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Full Length Research Paper

Screening of antiproliferative effect of *Limonia acidissima* Linn. fruit extracts on human breast cancer cell lines

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The aim of the study is to evaluate the anticancer effect of *Limonia acidissima* Linn. (Family Rutaceae) which has long been prescribed for various infectious and malignant diseases. The fruit extract of *L. acidissima* Linn. has been carried out by following due procedure. Cell lines, cultures were incorporated and procured for *in-vitro* and *in-vivo* study with reference to cell proliferation assay, cell viability assay and cell cycle analysis. The bio-assays of extracts from *L. acidissima* Linn. showed that a fraction (fraction 3) from an ethanolic extract had an anticancer effect on SKBR3 and MDA-MB-435 human breast cancer cells. The ED_{50} value of *L. acidissima* Linn. fraction 3 was 56.07 and 30.61 µg/ml for SKBR3 and MDA-MB-435, respectively. After 48 h of exposure, this fraction at a concentration of 100 µg/ml, significantly reduced cell proliferation in both cancer cells. In MDA-MB-435 cells, cell cycle analysis showed that the fruit extract fraction 3 induced the accumulation of cells in G2/M phase, whereas no significant change in cell cycle was detected in SKBR3 cells. The results indicated that the extract fraction could induce cell cycle arrest in some way. However, further investigation is needed to assess the molecular mechanisms that mediated anticancer activities of this plant.

Key words: Anticancer; SKBR3, MDA-MB-435, *Limonia acidissima* Linn., G2/M arrest.

INTRODUCTION

Breast cancer is one of the most prevalent malignancies in women in many countries worldwide. In India, breast cancer is the second in frequency after cervical cancer, with an estimated incidence rate of 17.2 per 100,000 which has increased in all parts of the country during the past decade. Though the breast cancer therapy which is usually multimodality treatment is in advance, cytotoxic drugs still play the important roles for increasing survival rate together with good quality of life (Senthilkumar et al., 2010; Fargeot et al., 2004). Therefore, the development and search for novel and effective anticancer agents have become very important issues (Cameron and Bell, 2004). To date, many cytotoxic agents, including natural products isolated from plant sources have been investigated for the discovery of the potential novel anticancer drugs (Kirtikar and Basu, 2005). Higher plants have long been shown to be excellent and reliable sources for the development of novel anticancer drugs. In India, many plants have been used for treatment of various malignancies over centuries. *Limonia acidissima* Linn, syn. *Feronia limonia* (Rutaceae) is a moderate-sized deciduous tree grown throughout India. The fruits are woody, rough and used as a substitute for bael in diarrheoa and dysentery (Senthilkumar et al., 2010). The bark and leaves of the plant are used for vitiated conditions of vata and pita while the fruits are used for tumours, asthma, wounds, cardiac debility and hepatitis. The fruit contains flavonoids, glycosides, saponins and tannins (Kirtikar and Basu, 2005). Some coumarins (Kirtikar and Basu, 2005) and tyramine derivative (Llango and Chitra, 2010) have also been isolated from the fruits

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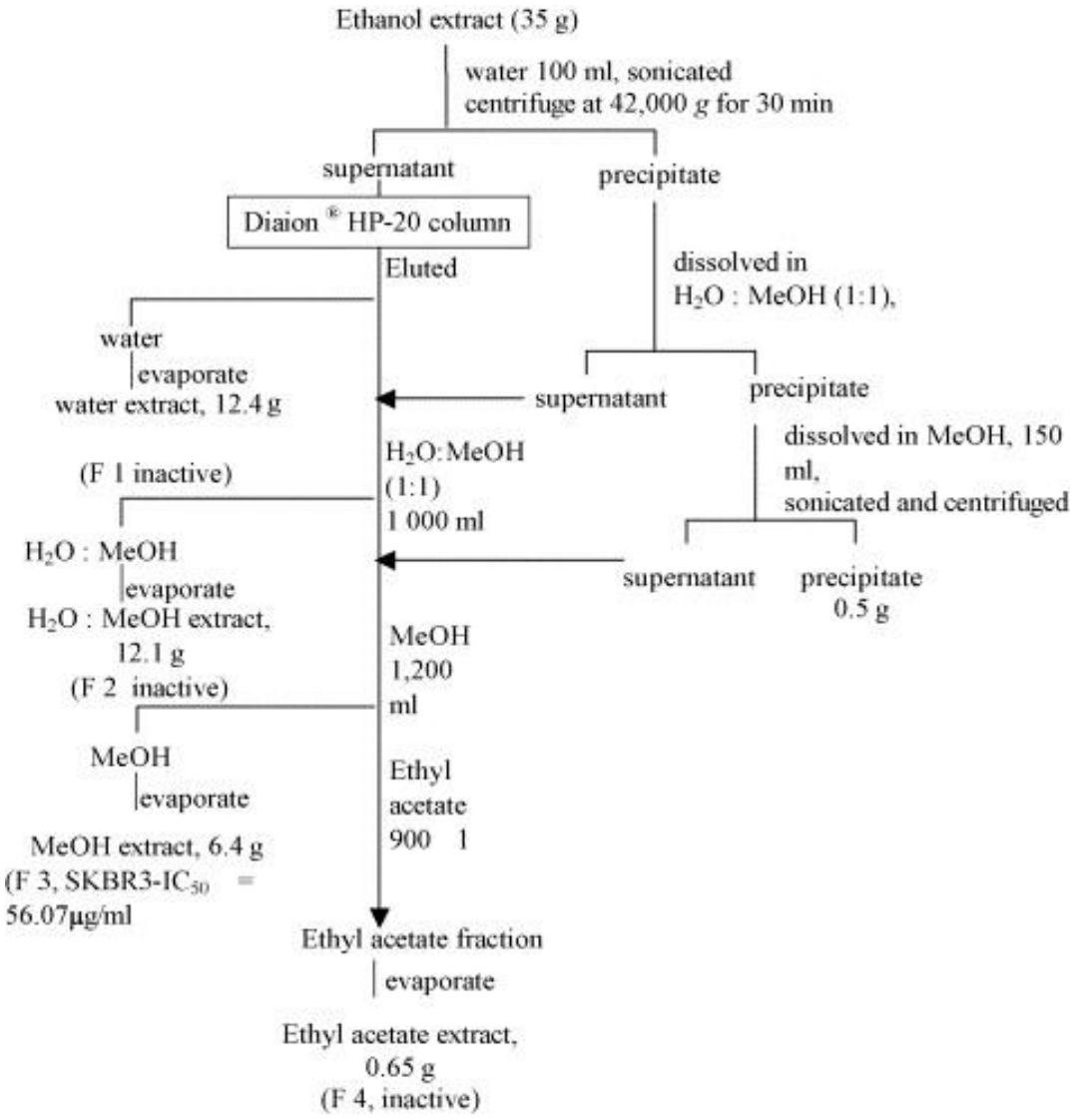

Figure 1. Bioactivity guided fractionation on Diaion® HP-20 column chromatography of the ethanolic extract.

of Limonia. The leaves were reported to possess hepatoprotective activity (Rang et al., 1999) while the fruit shells contain antifungal compounds, namely, psoralene, xanthotoxin, 2, 6-dimethoxybenzoquinone and osthenol (Rubinstein et al., 1990). However, the property of this plant, especially its anticancer activity, has not yet been investigated. Therefore, this prompted us to investigate the inhibitory growth effect of this plant on two different breast cancer cell lines, SKBR3 and MDA-MB-435.

MATERIALS AND METHODS

Plant material and extraction procedures

L. acidissima Linn. fruits were collected from forest division, Bhubaneswar hill area situated in the Eastern part of India, and was identified by Dr S. K. Sahu, a taxonomist at the Utkal University, Vanivihar, Orissa. A voucher specimen No. 161 was deposited at the same place. The fruits of this plant were cut into small pieces and ground into powder. The powder (1.5 kg) was macerated three times with 15 L of 95% ethanol for 7 days each (Llango and Chitra, 2010). The extracts were concentrated under reduced pressure resulting in 80.50 g extract (DER = 25.5:1).

The ethanolic fruit extract (35 g) was dissolved in distilled water (100 ml) and column chromatographed was done for total volume of 15 ml which was dry packed using the glass column (4 cm inner diameter and 60 cm long) and equilibrated with water (100 ml) and sonicated for 15 min in an ultrasonic bath. The suspension was centrifuged at 20,000 rpm (about 42,000 ×g) for 30 min. The supernatant was applied onto the Diaion® HP-20 column, and the eluent was collected to get the water soluble fraction (fraction 1) (Figure 1). The precipitate was dissolved in ethanol-water (1:1/100 ml) and ethanol (150 ml) to get the fractions 2 and 3, respectively. Each supernatant was repeatedly performed as described earlier. The precipitate was dissolved in ethyl acetate to

get fraction 4. The chemical composition of each fraction was monitored on thin layer chromatography (TLC). The fractions were on to silica gel GF_{254} Alufolien (Merck) and developed with a chloroform/ethanol (90:10, v/v). The phytosteryl glucoside was used as reference. After developing, the TLC was sprayed with anisaldehyde-sulfuric acid and heated in an oven at 100°C for 1 to 2 min. The ethanol fraction revealed eight bands. Three bands were yellow to orange with R_f values of 0.12, 0.25 and 0.30. Five bands were violet to blue with R_f values of 0.17, 0.38, 0.41, 0.56 and 0.61. The violet to blue reference compound appeared at the R_f value of 0.17.

Preparation of *L. acidissima* Linn. fruit extracts

All *L. acidissima* Linn. fruit extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, USA) except for the water fraction (fraction 1) which was dissolved in water. For all experiments, final concentrations of the tested compounds were prepared by diluting the stock with the culture medium.

Cell lines and culture

All the breast cancer cell lines, SKBR-3 (ATCC No. HTB-30) and MDA-MB-435 (ATCC No. HTB-129), were procured from New Scientific Agencies, Bangalore, India. SKBR-3 is human breast cancer cell line with over-expression of HER2/*neu* receptor and absence of ER receptor whereas MDA-MB-435 breast cancer cell line is absent ER receptor and also HER2/*neu* expression (Saima et al., 2000). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Promo Cell, Germany) supplemented with 10% heat-inactivated foetal bovine serum (Promo Cell, Germany) and 1% penicillin-streptomycin (Promo Cell, Germany). The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Cell proliferation assay

Cell proliferation assay is done by taking a total of 1×10^4 cells per well was seeded in a 96-well plate. After 36 h of incubation, various concentrations of fruit extracts were added to the wells to get the final concentration of 1, 10, 100 and 500 µg/ml. Control groups were added with DMSO to get the final concentration of 1%. Doxorubicin was used as positive control. Then, cells were incubated for an additional 48 h. After 2 days, 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) 1 mg/ml in phosphate buffer saline (PBS) was added to each well and incubated for 4 h at 37°C. The medium was removed and formazan was dissolved in DMSO and the optical density was measured at 590 mm using a Bio-assay reader (Biorad, USA) (Rubinstein et al., 1990).

Cell viability assay

The cells were suspended in a 96-well plate at the same concentration as the cell proliferation assay and incubated for 24 h at 37°C with 5% CO_2. Various concentrations of fruit extracts as indicated were added to each well including DMSO as control groups. After 48 h incubation, cells were trypsinized and viable cell counting was performed by enumerating cells which excluded trypan blue dye using a haemocytometer.

Cell cycle analysis

To determine the cell cycle, 1×10^6 cells were suspended in each tissue culture dish and treated with fruit extracts at a final concentration of 80 and 160 µg/ml for SKBR3. For MDA-MB-435, cells were treated with fruit extracts at a final concentration of 60 and 120 µg/ml. 1% DMSO was added to the control group. After treatment for 48 h, cells were collected and incubated with reagents as described in the protocol of the Cycle TEST™ PLUS DNA reagent kit (Becton Dickinson Immunocytometry System). The DNA content of cells was measured by flow cytometry (Saima et al., 2000). All samples were analysed within 3 h by FACS carlibur using Cell Quest software.

Statistical analysis

The experiments were performed in triplicate. The data regarding cell proliferation and viability assay were expressed as means ± standard deviation. The values of ED_{50} were calculated by using R^2 equation. A P value less than 0.05 were considered significant.

RESULTS

The percentage of growth inhibition of the fruit extract on human breast cancer cells is as shown in Figure 2A and B. Fruit extracts from fractions 1 to 4 and also the crude extract (ethanolic extract) were used to determine the ED_{50} value (50% inhibition of cancer cell growth) in two different breast cancer cell lines, SKBR3 and MDA-MB-435, with a final concentration ranging from 1 to 500 µg/ml. At 48 h, the ED_{50} values of fraction 3 were 56.07 ± 3.77 and 30.61 ± 1.49 µg/ml for SKBR3 and MDA-MB-435, respectively. As fraction 3 was found to have an inhibitory growth effect on these cell lines, this fraction was used to investigate the further effects of this plant on these cells.

Effect of extract on cell viability

We also characterized the cytotoxic effect of *L. acidissima* Linn. on SKBR3 and MDA-MB-435 by conducting a cell viability assay stained with trypan blue. The culture of these two breast cancer cell lines was treated with fruit extract fraction 3 at final concentrations of 1, 10, 100 and 500 µg/ml for 48 h. The results indicated that at a concentration of 100 µg/ml. Both cell lines showed low viability of less than 50% as compared to that of the control group. No cell inhibition was observed at 10 µg/ml.

Cell cycle induced by extract

The effect of the fruit extract on cell cycle progression on SKBR3 and MDA-MB-435 was determined by flow cytometry. SKBR3 treated with fruit extract fraction 3 at final concentrations of 160 µg/ml, as shown in Figure 3A and B, showed slightly S phase block by decreasing the population of the S phase from 12.89 to 7.19%. For MDA-MB-435 cell cycle progression, the result showed that the

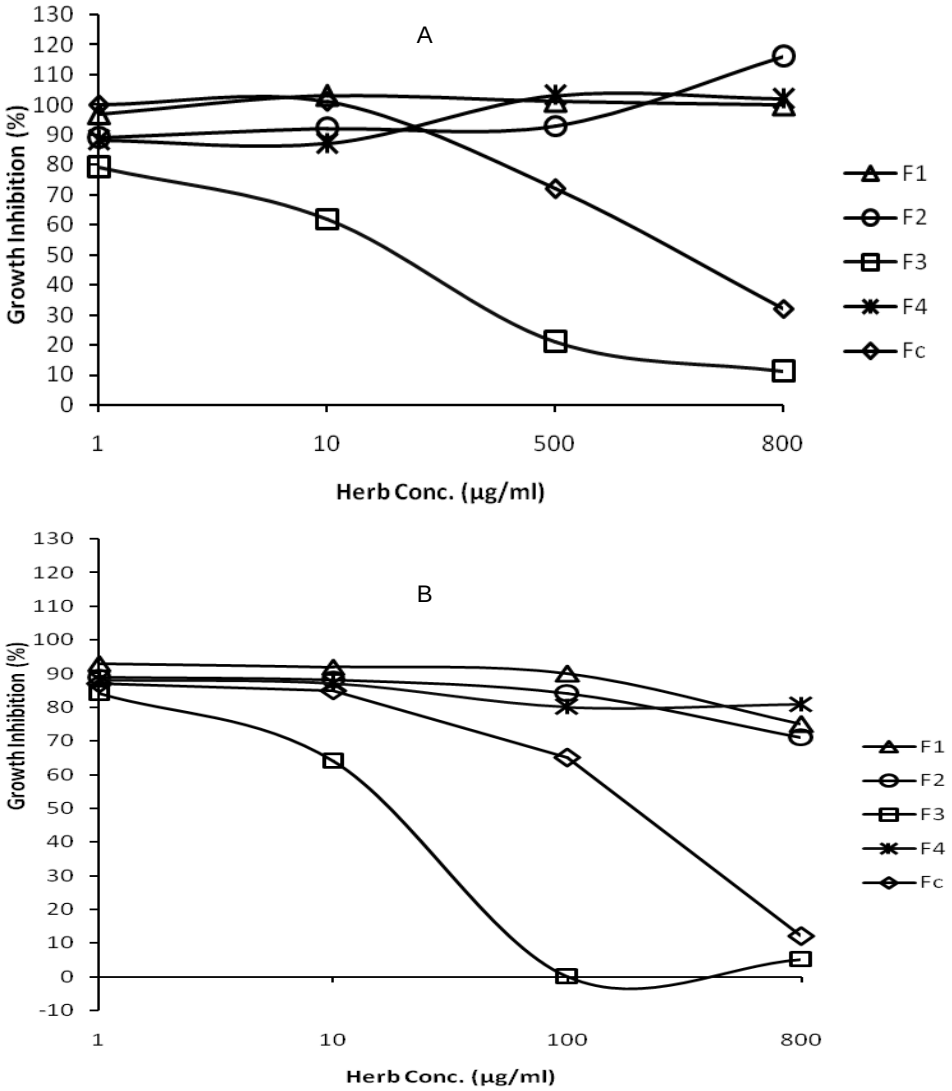

Figure 2. Inhibitory growth effect of *L. acidissima* extracts on SKBR3 (A) and MDA-MB-435 (B) breast cancer cell lines. Each cell line was treated with plant extracts, fractions 1 to 4 (F1 to 4) including the crude extract (Fc), at different concentrations of 1, 10, 100 and 500 µg/ml for 48 h and the growth inhibition was determined using the MTT assay. The growth inhibition was calculated as the percentage of inhibition compared with that of the control.

treated MDA-MB-435 cells at a concentration of 120 µg/ml increased the population of the G2/M phase from 24.41 to 45.65% when compared with that of the control groups (Figure 4A and B).

DISCUSSION

The plant ethanolic extract was fractionated in column chromatography, according to the compound polarity. The eluting solvents were transferred to the column, starting from water, water-ethanol (1:1), ethanol and ethyl acetate. They were subjected to the MTT test for antiproliferative activities. The water soluble fraction comprised the most polar compounds, that is, inorganic salts, sugars, amino acids and saponins, the water-ethanol soluble fraction contained the glycosides and the ethyl acetate is composed of the aglycones. The ethanolic extract showed the cytotoxicity against the SKBR3 and MDA-MB-435 breast cancer cell lines with the ED_{50} values of 56.07 ± 3.77 and 30.61 ± 1.49 µg/ml, respectively. This inhibitory growth activity of the treated cells acted in a dose-dependent manner which was also confirmed by the cell viability with the trypan blue exclusion assay. Such observation, demonstrated that some active components of this plant should be in the ethanolic fraction.

Figure 3A and B shows that SKBR3 cells treated with

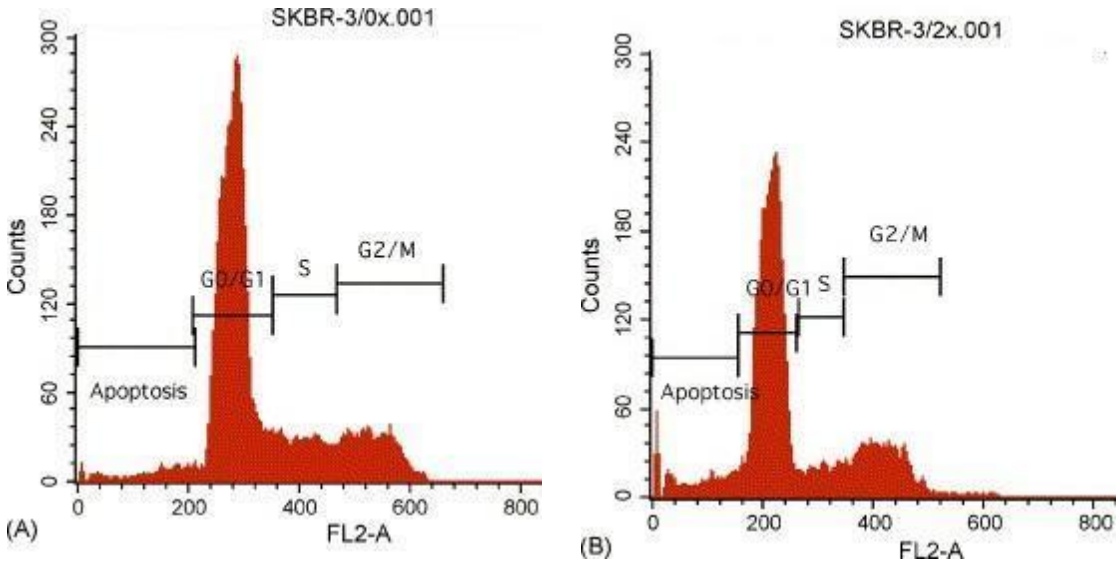

Figure 3. Inhibition of cell cycle progression in breast cancer cell lines analysed by flow cytometry. (A) Cell cycle analysis of SKBR3 cells without the plant extract at 48 h. GO/G1 = 69.41%, S = 12.89%, G2/M = 15.12%. (B) Cell cycle analysis of SKBR3 cells after treatment with plant extract fraction 3 for 48 h at 160 µg/ml. GO/G1 = 66.16%, S = 7.19%, G2/M = 19.61%.

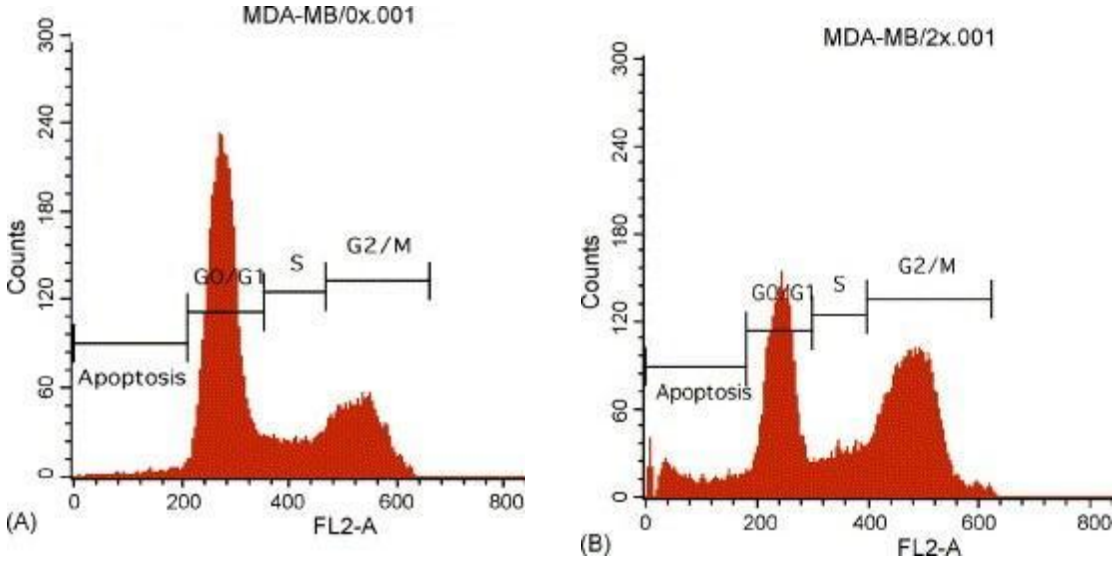

Figure 4. (A) Cell cycle analysis of MDA-MB-435 cells without treatment with the plant extract. GO/G1 = 65.75%, S = 9.01%, G2/M = 24.41%. (B) Cell cycle analysis of MDA-MB-435 cells following treatment with the plant extract fraction 3 at 120 µg/ml for 48 h. GO/G1 = 36.80%, S = 10.11%, G2/M = 45.65%.

the fruit extract for 48 h slightly decreased in the number of cells in S phase, whereas MDA-MB-435 treated with the extract for 48 h remarkably accumulated in the G2/M phase of the cell cycle up to 45% as shown in Figure 4A and B. The results indicated that the fruit extract fraction 3 could suppress breast cancer cell lines proliferation especially MDA-MB-435 via the cell cycle blockage.

Many anticancer agents from plants that have been prescribed for treating malignancies nowadays inhibit cancer cell growth through cell cycle regulation, including the G2/M accumulation (Schiff et al., 1997). It is well known that agents that affect the G2/M phase cell cycle arrest interact by targeting tubulin or disrupting the tubulin-microtubule equilibrium (Hadfield et al., 2003 and

Hardman et al., 1996; Tanaka et al., 2004). According to our results, *L. acidissima* Linn. Exhibited antiproliferative effect against MDA-MB-435 breast cancer cells via G2/M cell cycle arrest, thus it should interact with tubulin to the same extent as the plant-derived chemotherapeutic agents. However, the antiproliferative activities of this plant might be possibly dependent on cell types including the culture conditions.

Conclusion

L. acidissima Linn. fraction 3 could inhibit the proliferation of human breast cancer cell lines, SKBR3 and MDA-MB-435. Our results demonstrated that the cell cycle via G2/M blockage plays some roles in *L. acidissima* Linn. which induced antiproliferative activities in MDA-MB-435. However, further chemical work and investigations at molecular level are required to identify the active components that could induce growth inhibition and to establish the possible correlation among the mentioned activities of the fruit extract.

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REFERENCES

Cameron D, Bell R (2004). Optimizing treatment for patients with breast cancer. Oncology, 31: 4-5.

Fargeot P, Bonneterre J, Roche H, Lortholary A, Campone M, Praagh IV, Monnier A, Namer M, Schraub M, Barats JC (2004). Disease-free advantage of weekly Epirubicin plus Tamoxifen versus Tamoxifen alone as adjuvant treatment of operable, node-positive, elderly breast cancer patients. J. Cln. Oncol., 22: 4622-4630.

Hadfield JA, Ducki S, Hirst N, McGown AT (2003). Tubulin and microtubules as targets for anticancer drugs. Progress Cell Cycle Res., 44: 309-325.

Hardman JG, Limbird JE, Molinoff PB, Ruddon RW, Gillman AG, editors (1996). Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed. New York: Mc Graw-Hill Companies, pp. 461-86.

Kirtikar KR, Basu BD (2005). Indian Medicinal Plants. Dehradun, India, International Book Distributors, I: 478-479.

Llango K, Chitra V (2010). Wound Healing and Anti-oxidant Activities of the Fruit pulp of Limonia acidissima Linn (Rutaceae) in Rats. Trop. J. Phys. Res., 37: 120-125.

Rang HP, Dale MM, Ritter JM (1999). Pharmacology. Edinberg. Chuchi ll Livingstone, p. 697-699.

Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, Scudiero DA, Monks A, Boyd MR (1990). Comparison of *in-vitro* anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J Nat. Cancer Inst., 82: 111-116.

Saima Y, Das AK, Sarkar KK, Sen AK, Sur P (2000). An antitumor pectic polysaccharide from Feronia limonia, Int. J. Biol. Macromol., 27: 333-335.

Schiff PB, Fant J, Horwiz SB (1997). Promotion of microtubule assembly *in-vitro* by Taxol. Nature, 277: 655-657.

Senthilkumar KL, Kumawat KB, Rajkumar M, Senthilkumar (2010). Antidiarrhoeal activity of bark extracts of Limonia acidissima Linn. Res. J. Pharm. Biol. Chem. Sci,, 1(4): 550-553.

Tanaka Y, Fujiwara K, Tanaka H, Meheta K, Kohno I (2004). Paclitaxel inhibits expression of heat shock protein 27 in ovarian and uterine cancer cells. Int. J Gyn. Cancer., 14: 616-620.