

ORIGINAL ARTICLE

Anti-proliferative and Apoptosis-Inducing Effects of *Morinda citrifolia* L. Shoot on Breast, Liver, and Colorectal Cancer Cell Lines

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ABSTRACT

Introduction: *Morinda citrifolia* or also known as noni is commonly consumed raw or blanched as side dishes or 'ulam'. As cancer is one of the most leading causes of death in the world, we aimed to evaluate the anti-proliferative potential of noni shoot against various types of cancer cell lines. **Methods:** The breast cancer (MDA-MB-231), liver cancer (HepG2), and colorectal cancer (HT-29) cell lines were treated with 70% ethanol extract of noni shoot for cytotoxicity testing using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis induction effects were examined using AO/PI dual fluorescent assay and cell cycle analysis using flow cytometry. Gas chromatography-mass spectrometry (GCMS) was also carried out to characterize the active compounds in noni shoot. **Results:** The cytotoxicity assay demonstrated noni shoot had IC₅₀ of 49.72 µg/mL, 307.5 µg/mL and 65.43 µg/mL against MDA-MB-231, HepG2, and HT-29 cell lines, respectively. The AO/PI staining showed apoptotic bodies such as cell blebbing, chromatin condensation, and nuclear fragmentation was markedly induced in the selected cancer cell lines-treated with noni shoot extract. Apoptosis induction by noni shoot was showed by a significant increase in sub G₀/G₁ phase in MDA-MB-231 and HT-29 cell lines of cell cycle analysis. It was found that noni shoot extract contained mostly acetic acid and ethriol that may contribute to its anti-cancer properties. **Conclusion:** These findings showed the potential anticancer properties of noni shoot extract thereby, further studies are needed to understand the mechanism of noni as anti-cancer agent and possibility to be developed as a nutraceutical or functional food products.

Keywords: *Morinda citrifolia*, Shoot, Anti-cancer, Apoptosis, Phytochemicals

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INTRODUCTION

Cancer is a leading cause of death in both more and less developed countries (1). Cancer can be defined as a disease where abnormal cells continue to grow in an uncontrolled manner and disregard the normal rules of cell division thus leading to activation of numerous gene that involved in survival, cell cycle, metastasis, and angiogenesis (2). From 2007 to 2011, it is reported that there are 103507 new cancer cases was diagnosed in Malaysia (3). From the number of new cases, 45.2 % (46794) cases were male patients

and 54.8 % (56713) are females. Breast cancer, liver cancer, and colorectal cancer are most common cancer worldwide and all three types of cancer placed in top five in both men and women worldwide and global incidence were remarkably rising (4). There are many types of cancer treatment that provided to the patients that include surgery, radiotherapy, and applications of chemotherapy drugs, the biological molecule as well as immune-mediated therapies (5). Recently, many research has targeted the stimulation of programmed cell death also known as apoptosis as a prime molecular target for chemoprevention and treatment of cancer (6). Using plant-derived compounds or phytochemicals therapeutics approach for cancer have been applied extensively and natural compounds that isolated from medicinal plants are the lead compounds as the anti-cancer agents (7).

Morinda citrifolia L. or noni is originated in Southeast Asia and the plant has been brought to another tropic region such as Australia, Asia Pacific and Caribbean (8). Noni was claimed to possess medicinal properties by old folks throughout generations although there is no or little scientific evidence to prove the claims (9). Noni leaves are used to treat bacterial infections, inflammation, hemorrhage, burns wounds, fever (10), rheumatic aches and swelling of joints (11). In addition, noni leaves are rich in carotenoids which is important to treat vitamin A deficiency (12). Thani et al. (2010) have also shown that noni extract exhibited anti-cancer properties against human epidermoid carcinoma (KB) and human cervical carcinoma (HeLa) cells (13). Aside from leaves, it is also reported that other parts of the noni also possess health benefits. Noni fruit juice is used in alternative medicine for the treatment of diabetes, arthritis, digestion problems, atherosclerosis and many more (14). Furthermore, noni fruits may suppress tumour growth through activation of the immune system in Lewis lung (LLC) peritoneal carcinomatosis model (15), neuroblastoma (36 %) and breast cancer (29 %) (16).

Nowadays, most of the pharmaceutical agencies were mostly relies on natural products that originated from traditional medicinal plants and always exploring for possible traditional plants that might have medicinal properties. The current study demonstrates the potential anti-proliferative effects of *Morinda citrifolia* shoot extract towards breast cancer (MDA-MB-231), liver cancer (HepG2), and colorectal cancer (HT-29) cell lines.

MATERIALS AND METHODS

Materials and chemicals

Ethanol was bought from Fisher Scientific (Loughborough, Leicestershire, UK). MTT, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and doxorubicin were purchased from Sigma (St. Louis, MO, USA). BD Cycletest™ Plus DNA was obtained from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). Roswell Park Memorial Institute (RPMI)-1640 medium were purchased from Nacalai Tesque Co. (Kyoto, Japan). Penicillin and streptomycin (100 x), fetal bovine serum (FBS), and trypsin-EDTA (1 x) were purchased from PAA Laboratories GmbH (Pasching, Austria).

Sample preparation

Fresh noni shoot was obtained from University Agriculture Park, Universiti Putra Malaysia (UPM) and identified by Dr. Mohd Firdaus Ismail from Biodiversity Unit, Institute of Bioscience, UPM, Selangor, Malaysia (Voucher no: SK 3217/17). The noni shoots were washed and lyophilized for 48 h. The samples were extracted using 70 % ethanol (17, 18). Briefly, 2g of the freeze-dried sample was mixed with 100 milliliters (100 ml) of 70 % ethanol. The mixture then centrifuged

at 1536 x g for 5 minutes. The supernatant was removed using a rotary evaporator and the extract was reserved for analysis.

Cell culture

Human breast cancer (MDA-MB-231), hepatocellular cancer (HepG2), colorectal cancer (HT-29), and mouse fibroblast (BALB/c 3T3) cell lines were grown in RPMI supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin.

MTT cytotoxic assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to examine cell proliferation (19). Briefly, noni shoot extract was incubated in the selected cell lines for 72 hours with various concentrations of the extract and the absorbance was read at 570 nm. The endpoint criteria for anti-proliferative activity was selected according to the cytotoxic activity of IC₅₀ values that below 100 µg/ml for crude extracts (20). Therefore, for further analysis, only selected cancer cells with low IC₅₀ values (< 100 µg/mL) which showed promising and greater anti-proliferative effects were chosen.

Acridine orange/Propidium iodide (AO/PI) dual fluorescent assay

The selected cancer cell lines were treated with IC₅₀ concentrations obtained from the MTT assay and incubated for 24, 48, and 72 hours. Cells were stained with 1 mg/ml of acridine orange (AO) and propidium iodide (PI) and observed using a fluorescence microscope (21).

Cell cycle

BD Cycletest™ Plus DNA reagent kit was used to determine cell cycle distribution (BD Biosciences, San Jose, CA, USA). The MDA-MB-231 cells were incubated with 25 µg/mL, 50 µg/mL, and 75 µg/mL of noni shoot extract for 72 hours of incubation. The HT-29 cell lines were incubated with 33 µg/mL, 65 µg/mL and 98 µg/mL of sample extracts for 72 hours. Data were analysed using the FACS Calibur flow cytometer with Cell Quest 3.3 software (22).

Gas Chromatography-Mass Spectrophotometry (GCMS)

GCMS analysis was carried out on Shimadzu GCMS QP2010 Plus (Kyoto, Japan) equipped with SGE BPX5 column (30 m length x 0.25 mm I.D x 0.25 µm film thickness) (23). Oven temperature was set at initial temperature of 50 °C /1 minute and increased to 240 °C with 5 °C/1 minute increment followed by a ramp of 10 °C/1 minute to 280°C/15 min. The inlet temperature was 250 °C and the transfer line temperature was 280 °C. The mobile phase was using helium as a carrier gas and set a flow rate of 1.0 ml/1 minute. The spectral data were compared with the National Institute Standard and

Technology (NIST) NIST08 GCMS library database.

Data analysis

Data were expressed as the mean \pm standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple range test. A p-value of <0.05 was considered significant.

RESULTS

Cytotoxic effects

The MDA-MB-231, HepG2, HT-29 and BALB/c 3T3 cell lines were initially incubated with various concentrations of the extract (0 - 1000 $\mu\text{g}/\text{mL}$) for 72 hours. Table I shows the IC_{50} values of noni extract in MDA-MB-231, HepG2, HT-29, and 3T3 cell lines. Noni shoot extract had demonstrated the lowest IC_{50} value in MDA-MB-231 (49.72 $\mu\text{g}/\text{mL}$), followed by HT-29 (65.43 $\mu\text{g}/\text{mL}$) and HepG2 (307.5 $\mu\text{g}/\text{mL}$), indicating its marked and highly cytotoxic effects on breast cancer and colorectal cancer cell lines and less cytotoxic in liver cancer cell lines. The lower the IC_{50} value of extract showing the higher cytotoxicity effects, thereby has potential as anti-proliferative agents. In addition, the results showed that no cytotoxic effect was observed on BALB/c 3T3 cell lines after incubated with noni shoot extract for 72 hours (IC_{50} cannot be determined; $>80\%$ of cells growth). These findings showed that the noni shoot extract is selective anti-cancer agents because it only caused the cell death in cancer cell lines without affecting the normal cell.

Doxorubicin that was used as positive control in this study, is one of the chemotherapy drugs capable to induce toxicity in MDA-MB-231, HepG2, and HT-29 cell lines (IC_{50} value of 1.87, 2.29, and 1.93 $\mu\text{g}/\text{mL}$ respectively; Table I). Nonetheless, doxorubicin also showed a high cytotoxic effect in BALB/c 3T3 normal mouse fibroblast cells.

Acridine orange and propidium iodide double staining

The MDA-MB-231 cell lines were incubated with noni shoot extract (50 $\mu\text{g}/\text{mL}$) for 24, 48 and 72 hours of incubations. The MDA-MB-231 cell lines-incubated

Table I: IC_{50} values of noni shoot extract and doxorubicin in breast cancer (MDA-MB-231), liver cancer (HepG2), colorectal cancer (HT-29), and normal mouse fibroblast (BALB/c 3T3) cell lines

Cell lines	IC_{50} value ($\mu\text{g}/\text{mL}$)	
	Noni shoot extract	Doxorubicin
MDA-MB-231	49.72	1.87
HepG2	307.50	2.29
HT-29	65.43	1.93
BALB/c 3T3	N/A	1.96

N/A= IC_{50} cannot be determined

with noni shoot extract had demonstrated the early apoptosis features such as cell blebbing and chromatin condensation (Fig. 1B) after 24 hours of incubation. The MDA-MB-231 cell lines showed both early apoptosis and late apoptotic cells which indicated by orange colour emission after 48 hours (Fig. 1C). After 72 hours, there were signs of late apoptotic cells and secondary necrosis cells in MDA-MB-231 cells (Fig. 1D).

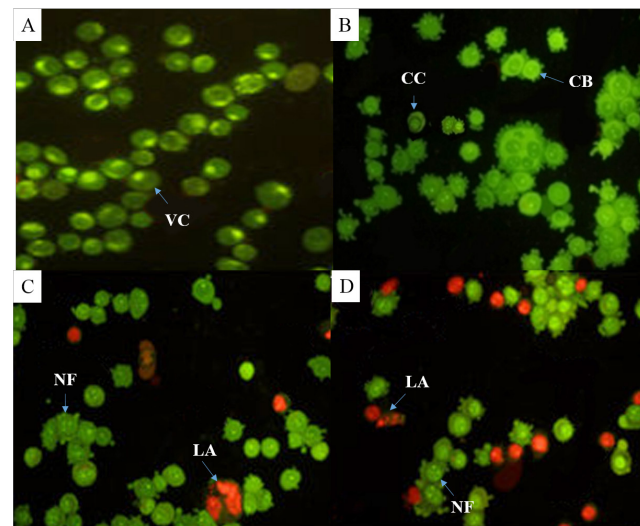


Figure 1: Morphological characterization of breast cancer (MDA-MB-231) cell lines after incubation with noni shoot extract using acridine orange (AO) and propidium iodide (PI) staining. Breast cancer, MDA-MB-231 cell lines were either (A) untreated (VC: viable cell) or treated with noni shoot extract (49.72 $\mu\text{g}/\text{mL}$) after 24 h (B), 48 h (C) and 72 h (D) of incubations. Incubation of MDA-MB-231 cell lines with noni shoot extract showed the characteristics of apoptosis such as chromatin condensation (CC), nuclear fragmentation (NF), cell blebbing (CB) and late apoptosis (LA) (Magnification 400 x).

The HT-29 cell lines were incubated with noni shoot extract (65 $\mu\text{g}/\text{mL}$) for 24, 48 and 72 hours of incubations. As shown in Fig. 2, the HT-29 cell lines exhibited early apoptotic features i.e. chromatin condensation and cell blebbing after 24 hours of incubation with noni extract (Fig. 2B). When compared to control (untreated cells), the cells were appeared to be healthy as they exhibited green colour (Fig. 2A). The late apoptosis (orange colour) were also detected only after 24 hours (Fig. 2B). In addition, the cells that exhibited early and late apoptotic features were increased after 48 hours (Fig. 2C). After 72 hours, cells with secondary necrosis were observed in addition to early and late apoptotic cells (Fig. 2D).

Cell cycle analysis

The MDA-MB-231 cell lines were incubated with noni shoot extract (25, 50, 75 $\mu\text{g}/\text{mL}$) for 72 hours. As shown in Fig. 3, there was an increment of the cell population in Sub- G_0/G_1 phase following the incubation with noni shoot extract when compared with control. In addition, the proportion of cells in G_0/G_1 phase was decreased following the increment of concentrations of noni shoot extract. The cell population of the control (untreated cells) were as followed; sub G_0/G_1 (6.71 + 4.45 %), G_0/G_1 (67.36 + 6.21 %), S (9.42 \pm 3.24 %), and M (14.15 \pm 8.25 %). After 72 h, there was significant increased

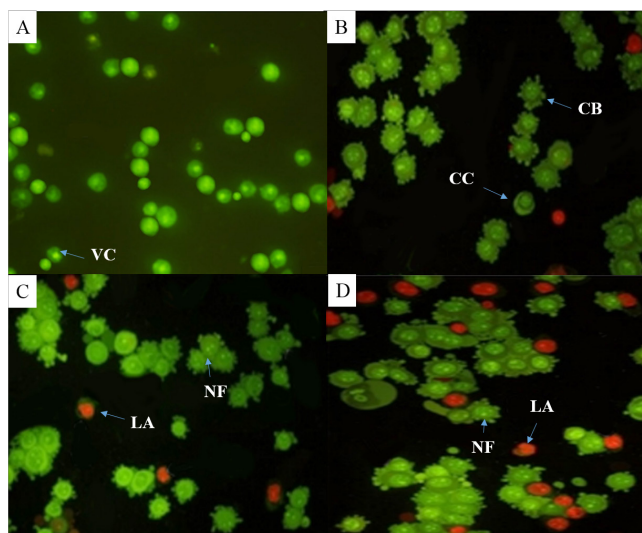


Figure 2: Morphological characterization of colorectal cancer (HT-29) cell lines after incubation with noni shoot extract using acridine orange (AO) and propidium iodide (PI) staining. Colorectal cancer, HT-29 cell lines were either (A) untreated (VC: viable cell) or treated with noni shoot extract (65.43 µg/ml) after 24 h (B), 48 h (C) and 72 h (D) of incubations. Incubation of HT-29 cells with noni shoot extract showed the characteristics of apoptosis such as chromatin condensation (CC), nuclear fragmentation (NF), cell blebbing (CB) and late apoptosis (LA) (Magnification 400 x).

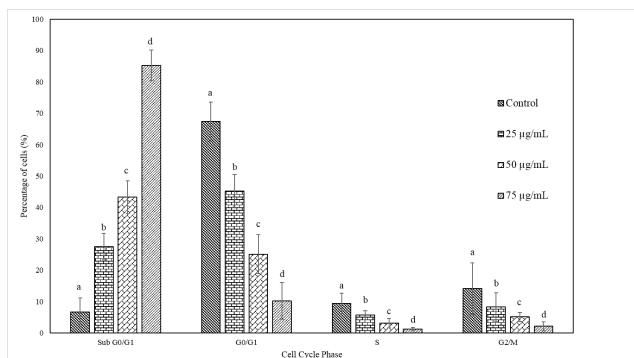


Figure 3: Cell cycle distributions in MDA-MB-231 cell lines after incubation with three different concentrations (25, 50, and 75 µg/mL) of noni shoot extract for 72h. The cell cycle kinetics were analysed using flow cytometry. Values are reported as mean ± SD (n=3) and different letters are significant relative to respective control by Tukey's test at p<0.05.

(p<0.05) of the cell proportion in the sub G₀/G₁ for the treated-cells with noni shoot extract (25, 50, 75 µg/mL) when compared to the control in dose-dependent manner (27.47 ± 4.23 %, 43.26 ± 5.24 %, and 85.22 ± 4.89 %, respectively). Besides, the proportion of cells in other phases (G₀/G₁, S, and G₂/M) were significantly decreased (p<0.05) in a dose-dependent manner (Fig. 3).

As shown in Fig. 4, there were increased in the cell population of the sub G₀/G₁ phase of HT-29 following treatment with noni extracts. The proportion of cells in sub G₀/G₁ phase was significantly increased (p<0.05) in HT-29-treated with noni shoot extract (33, 65, 98 µg/ml; 26.32 ± 4.17 %, 55.14 ± 5.45 %, and 83.42 ± 7.45 % respectively) when compared to the control (only 4.21 ± 1.42 %). In addition, there was a significant increase

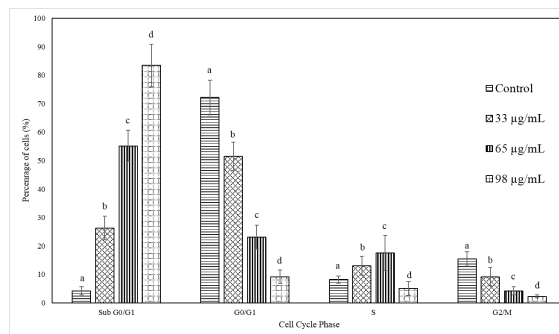


Figure 4: Cell cycle distributions in HT-29 cell lines after incubation with three different concentrations (33, 65, and 98 µg/mL) of noni shoot extract for 72h. The cell cycle kinetics were analysed using flow cytometry. Values are reported as mean ± SD (n=3) and different letters are significant relative to respective control by Tukey's test at p<0.05.

(p<0.05) in the proportion of cells in the S phase of the cell cycle for HT-29 cell lines-treated with noni shoot extract (33 and 65 µg/ml), as shown in Fig. 4.

Gas Chromatography-Mass Spectrophotometry (GCMS) analysis of noni shoot extract

The result of the GCMS analysis of an ethanolic extract of noni shoot extract was shown in Table II. The most abundant compounds in noni shoot extract are acetic acid (17.98 %) and ethriol (16.59 %).

Table II: Compounds that identified in noni shoot extract using GCMS

Peak	Compound	Retention Time	Area (%)
1	1H-Pyrrole	3.21	0.24
2	Toluene	3.63	0.01
3	Propenoic acid	3.97	0.24
4	Acetic acid	4.31	0.30
5	Glycerin	4.63	0.81
6	2-Hexanone	5.47	0.08
7	Propanal	5.8	0.28
8	m-xylene	6.04	0.01
9	Glyceraldehyde	6.19	0.17
10	3-Furanmethanol	7.05	0.18
11	Butanoic acid	7.53	0.56
12	Ethanimidic acid	7.92	0.18
13	Butyrolactone	8.11	0.26
14	Piperidine, Glutamic acid	9.75	1.91
15	Phosphonic acid	10.35	0.02
16	Carbolic acid	10.54	0.40
17	Sorbitol	10.76	0.11
18	Octadecanoic acid	11.91	0.14
19	Hyacinthin	12.08	0.03
20	Tetradecanoic acid	12.29	0.09
21	Allenton	13.36	0.46
22	Cyclopropyl carbinol	13.68	0.56
23	Valeric acid aldehyde	14.82	0.32
24	Acetoacetic acid	14.87	4.01
25	Glycerin	15.37	7.21
26	Glyceritol	15.68	2.85
27	Benzoic acid	15.9	1.30
28	Glycerol formal	16.71	0.69
29	Pyrocatechol	17.33	0.87
30	Coumaran	18.07	2.86

(table continues)

Table II: Compounds that identified in noni shoot extract using GCMS (continued)

Peak	Compound	Retention Time	Area (%)
31	Glycerin monoacetate	19.04	0.80
32	Pyrazine	19.76	1.09
33	Ursolic acid	21.75	1.32
34	Fourrine	21.75	0.67
35	Trimethylene Borate	21.96	1.49
36	Butanamide	22.74	0.04
37	Isobutylglycerol	23.09	0.34
38	Ethriol	23.52	16.59
39	Phenol	24.5	0.07
40	Succinic Acid	24.51	0.05
41	m-Salicyclic acid	24.75	0.49
42	Levogluconan	24.87	1.61
43	D-allose	25.16	0.24
44	Oxalic acid	26.07	0.08
45	Ethanone	26.2	0.05
46	Fumaric acid	26.29	0.05
47	3-Deoxy-d-mannonic acid	26.45	0.19
48	Vitamin E	26.95	0.05
49	.beta.-D-glucopyranoside	27.38	0.26
50	Cyclohexanol	27.57	5.93
51	Pentanoic acid	28.03	8.22
52	Acetin	28.05	1.92
53	Muco-Inositol	28.16	1.28
54	6-Hydroxycoumarin	28.96	2.52
55	Carbamic acid	29.11	0.05
56	myo-inositol	29.13	0.02
57	Benzoic acid	31.14	0.04
58	Ascorbic acid	31.3	2.74
59	Pentadecanoic acid	31.51	1.19
60	Hexadecanoic acid	31.59	0.13
61	Phytol	31.84	0.20
62	Linolenic acid	33.26	0.03
63	Heptadecanoic acid	34.36	0.09
64	Scopoletin	34.86	1.46
65	Hexadecanoic acid	37.03	0.78
66	Acetic acid	37.77	17.98
67	Octanoic acid	38.00	1.02
68	Squalene	38.13	0.37
69	Gamma-tocopherol	38.53	0.92
70	Heptacosane	39.99	0.05
71	Campesterol	45.19	0.04
72	Vitamin E	48.46	0.32
73	Eicosanoic acid	54.96	0.11

DISCUSSION

As shown in Table I, noni shoot extract markedly reduced the proliferation of all types of cancer cells and had markedly higher cytotoxicity effects against MDA-MB-231 and HT-29 cell lines. Several other studies had reported the potential cytotoxic effect of noni in various cancer cell lines such as in Jurkat cell lines with IC_{50} of 14.5 to 15.0 $\mu\text{g/mL}$ (24). Another study of a various solvent extract of noni leaves showed cytotoxicity of the extract in cancer cells i.e., the ethanol extract of noni leaves had an IC_{50} value of 169.17 $\mu\text{g/mL}$ and 310.0 $\mu\text{g/mL}$ in human epidermoid carcinoma (KB)

cells and human cervical carcinoma (HeLa) cells lines, respectively. In addition, the same study also reported that the 95% ethanol extract of noni had IC_{50} higher than 600 $\mu\text{g/mL}$ in liver cancer (HepG2) and breast cancer (MCF-7) cell lines (13). In comparison with the present study, the IC_{50} value of noni extract in breast cancer cells, MDA-MB-231 and liver cancer cell lines, HepG2 was much lower (Table I) than that obtained in the previous study (13) possibly due to a different percentage of extraction solvent and different parts of the plant used.

A previous study had shown that damnacanthal and nordamnacanthal, compounds that extract from noni roots were able to induce apoptosis through intrinsic pathways and arrest the cell cycle in oral squamous cell carcinoma (OSCC) cells (25). Another study also reported that ethyl acetate extract of noni fruits demonstrated the cell cycle arrest at the G1/S phase and G0/G1 phase in MCF-7 and MDA-MB-231 cells, respectively (26).

All cells-treated with noni extracts (Fig. 3 and 4) markedly increase the cell population of sub G0/G1 phase indicating DNA fragmentation in apoptotic cells. Moreover, the proportion of cell death was significantly increased in a dose-dependent manner in cancer cells-treated with noni shoot extracts. The increment of cell count in sub G0/G1 phase showed that cells undergo apoptosis and cause the cleavage of nuclear DNA into multiple fragments (27). These findings suggest that noni shoot extract only induced the apoptosis in MDA-MB-231. However, in HT-29 cells-treated with noni caused cell cycle arrest at S phase and thereby an inhibition of DNA replication.

The most abundant compounds in noni shoot extracts are acetic acid and ethriol (Table II). Acetic acid is the simplest carboxylic acid and it is characterized by its sour and pungent smell (28). The effectiveness of acetic acid was shown to induce apoptosis in human gastric cancer cell lines. Hence, it's demonstrated that acetic acid has potential as an anti-cancer agent (29). Ethriol is the organic compound in triol group where it has three hydroxyl functional groups and may act as antidiabetic and anticancer agent (30).

CONCLUSION

It was found that noni shoot extract was cytotoxic on MDA-MB-231 and HT-29 cell lines. Furthermore, noni shoot extract had less toxic effects on 3T3 cell lines. This implies noni shoot extract possess potential anti-proliferative activities without affecting normal cells. Noni shoot extract induced apoptosis in MDA-MB-231 and HT-29 cell lines. Moreover, the identified compounds in noni (acetic acid and ethriol) shoot extract that was shown in this present study can be further investigated for their medicinal properties particularly their anti-cancer effects. Noni shoot extract

possesses anti-cancer properties in selected cancer cells via induction of apoptosis pathway and further research can provide more information about noni as one of the possible nutraceutical or functional food product.

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