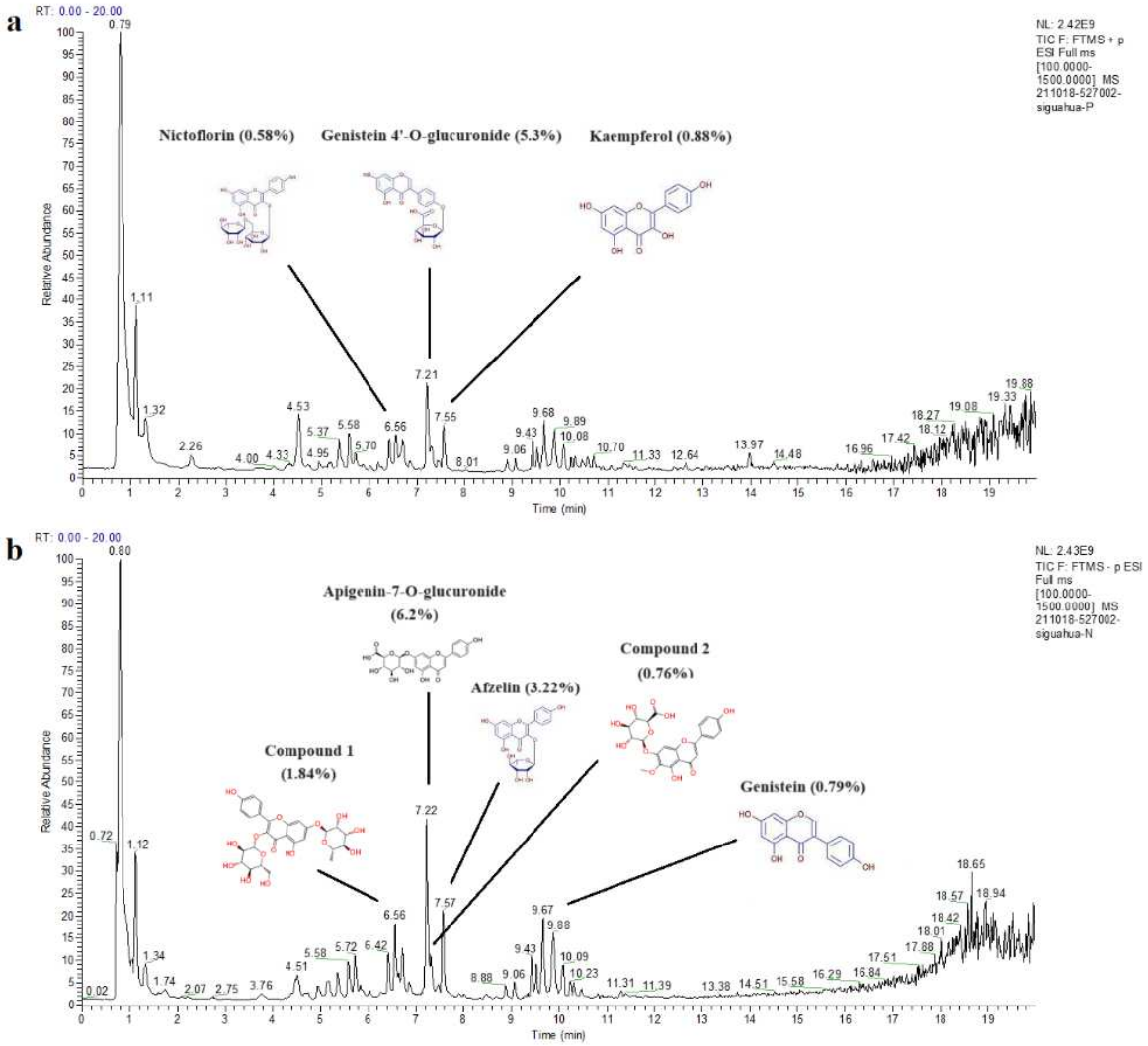

Fig. 7: Total ion current (TIC) of LCFE in LC-MS under positive ion mode (a) and negative ion mode (b). The high content of flavonoids was displayed with their relative content and structure.

cells. But the way LCFE triggered MDA-MB-231 cells was different. ZFP36 mRNA and protein levels were increased, while BMP4 levels were decreased in MDA-MB-231 cells (fig. 6). Caspase 3 was also cleaved and activated, but Bcl-2 expression remained unchanged (fig. 6). BMP4 regulates cell proliferation and differentiation, and its downregulation was linked to apoptosis induction by *Antrodia camphorata* extract treatment (Ding *et al.,* 2021). Hence, LCFE may trigger apoptosis via the ZFP36/BMP4 axis in MDA-MB-231 cells.

Aside from apoptosis, autophagy, a type II programmed cell death (PCD), has a close connection with tumorigenesis and treatment. Autophagy may be triggered by radiation or chemotherapy in response to therapeutic stress in cancer cells (White, 2012). On the other hand, overinduction of autophagy during cancer treatment may result in autophagic cell death (Linder and Kogel, 2019). Kaempferol, a flavonoid compound, has been verified to activate autophagic death via the IRE1-JNK-CHOP pathway in gastric cancer cells (Kim *et al.,* 2018). VMP1 expression was upregulated after LCFE treatment in MCF-7 cells (fig. 5d). Furthermore, LC3 was upregulated, and LC3-II was activated after the extract treatment, indicating the induction of autophagy (fig. 5e and f). VMP1 can colocalize with LC3 and plays a role in autophagy initiation by interacting with beclin-1 protein (Ropolo *et al.,* 2007). Based on these findings, LCFE can activate cell autophagy in MCF-7 cells by upregulating VMP1 and activating LC3. The expression of TBC1D14 was reduced in MDA-MB-231 cells, while VAMP8 was clearly increased after LCFE treatment (fig. 6d and e). Meanwhile, both ATG5 and LC3 protein levels were upregulated after treatment (fig. 6f and g). TBC1D14 inhibits autophagy by interacting with ULK1, an autophagy kinase in the autophagy initiation complex ULK1/ATG13 (Longatti *et al.,* 2012). The key step of autophagosome formation is vesicle elongation, which is dependent on ATG5-ATG12 and LC3 systems (Ohsumi, 2001). Besides, VAMP8 is an integral membrane protein that participates in

autophagosome maturation and fusion with the lysosome (Huang *et al.,* 2021). Therefore, our results strongly suggest that LCFE can suppress TBC1D14 expression, enhance ATG5 expression and active LC3-II for autophagosome formation, and increase VAMP8 expression to facilitate autophagosome maturation and autophagosome-lysosome fusion in MDA-MB-231 cells.

Plant metabolites are important sources for screening new pharmacological chemicals (Roumani *et al.,* 2020). LCFE contained a high concentration of metabolites when analyzed using LC-MS. But, Cucurbitacin B, the major constituent in the fruit of the Cucurbitaceae plant with anti-cancer activities (Mukherjee *et al.,* 2022), was not found in the extract. Whereas, LCFE contained a high content of other metabolites with anti-cancer properties, like flavonoids, isoflavonoids, coumarins and derivatives (fig. 7). These phytochemical compounds have been proved to induce apoptosis or affect related gene expression in cancer cells (Seca and Pinto, 2018). Most notably, two undefined flavonoid compounds with high content in the extract were detected. These undefined flavonoids have potential values for anticancer agent screening and need further investigation.

CONCLUSION

In summary, LCFE suppresses breast cancer cells proliferation and migration, but has no effect on non-cancerous MCF-10A cells. Furthermore, it contains a high concentration of bioactive compounds with anticancer potential and induces cell apoptosis and autophagy by regulating apoptosis and autophagy related gene expression in breast cancer cells. These findings highlight the potential of LCFE as a natural agent in human breast cancer treatment.

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Authors' contributions
Chang Liu: Sample acquisition and preparation, Cell culture, Cell proliferation assay, Writing – original draft. Jun Yuan: Hoechst staining, qRT-PCR, Western blot, Writing – original draft. Deng Zou: qRT-PCR, Western blot, Software, Analyze experimental data. Xiaojie Jiang: Analyze experimental data. Jun Li: Analyze experimental data. Haijun Zhang: Project management, Supervision. Yuxuan Sun: Methodology, Supervision, Writing – review & editing.

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Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval
The ethical approval was granted by the Huaibei Normal University Ethical Review Committee with ethical approval number (CHNU-EC-20240325).

Conflict of interest
The authors declare that they have no financial conflicts of interest.

Supplementary data
https://www.pjps.pk/uploads/2025/12/SUP1765535832.pdf

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