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Cytotoxic and Antimigration Effects of Different Parts of *Oroxylum Indicum* Extract on Human Breast Cancer MCF-7 Cells

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ABSTRACT

The objective of this research was to investigate the effects of *Oroxylum indicum* extracts on breast cancer MCF-7 cell proliferation and migration. Four parts of O. *indicum*, including leaf, bark, pod, and seed, were used. The total phenolic and flavonoid contents were determined to be at high concentrations in the four parts of *O. indicum* with the seed extract showing the highest levels. For MCF-7 cell death and proliferation, all *O. indicum* extracts caused stimulating cancer cell death and inhibiting cancer cell proliferation in dose- and time-dependent manners, surprisingly; the seed extract had the highest effects to inhibit cell proliferation. The IC_{50} values of cell viability of *O. indicum* extracts were demonstrated as 161.2±8.63, 286.73±33.01, 149.03±8.81, and 107.06±5.66 µg/mL for leaf, pod, bark, and seed, respectively. Cell counts by crystal violet staining showed that the seed extract stimulated cell death at the lowest concentration. Moreover, all of the *O. indicum* extracts decreased MCF-7 cell colony formation. Finally, we found that *O. indicum* extracts could inhibit cancer cell migration in a dose-dependent manner. In conclusion, our results showed that the seed extract of *O. indicum* showed the highest cytotoxic and anti-migratory activity, which was more than in the leaf, pod, and bark, on breast MCF-7 cell cancer and *O. indicum* can be an anticancer agent for breast cancer patients.

Keywords: Breast cancer; *Oroxylum indicum*; Antiproliferation; Cell migration; Cell grow

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1. Introduction

Cancer is uncontrolled cell proliferation and division, which cannot be completely abolished by surgery, chemotherapy, or radiotherapy. Breast cancer is the most common cancer among women and it is a major cause of mortality in breast cancer patients [1, 2]. For the treatment failure, the two major problems are drug resistance and drug toxicity. Drug resistance to the standard anticancer agents, such as anthracyclines (doxorubicin) and taxanes (paclitaxel and docetaxel), is the primary problem in current breast cancer [3]. For drug toxicity, the anticancer agents are not only toxic for the cancer cells but can also affect normal cells. Therefore, cancer is a fatal disease because of a lack of availability and effective drugs for treatment. Consequently, researchers are aiming to discover new anticancer drugs from plants, such as vegetables, which will eventually be useful in the treatment of breast cancer [4].

Oroxylum indicum belongs to Bignoniaceae family and has been used as a herbal medicine in Asia for the prevention and treatment of many diseases, such as rheumatoid arthritis, respiratory diseases, diabetes mellitus, and diarrhea [5]. *O. indicum* has been testified to be a treatment for cancer, regardless of a lack of knowledge on the mechanism of action for therapeutic modality [6]. Several parts of *O. indicum* can be used for cancer treatment, such as the root and bark. Furthermore, *O. indicum* bark extracts were reported to inhibit human breast cancer cell proliferation [7]. Oroxylin A is a major flavonoid in the stems and root bark of *O. indicum* and it has been reported to inhibit the growth and proliferation of many cancer cell types, such as lung, breast, colon, and liver [8-11].

However, there has not been a previous comparative study on the four parts of *O. indicum* on breast cancer MCF-7 cell proliferation and migration. This study, to the best of our knowledge, is the first to present data to explore the four parts of *O. indicum* on human breast cancer cell death, cell viability, cell replication, and cell migration. We extracted the four parts with 80% ethanol and tested them on MCF-7 cells by sulforhodamine B (SRB), cell count, colony formation, and cell migration.

2. Material and Methods

2.1 *O. indicum* extraction

O. indicum was collected from Maha Sarakham Province, Thailand, in January-December 2017, and divided into four parts. Identification was performed by the Applied Thai Traditional Medicine, Faculty of Medicine, Mahasarakham University (specimen no. MSUT_7226). For the extraction methods, the four parts of *O. indicum*, including leaf, bark, pod, and seed, were dried, weighed, sliced, and macerated in 80% ethanol at room temperature for seven days. Afterwards, the four extracts were filtered and concentrated using a rotary evaporator and then lyophilized. The % yields of the extracts were 27.01%, 22.89%, 6.79%, and 14.75% for the leaf, pod, bark, and seed, respectively, per dry weight. The crude extracts of *O. indicum* were stored at -20°C until use.

2.2 Phenolic contents

The total phenolic contents of the *O. indicum* extracts were examined by the Folin-Ciocalteu method. Briefly, 0.5 mL of the four crude extracts from *O. indicum* (0.5 mg/mL) were mixed with 2 mL of Folin-Ciocalteu reagent for 5 min. Then, the reaction mixture was added to 2 mL of sodium carbonate (60 g/L) and incubated for 60 min. The absorbance intensity was measured at 630 nm using an Opsys MR^{TM} microplate reader (Dynex Technologies, Chantilly, VA, USA). The total phenolic contents were calculated

from a calibration curve with results expressed as mg of gallic acid equivalent per g dry weight.

2.3 Flavonoid contents

The total flavonoid contents of the crude extracts were determined by the aluminium chloride colorimetric method. Briefly, 0.5 mL of the four crude extracts of *O. indicum* (1 mg/mL) were mixed with a 4.5 mL assay mixture, containing 10% aluminium chloride ($AlCl_3$) solution and 1 M potassium acetate, and then it was incubated for 30 min. The absorbance intensity was measured at 450 nm using an Opsys MR™ microplate reader (Dynex Technologies, Chantilly, VA, USA). The total flavonoid contents were calculated from a calibration curve with the results expressed as mg rutin equivalent per g dry weight.

2.4 Cell lines and cell ultures

Human breast cancer cell line, MCF-7, was obtained from the American Type Culture Collection (ATCC; USA) and maintained according to ATCC's recommendations. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and incubated at 37°C with 5% CO_2.

2.5 Sulforhodamine B (SRB) assay and cell count by crystal violet staining

SRB was used to measure the effect of the *O. indicum* extracts on the cell death of MCF-7 cells, as in a previously described method [12]. MCF-7 cells ($1x10^4$ cells/well) were seeded in 96-well plates, and the next day new DMEM medium containing each of the four extracts (0-500 μg/mL) was added for 24-72 h. Then, the MCF-7 cell death was examined by SRB assay. Briefly, cells were fixed with 10% trichloroacetic acid, stained with 0.4% SRB, washed with tap water, and solubilized with 10 mM Tris base. The optical density was measured by spectrophotometer at 540 nm. The IC_{50} concentration (50% inhibition of cell viability) was calculated from dose-response curves.

Crystal violet staining was used to confirm the SRB assay on the cell viability or cell death. MCF-7 cells were cultured with extracts for 72 h. Then, cells were fixed with 100% methanol for 30 min, stained with 0.5% crystal violet for 1 h, washed with tap water several times, air dried, captured, and counted.

2.6 Colony formation assay

A colony formation assay was used to measure the effect of *O. indicum* extracts on the cell growth of MCF-7 cells, as in a previously described method [12]. MCF-7 cells (500 cells/well) were seeded in 6-well plates and the next day new DMEM medium containing each of the four extracts (0-500 μg/mL) was added for 24 h and cultured for 14 days. Then, MCF-7 cell colony formation was examined with the colony formation assay. Briefly, cells were fixed with 100% methanol, stained with 0.5% crystal violet, washed with tap water, captured, and counted.

2.7 Wound healing assay

A wound healing assay was used to measure the effect of *O. indicum* extracts on the cell migration of MCF-7 cells, as in a previously described method [12]. MCF-7 cells (500 cells/well) were seeded in 24-well plates and the next day cells were scratched with a 0.2 mL pipette tip and new DMEM medium containing each of the four extracts (0-500 μg/mL) was added for 72 h.

Then, the cells were fixed with 100% methanol, stained with 0.5% crystal violet, washed with tap water, and captured. The wound distance was calculated by dividing the area by the length of the scratch.

2.8 Statistical analysis

Control and treatment groups were statistically compared using Student's t-test. Results were considered to be statistically significant if p values were less than 0.05.

3. Results and discussion

3.1 Total phenolic and flavonoid contents from four parts of *O. indicum*

The *O. indicum* extracts from the leaf, pod, bark, and seed were extracted by 80% ethanol because from our preliminary data found that *O. indicum* extracts by 80% ethanol had the higher potency of anticancer activity more than distilled water (data not shown). The *O. indicum* extracts were standardized using colorimetric methods to quantify the gallic acid and rutin contents represented as the phenolic and flavonoid contents. Our results showed that the four *O. indicum* extracts showed high levels of phenolic contenets and flavonoid content; the leaf extract had the highest phenolic contenets with 154.74±34.97 mg/g and the seed extract had the highest flavonoid contents with 67.81±9.82 mg/g (Table 1).

High levels of phenolic and flavonoid contents are also acknowledged to play pivotal roles in anticancer effects. Our results showed that the seed extract had the highest levels of flavonoid contents and correlated with the greatest efficacy for inhibiting MCF-7 cell proliferation, more than the extracts from the other parts of *O. indicum*. Within different plants, flavonoids and phenolics occur in every part, but they are usually concentrated in the leaves and flowers [13, 14]. The anticancer efficacy of flavonoids is due to their ability to induce apoptosis of cancer cells, which may also include breast cancer [15-17].

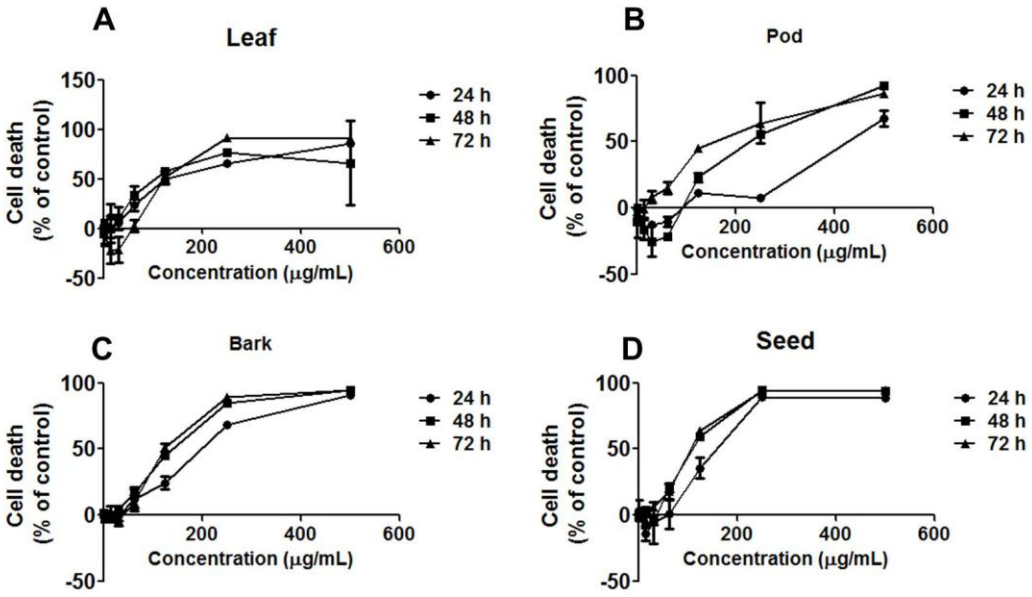
3.2 Effects of *O. indicum* extract on MCF-7 cell death

The cytotoxicity was estimated by the SRB assay and cell counting. All four tested extracts of *O. indicum* from the leaf, pod, bark, and seed, showed concentration- and time-dependent effects with low IC_{50} values (Fig. 1A-E). From our results, the seed had the highest efficacy to induce MCF-7 cells death with an IC_{50} value of 107.26±5.66 μg/mL for 72 h, which was followed by bark, leaf, and pod with IC_{50} values of 123.45±15.23, 138.22±5.23, and 164.21±1.23 μg/mL, respectively. Next, to confirm the effects of the *O. indicum* extracts, we examined the cancer cell viability by cell counting. After the cells were treated with the *O. indicum* extracts, cells were stained with 0.5% crystal violet, captured, and counted, after which they were compared with the control groups. From our results, we found that all the *O. indicum* extracts could inhibit MCF-7 viability in a dose- dependent manner.

Table 1. % Yield, total phenolic and flavonoid contents of four parts of *O. indicum*.

Parts of *O. indicum*	% Yield	Total phenolic contenets (mg gallic acid/g dry weight extract)	Total flavonoid contents (mg rutin /g dry weight extract)
Leaf	27.01	154.74 ± 34.97	56.97 ± 5.89
Pod	22.89	33.55 ± 2.97	17.09 ± 1.2
Bark	6.79	83.67 ± 14.38	58.22 ± 8.73
Seed	14.75	107.35 ± 20.45	67.81 ± 9.82

Moreover, we confirmed the results from the SRB assay that the seed extract had the greatest activity on breast cancer cells with an IC_{50} value of 64.54±2.72 μg/mL, which was more than those of the leaf, pod, and bark extracts (IC_{50} values of 101.36±6.45, 122.43±2.07, and 125.14±9.95 μg/mL, respectively) (Fig. 2A-D). In conclusion, all four parts of *O. indicum* extracted significantly inhibited MCF-7 cell proliferation and also induced cell death.



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Part of *O. indicum*	IC_{50} of cell viability (μg/mL)		
	24 h	48 h	72 h
Leaf	166.43 ± 12.72	156.83 ± 27.34	138.22 ± 5.23
Pod	706.06 ± 71.33	225.56±25.31	164.21±1.23
Bark	222.4 ± 25.84	135.23±6.34	123.45±15.23
Seed	166.7 ± 32	113.06 ± 7.27	107.06 ± 5.66

Fig. 1. Effects of *O. indicum* extracts on MCF-7 cell death. (A-E) MCF-7 cells had four extracts of *O. indicum* added at various doses for 24-72 h and measured cancer cell deaths by SRB assay. Data represent mean±SEM of three independent experiments.

Our study showed a comparison between the extracts from four parts of *O. indicum* on breast MCF-7 cell death. This is the first research to compare the edible parts of *O. indicum*, including leaf and pod, and non-edible parts, including bark and seed. We found that the seed extract had the highest activity of more than pod > leaf > bark. From our results we suspect that the effects on MCF-7 cell death may relate to the levels of phenolic and flavonoid contents.

Previously, Dhru et al showed that the n-butanol fraction of the root bark of *O. indicum* had the highest toxicity on the MCF-7 cell line with 70.41% inhibition in the MTT assay, which was greater than the chloroform or ethylacetate fractions [18]. Previously, the bark of *O. indicum* has been used to demonstrate apoptotic activity, albeit from polar extracts [19, 20]. Hence, the results imply that *O. indicum* extracts from four parts (including seed, leaf, and pod) were able to selectively target cancer cells and inhibit cell proliferative. *O. indicum* needs further study on the mechanism of action that induces MCF-7 cell death.

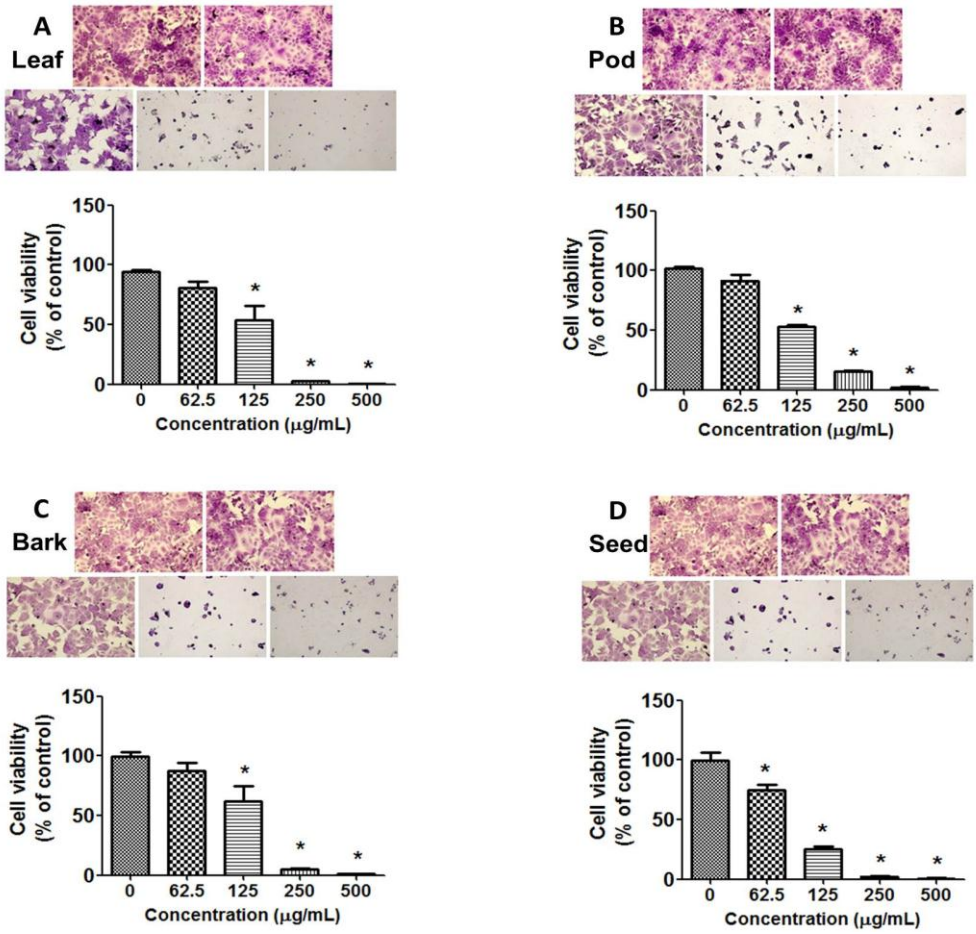

Fig. 2. Effects of *O. indicum* extracts on MCF-7 cell viability determined by crystal violet staining. (A-D) MCF-7 cells had four extracts of *O. indicum* added at various doses for 72 h and measured cancer cell viability by crystal violet staining and counting. Data represent mean±SEM of three independent experiments. *$p < 0.05$ vs. control.

3.3 Effects of *O. indicum* extract on MCF-7 cell colony formation

To test the effects of the four *O. indicum* extracts on cancer cells' replicative activity, colony formation was used. MCF-7 cells were grown for 14 days, stained with crystal violet, counted, and compared with control groups. Our results concluded that the four *O. indicum* extracts could inhibit colony formation at low concentrations.

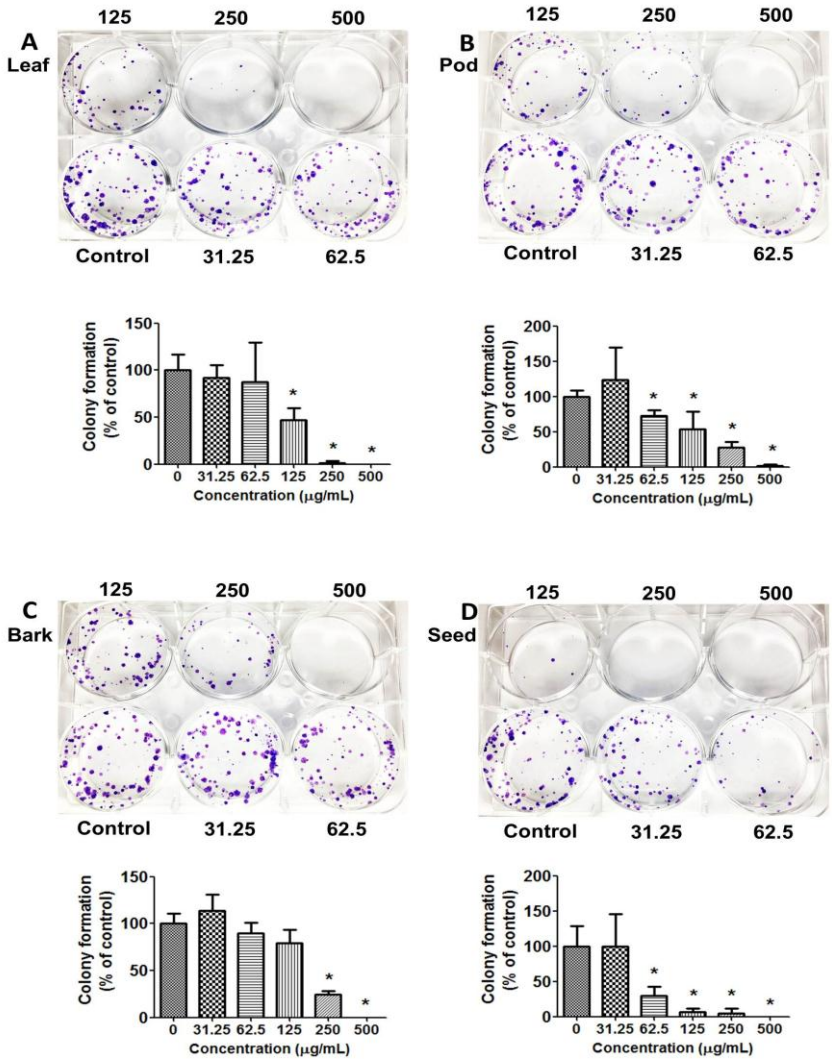

Fig. 3. Effects of *O. indicum* extracts on MCF-7 cell colony formation. (A-D) MCF-7 cells had four extracts of *O. indicum* added at various doses for 24 h and measured cancer cell colony formation after 14 days of treatment. Data represent mean±SEM of three independent experiments. *$p < 0.05$ vs. control.

Interestingly, the seed extract had the lowest IC_{50} value (49.66±19.63 μg/mL), which was followed by the pod (157.69±51.21 μg/mL), leaf (161.17±29.81 μg/mL), and bark (330.50±16.17 μg/mL) extracts (Fig. 3A-D). In conclusion, four parts of *O. indicum* extracts significantly inhibited MCF-7 cell colony formation. In our previous studied we tested the effects of *Cratoxy formosum* on both breast (MCF-7) and liver (HepG2) cancer cells and the results showed that C. *formosum* had high levels of phenolic and flavonoids

that were related to colony formation [12, 21]. The mechanism of action may be through significantly induced ROS formation, increased caspase 3 activities, and reduced mitochondrial membrane potential, thereby leading to cancer cell apoptosis and cell death. Moreover, non-polar extracts of *O. indicum* can effectively target ER-negative breast cancer cells to induce apoptosis, without harming normal cells due to cancer specific cytotoxicity [6].

3.4 Effects of *O. indicum* extracts on MCF-7 cell migration

To examine the effects of the four *O. indicum* extracts on cancer cell migration a wound healing assay was used. The size of a scratch wound was observed after treatment with each extract for 72 h. Our results indicated that the four *O. indicum* extracts can inhibit MCF-7 cell migration in a dose-dependent manner (Fig. 4A-D). The IC_{50} values of the four O. *indicum* extracts on MCF-7 cell migration inhibition were 79.09±7.65, 200.23±36.71, 205.87±57.74, and 208.03±26.19 μg/mL for seed, pod, leaf and bark, respectively. In conclusion, the four extracts of *O. indicum* parts significantly inhibited MCF-7 cell migration. Kumar et al and Yodkeeree et al found that *O. indicum* from a petroleum ether hot extract could inhibit breast cancer MDA-MB-231 cell migration [6, 22]. *O. indicum* extracts might be a suitable source for deriving precursors to be used in targeted therapy of malignant breast cancer, especially for the seed extract. Furthermore, we need to explore the differences in the active compounds between the four extracts and their mechanisms of action.

4. Conclusion

In conclusion, the ethanolic extract of *O. indicum* exhibited effective cytotoxic activity against human breast cancer MCF-7 cells by inhibiting cell proliferation, stimulating cancer cell death, decreasing colony formation as well as inhibiting cell migration with the lowest concentration, especially for the seed extract. This may provide a new approach for breast cancer prevention and/or therapy. We need to explore the mechanism of action in more detail via further studies in vivo and in clinical trials to establish whether *O. indicum* extracts can be used as a safe drug for cancer therapy. In addition, studies are necessary for the chemical characterization of the active principles and more extensive biological evaluations.

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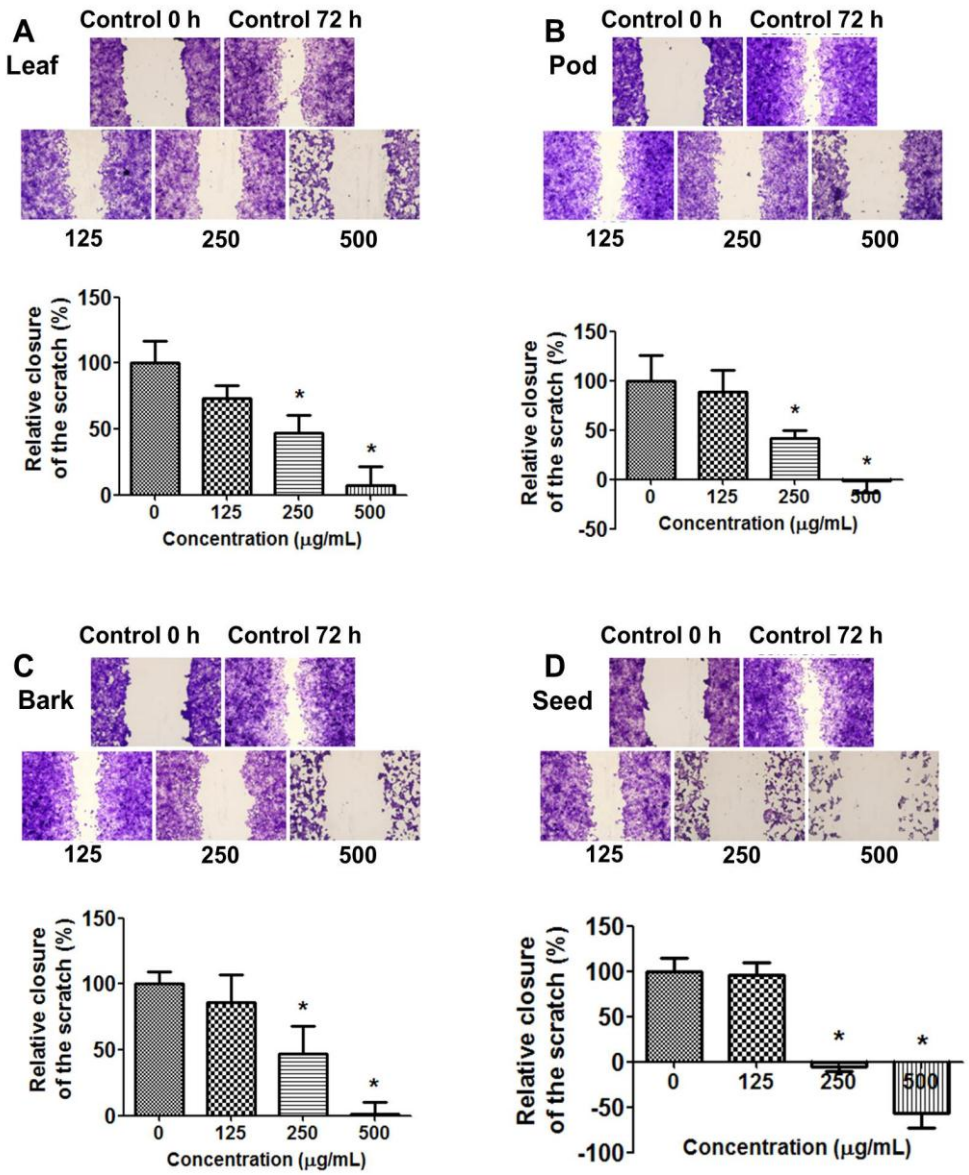

Fig. 4. Effects of *O. indicum* extracts on MCF-7 cell migration. (A-D) MCF-7 cells were added four extracts of *O. indicum* at various doses for 72 h and measured cancer cell migration. Data represent mean±SEM of three independent experiments. *$p < 0.05$ vs. control.

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