Cytotoxicity Screening of Plants of Genus *Piper* in Breast Cancer Cell Lines

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**Abstract**

**Purpose:** To examine whether seven species of plants of genus *Piper* possess anti-cancer effects.

**Methods:** One normal breast and three breast cancer cell lines were used to test cytotoxic effects over a period of 72 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dried plants were extracted with methanol and dichloromethane, and the effective extract isolated by crystallization, acid/base extraction and column chromatography techniques. Fragmented DNA was purified by phenol/chloroform/isoamyl alcohol.

**Results:** Methanol and dichloromethane extracts of *Piper* retrofractum, *Piper betle*, especially *Piper nigrum*, exhibited strong effect on MDA-MB-468. When the crude extract of *P. nigrum* was then separated by column chromatography, fraction D showed activity against both MCF-7 and MDA-MB-468 cells. Fraction DE that was isolated from D demonstrated a highly cytotoxic effect with IC₅₀ values of 8.33 ± 1.27 and 7.48 ± 0.57 µg/ml on MCF-7 and MDA-MB-468 cells, respectively. Furthermore, fraction DF exhibited a strong cytotoxic effect only on MCF-7 with IC₅₀ value of 8.51 ± 0.39 µg/ml. DNA smears of MCF-7 and MDA-MB-468 cells treated with fraction DE and DF were observed within 7 days.

**Conclusions:** These results indicate that the compounds isolated from *P. nigrum*, viz, DE and DF, have cytotoxic effect on breast cancer cell lines. These fractions could be promising agent for breast cancer treatment. Further studies on the isolation, structural and mechanism elucidation of the active compound are still needed being carried out.

**Keywords:** Cytotoxicity, Breast cancer, *P. nigrum*, DNA fragmentation

**INTRODUCTION**

Breast cancer is a major cause of death in the female population. According to worldwide data in 2008 of American Cancer Society, there are 1,383,500 new cancer cases (23 %) and 458,400 cancer deaths (14 %) [1]. The most common treatment for breast cancer is radiotherapy, hormonal therapy and chemotherapy. The patients often experience many extreme side effects from anti-cancer drugs. Intensive treatment with radiotherapy or chemotherapy is usually associated with adverse side effects ranging from nausea to bone marrow failure [2]. Moreover, tumor cells are usually resistant to chemotherapy [3]. Therefore, phytochemicals from medicinal plants may be an option for treatment.

Numerous studies have reported that phytochemicals isolated from plants of the *Piper* genus are potent biological agents with...
Extraction procedure

Five hundred grams of *P. nigrum* powder were isolated into a brown pellet (P1) via crystallization according to Reshmi et al [4]. Briefly, the powder of *P. nigrum* was mixed into dichloromethane with 1:1.2 volumetric mixing ratio. Then, the mixture was incubated in a shaking incubator at 35 °C for 2 h. The extract was filtered and evaporated on a rotary evaporator. One hundred milliliters of cold diethyl ether was added into the residue and shook for 5 min after the residue was cooled in an ice bath. The mixture was evaporated and cooled again. In recrystallization step, the cold diethyl ether was added and the mixture was shook for 25 min. Then, the yellow crystal (P2) was separated by filtration through filter paper. And the solvent was evaporated on a rotary evaporator to obtain extract CP2, which was divided into two groups. The first group was extracted using acid/base extraction (10 % hydrochloric acids and saturated sodium hydroxide) to obtain two parts, P3 and CP3. Then, the second group of CP2 was purified by column chromatography using a silica gel plate (Merck Kieselgel 60) with 100 % dichloromethane and dichloromethane/methanol (9:1) was used as an eluting solvents. Eleven fractions (A-K) were collected according to TLC spots. The active fraction D was then processed by column chromatography and eluted with ethyl acetate/hexane (2:3). Finally, the fractions DE and DF were obtained and tested on cancer cell lines.

Thin layer chromatography (TLC) analysis

The samples of each *P. nigrum* fraction were submitted to TLC plates (a silica coated plate, Merck). For fractions A to K, the bands were separated using 100 % dichloromethane as mobile phase. Fractions F to K were eluted again with 5 % methanol in dichloromethane to observe their chromatogram legibly. The obtained chromatogram was revealed with Dragendorff's reagent. The fractions containing alkaloids were indicated by the presence of an orange band [11]. The TLC-patterns were used to select the fractions for testing cytotoxicity.

Table 1: Plant species investigated

| Plant                        | Family      | Part of plant | Voucher specimens no. |
|------------------------------|-------------|---------------|-----------------------|
| *Piper nigrum* L.           | Piperaceae  | Fruit         | SKP 146161401          |
| *Piper retrofractum* Vahl   | Piperaceae  | Fruit         | SKP 146161801          |
| *Piper ribesoides* Wall.    | Piperaceae  | Stem          | SKP 146161802          |
| *Piper betle* L.            | Piperaceae  | Leaf          | SKP 146160201          |
| *Piper samentosum* Roxb. Ex Hunt. | Piperaceae | Leaf          | SKP 146161901          |
| *Piper cubeba* L.           | Piperaceae  | Fruit         | SKP 146160302          |
| *Piper porphyrophyllum* N.E.Br. | Piperaceae | Leaf          | SKP 146161001          |
Cell culture conditions

MCF-7, MDA-MB-231, MDA-MB-468 and MCF-12A cells were obtained from ATCC (Manassas, VA, USA). MCF-7 cells were grown in RPMI 1640 (Invitrogen) containing 10 % fetal bovine serum (Invitrogen) with 50 units/ml of penicillin (Invitrogen) and 50 µg/ml of streptomycin (Invitrogen). MDA-MB-231 and MDA-MB-468 cells were grown in DMEM (Invitrogen) supplemented with 50 units/ml of penicillin (Invitrogen) and 100 µg/ml of streptomycin (Invitrogen). MCF-12A cells were grown in a 1:1 mixture of DMEM and Ham’s F12 medium (PAA Laboratories GmbH) containing 5 % horse serum (Invitrogen) with 20 ng/ml human epidermal growth factor (Invitrogen), 100 ng/ml bovine insulin (Sigma) and 500 ng/ml hydrocortisone, 95 % (Sigma). All cells were maintained in a humidified incubator at 37 °C and 5 % CO₂.

Cytotoxic assay

MCF-7, MDA-MB-231, MDA-MB-468 and MCF-12A cells were seeded in 96-well plates at a density of 2 × 10⁵ cells/well. The cell lines were exposed to crude extracts in a concentration range of 0-80 µg/ml and piperine in a concentration range of 1.25-20 µg/ml for 72 h. Then the cells were washed with 1X PBS and incubated in 100 µl of 0.5 mg/ml MTT at 37 °C for 30 min. Under light protection, the dark blue crystals of formazan (MTT metabolites) were dissolved with 100 µl DMSO and incubate at 37 °C for 30 min. Absorbance was measured at 570 and 650 nm using a microplate reader spectrophotometer. Viable cells were represented as a percentage of survival and calculated as in Eq 1 (n = 3).

\[
\text{Survival (\%)} = \frac{(A_{570} - A_{650})/(A_{570}^c - A_{650}^c))}{100} \cdot (1)
\]

where \(A_{570}^c\), \(A_{650}^c\), \(A_{570}\), and \(A_{650}\) are the absorbance of the test samples (at 570 and 650 nm) and negative control (at 570 and 650 nm), respectively.

According to US NCI plant screening program, a crude extract is generally considered to have in vitro cytotoxic activity with IC₅₀ value ≤ 20 µg/ml, while this value was deemed at ≤ 4 µg/ml for a pure compound [12].

DNA fragmentation

The cells were treated with the crude extracts and positive controls, doxorubicin and curcumin. The treated cells were incubated up to 7 days and collected everyday by trypsinization and centrifugation. The DNA was extracted with phenol/chloroform/isooamyl alcohol and precipitated with 2:3 volume of isopropanol. The precipitated DNA was washed once with 70 % ethanol (v/v) and air-dried for 30 min. DNA was loaded onto a 1.5 % agarose gel and visualized with ethidium bromide under UV light.

Statistical analysis

Median inhibition concentration (IC₅₀) was obtained by SoftMax® Pro 5 program (MDS Analytical Technologies Inc., California, USA) and expressed as the mean ± standard deviation.

RESULTS

Cytotoxic effect of crude extracts on breast cancer cells

Three breast cancer (MCF-7, MDA-MB-231, MDA-MB-468) and one normal breast (MCF-12A) cell lines were used to test the cytotoxic effect of seven Piper genus plants at 72 h. The cytotoxicity of the crude extracts of Piper plants is shown in Table 2. Regarding the methanolic crude extracts, P. nigrum and P. retrofractum revealed high effect on MDA-MB-468 cells with IC₅₀ value of 9.04 ± 0.71 µg/ml and 12.27 ± 2.14 µg/ml, respectively. However, P. ribesoides, P. betle and P. cubeba exhibited a lower cytotoxic effect on breast cancer cell lines. Concerning dichloromethane extracts, P. nigrum and P. betle demonstrated strong effect on MDA-MB-468 cells with IC₅₀ value of 7.94 ± 4.52 µg/ml and 11.26 ± 0.01 µg/ml, respectively. Moreover, P. betle showed cytotoxic on MBA-231 cells with IC₅₀ value of 19.76 ± 2.87 µg/ml. Both methanol and dichloromethane crude extracts of all tested plants exhibited low-level effect on MCF-12A cells. Our results indicated that P. nigrum possessed strong cytotoxic activity on breast cancer cell lines.

Cytotoxic effect of extracts of P. nigrum

Since P. nigrum represented strong cytotoxic activity on breast cancer cell lines, here we performed MTT assay to test whether the cytotoxic effect of crude extract of P. nigrum come from two pure compounds (piperine and pellitorine) or other compounds. Commercial piperine and pellitorine, major compound of P. nigrum, were used to test their cytotoxic effects and compared them with those curcumin, the active compound from turmeric, the cytotoxic effects of which on many cancer cells have been reported [7-9]. Our results revealed that both piperine and pellitorine had less effect than crude
### Table 2: Cytotoxicity of dichloromethane and methanol crude extracts of *Piper* on breast cancer and normal breast cell lines

| Plant | Methanol crude extract | IC<sub>50</sub> value ± SD (µg/ml)<sup>a</sup> | Dichloromethane crude extract | IC<sub>50</sub> value ± SD (µg/ml)<sup>a</sup> |
|-------|-------------------------|-----------------------------------------------|-------------------------------|-----------------------------------------------|
|       |                         | MCF-7 | MDA-MB-468 | MDA-MB-231 | MCF-12A | MCF-7 | MDA-MB-468 | MDA-MB-231 | MCF-12A |
| *P. nigrum* L. | 20.25 ± 0.01 | 9.04 ± 0.71<sup>b</sup> | 22.37 ± 2.31 | 46.31 ± 0.76 | 23.46 ± 1.10 | 7.94 ± 4.52<sup>b</sup> | 38.82 ± 0.23 | 35.65 ± 0.27 |
| *P. retrofractum* Vahl | 19.69 ± 0.88<sup>b</sup> | 12.27 ± 2.14<sup>b</sup> | 17.10 ± 0.46<sup>b</sup> | 32.41 ± 5.94 | 20.03 ± 2.85 | 17.34 ± 4.08<sup>b</sup> | 21.77 ± 2.32 | > 80 |
| *P. ribesoides* Wall. | 32.27 ± 0.46 | 25.24 ± 0.27 | > 80 | > 80 | 20.33 ± 3.02 | 38.25 ± 2.61 | > 80 | > 80 |
| *P. betle* L. | 19.30 ± 1.03<sup>b</sup> | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 |
| *P. sarmentosum* Roxb. | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 |
| *P. cubeba* L. | 26.63 ± 0.47 | 22.95 ± 2.09 | 59.19 ± 3.97 | > 80 | > 80 | > 80 | > 80 | > 80 |
| *P. porphyrophyllum* N.E.Br. | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 |

<sup>a</sup><sup>n</sup> Values are obtained from three independent experiments. <sup>b</sup> Values are obtained from three independent experiments. <sup>c</sup> The IC<sub>50</sub> is < 20 µg/ml, considered highly cytotoxic against to each cell line.

### Table 3: Cytotoxicity of from *P. nigrum* on breast cancer and normal breast cell lines

| Compound | IC<sub>50</sub> value ± SD (µg/ml)<sup>a</sup> | 1<sup>st</sup> column chromatography | 2<sup>nd</sup> column chromatography |
|----------|-----------------------------------------------|-----------------------------------|-----------------------------------|
|          | MCF-7 | MDA-MB-468 | MDA-MB-231 | MCF-12A | MCF-7 | MDA-MB-468 | MDA-MB-231 | MCF-12A |
| P1       | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 | > 80 |
| P2       | > 80 | 20.10 ± 3.83 | 53.62 ± 1.44 | > 80 | > 80 | > 80 |
| P3       | 61.35 ± 3.01 | 40.91 ± 6.22 | > 80 | > 80 | > 80 | > 80 |
| CP2      | 7.45 ± 1.59<sup>c</sup> | 18.19 ± 0.59<sup>c</sup> | 21.68 ± 1.69 | 48.07 ± 0.46 | > 80 | > 80 |
| CP3      | 17.74 ± 1.26<sup>c</sup> | 9.32 ± 3.65<sup>c</sup> | 25.88 ± 0.61 | > 80 | > 80 |
| Piperine<sup>b</sup> | > 20 | 19.07 ± 0.84<sup>c</sup> | 44.02 ± 3.18 | > 80 | > 80 | > 80 |
| Pellitorine<sup>b</sup> | > 20 | > 20 | > 20 | > 20 | > 20 |
| Curcumin<sup>b</sup> | 15.40 ± 4.26 | 3.91 ± 1.43<sup>d</sup> | 8.92 ± 1.84 | > 80 | > 80 |
| C        | 23.12 ± 2.29 | 16.79 ± 4.42<sup>c</sup> | 44.02 ± 3.18 | 39.38 ± 0.70 | > 80 | > 80 |
| D        | 5.82 ± 0.15<sup>c</sup> | 7.34 ± 0.51<sup>c</sup> | 24.39 ± 1.18 | 36.13 ± 6.67 | > 80 | > 80 |
| F        | 18.79 ± 1.09<sup>c</sup> | 21.75 ± 1.45 | 27.39 ± 2.66 | 34.46 ± 1.56 | > 80 | > 80 |
| G        | 65.17 ± 3.45 | > 80 | > 80 | > 80 | > 80 |
| I        | 48.17 ± 3.83 | 70.68 ± 6.25 | > 80 | > 80 | > 80 |
| DB       | 14.62 ± 0.53<sup>c</sup> | 24.29 ± 3.74 | 23.61 ± 1.20 | > 80 | > 80 |
| DC       | 23.09 ± 0.26 | 12.62 ± 3.31<sup>c</sup> | 28.83 ± 5.26 | 74.33 ± 2.45 | > 80 | > 80 |
| DD       | 14.73 ± 1.41<sup>c</sup> | 25.22 ± 1.98 | 23.39 ± 4.64 | 21.95 ± 4.30 | > 80 | > 80 |
| DE       | 8.33 ± 1.27<sup>c</sup> | 7.48 ± 0.57<sup>c</sup> | 20.29 ± 1.37 | 6.91 ± 0.01<sup>c</sup> | > 80 | > 80 |
| DF       | 6.51 ± 0.39<sup>c</sup> | 22.52 ± 1.07 | 33.02 ± 2.58 | 20.66 ± 2.80 | > 80 | > 80 |
| DG       | 29.52 ± 1.92 | 14.33 ± 3.56<sup>c</sup> | 30.80 ± 5.12 | 34.13 ± 1.72 | > 80 | > 80 |
| DH       | 22.05 ± 1.09 | 17.74 ± 1.22<sup>c</sup> | 26.87 ± 2.05 | > 80 | > 80 |

<sup>a</sup> Values are obtained from three independent experiments. <sup>b</sup> Piperine, pellitorine and curcumin were standard powder used to test cytotoxic effect with maximum concentration 20 µg/ml. <sup>c</sup> The IC<sub>50</sub> is < 20 µg/ml, considered highly cytotoxic against to each cell line. <sup>d</sup> The IC<sub>50</sub> is < 4 µg/ml, considered highly cytotoxic against to each cell line.
extract of *P. nigrum* on MDA-MB-468 cells. Moreover, curcumin showed strong effect on cancerous cell lines (MDA-MB-231 and MDA-MB-468) and also normal breast cell line (MCF-12A) (Table 3).

The compounds of *P. nigrum* were separated by crystallization and all the extracts were tested for cytotoxic activity. The finding suggested that the crude extract from crystallization, namely P1, did not effect to all cell lines, while the crude extract from recrystallization, namely P2, exhibited cytotoxic activity on MDA-MB-468 cells with IC₅₀ value of 20.10 ± 3.83 µg/ml; which is most likely piperine. In addition, the 1H-NMR profile of P2 was similar to that of commercial piperine (data not shown).

The supernatant from the recrystallization step was evaporated to obtain the crude extract, CP2. The CP2 was toxic to MCF-7 cells with IC₅₀ value of 7.45 ± 1.59 µg/ml. This crude extract was divided into two groups. The first group was processed by acid/base extraction to give two parts, P3 and CP3. CP3 killed MDA-MB-468 cells with IC₅₀ value of 9.32 ± 3.65 µg/ml, whereas P3 had little or no effect on MDA-MB-468 and MCF-7 cells. The IC₅₀ values of all the extracts are shown in Table 3. The second group (without piperine) was then isolated by column chromatography to give eleven fractions (A to K).

Five fractions (C, D, F, G, and I) from eleven fractions (A to K) separated by TLC were selected to test for cytotoxic activity on breast cancer cell lines. Fraction D had the best cytotoxic effect on three breast cancer cell lines; MCF-7 and MDA-MB-468 cells, with IC₅₀ values of 5.82 ± 0.15 µg/ml and 7.34 ± 0.51 µg/ml, respectively. The IC₅₀ values of the five fractions are shown in Table 3. All the fractions were visualized for alkaloids by the Dragendoeff’s reagent. Fractions D, E and F were observed as orange spots on the TLC plate (data not shown). The results showed that fractions D, E and F were alkaloids. Then, the fraction D was subjected to column chromatography on silica gel. Seven fractions (DB, DC, DD, DE, DF, DG and DH) from 9 fractions (DA to DI) were selected using TLC. A high-level reduction in MCF-7 cell survival was observed when they were treated with fractions DE and DF (IC₅₀ value at 8.33 ± 1.27 µg/ml and 6.51 ± 0.39 µg/ml). In addition, fraction DE exhibited a strong cytotoxic effect on MDA-MB-468 and MCF-12A cells (Table 3). These results indicated that fractions DE and DF were highly effective against breast cancer cell lines.

**DISCUSSION**

There are several reports which show that extracts from plants of genus *Piper* exhibit cytotoxic activity. Hot ethyl acetate and cold hexane:water extracts of *P. longum* showed a dose dependent cytotoxic effect on leukemia cell lines [13]. The methanolic extracts of *P. longum* was found to be cytotoxic for Dalton’s lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells with IC₅₀ value of 250 µg/ml and 100 µg/ml, respectively [7]. In addition, methanol extract of *Piper crocatum* Ruiz and Pav was a cytotoxic to on breast cancer (T47D) cells with IC₅₀ value of 44.25 µg/ml [5]. Three amides from *P. sarmentosum* Roxb showed a cytotoxic effect on human breast cancer (MCF-7 and MDA-MB-231), human ovarian carcinoma (SKOV3) and human colon/intestinal carcinoma cell lines at concentration of 20 µg/ml with percentage of cell survival > 50 % [14].

Our first aim of this study was to determine the cytotoxic activity of the methanol and dichloromethane extracts of the *Piper* plant Methanol extracts of *P. retrofractus* and *P. nigrum* were found to be highly active against MDA-MB-468 cells. For dichloromethane
extracts, *P. betle* and *P. nigrum* had strong cytotoxic effects on MDA-MB-468 cells. The dichloromethane extract of *P. nigrum* exhibited highly cytotoxic effects with IC$_{50}$ value of 7.94 ± 4.52 µg/ml. Consequently, it was chosen for further isolation of the cytotoxic compound with column chromatography.

Piperine is a major pungent alkaloids present in *Piper* plant such as *P. nigrum*. It has been reported to show cytotoxic activity towards cancer cell lines at high concentrations [6,7,14]. Piperine exerted cytotoxic activity against human leukemia (CEM and HL-60), murine melanoma (B16) and human colon (HCT-8) cell lines at concentration equal or higher than 17 µg/ml [6]. Similarly, our study produced results which corroborate the findings in previous studies. Piperine extracted from *P. nigrum* had less effect on breast cancer cell lines with IC$_{50}$ of 19 µg/ml. Therefore, we decided to eliminate piperine from the crude extracts using crystallization technique. After crystallization, crude extract P1 and supernatant were obtained. The IC$_{50}$ value and $^1$H-NMR profile of P1 showed that this extract was piperine. Therefore, CP2 that separated later should not contain piperine. Then, CP2 was further separated by column chromatography to get eleven fractions (A to K). The effective fractions (DE and DF) which were isolated from fraction D were alkaloids. Alkaloids often possess potent cytotoxicity to many cells, which make them interesting in terms of having potential anticancer properties. Thirty-eight natural pure compounds have been found in genus *Piper*; the major ones are alkaloids, terpenes and polyphenols [8]. The pepper alkaloids, piperine and piprartine, exhibited cytotoxic effects on HL-60, CEM, HCT-8 and B-16 cells [6].

In this present study, we used three breast cancer cell lines that have different characteristics. MCF-7 cell contains the p53 wild-type, while MDA-MB-468 and MDA-MB-231 cells represent p53 mutations. The results showed that fractions DE and DF had a higher cytotoxic effect on MCF-7 and MDA-MB-468 than MDA-MB-231 cells. In MDA-MB-468 cells, the p53 mutation occurred at codon 273 (Arg to His) [15], whereas the p53 mutation of MDA-MB-231 cells is found at codon 280 (Arg to Lys). The mutant p53 of MDA-MB-231 cells stabilized MDM2, the negative regulator of p53 hence these cells were protected from apoptosis [16]. Therefore,
fractions DE and DF may induce apoptosis in MCF7, MDA-MB-468 and MDA-MB-231 cells through different pathways.

Numerous studies of alkaloids isolated from plants have been reported including berberine, camptothecin and ellipticin. Berberine alkaloid isolated from Chinese herbs, induced apoptosis in MDM2-overexpressing cells. Down regulation of MDM2 by berberine occurs at a post-transcriptional level through modulation of death domain-associated protein followed by MDM2 self-ubiquitination and degradation [17]. The alkaloid used as antitumor drug, camptothecin, induce apoptosis which bind simultaneously both to the DNA and topoisomerase I and stabilizes the cleavage complex formed. This collision of cleavage complex with replication forks induces double-strand breaks that lead to apoptosis [18]. This alkaloid up regulates p21WAF1/CIP1 and induces apoptosis in breast cancer cell line in both p53-dependent (MCF-7) and -independent (MDA-MB-468) pathways [19]. Moreover, ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole), a cytotoxic plant alkaloid, is isolated from *Ochrosia elliptica* Labill. This compound is known to inhibit topoisomerase II and induces apoptosis by increasing the expression of p53 and KIP1/P27 that lead to Fas/APO-1 and Bax that accumulate in treated MCF-7 cells. In addition, ellipticine also increases Fas ligands (mFasL and sFasL) that cause the enhancing of Fas/APO-1 levels and caspase-8 activity, and increase the expression of Bax and decrease the expression of Bcl-2 and Bcl-XL leading to cytochrome c release from mitochondria, which activates caspase-9 [20].

**CONCLUSION**

The results suggest that *P. retrofractum*, *P. betle* and *P. nigrum* extracts have cytotoxic effect on breast cancer cell lines. The methanol and dichloromethane crude extracts of *P. nigrum* possess strong cytotoxic activity. In addition, fractions DE and DF from *P. nigrum* exhibit the highest cytotoxic activity on breast cancer cell lines. Both fractions also promoted DNA fragmentation which is related to the induction of cell death through apoptosis. However, the target molecules of the extracts are not known. Therefore, further studies should involve the isolation of the active agent(s) of *P. nigrum* as well as the investigation of the molecular mechanism of action of the compound(s) in human breast cancer cell lines.

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