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ORIGINAL ARTICLE – CANCER RESEARCH

Ginger extract has anti-leukemia and anti-drug resistant effects on malignant cells

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Abstract

Purpose Based on the poor prognosis of drug resistance in pediatric acute lymphoblastic leukemia (ALL) and adverse effects of chemotherapy, this study was aimed to evaluate the effect of several herbal extracts on leukemic cells.

Methods Two subtypes of T- and B-ALL cell lines, followed by ALL primary cells were treated with cinnamon, ginger, and green tea extracts, alone or in combination with methotrexate (MTX). Possible apoptosis was investigated using Annexin-V/PI double staining. Real-time PCR was applied to evaluate the expression levels of related ABC transporters upon combination therapy.

Results The IC_{50}s for cinnamon, ginger and green tea extracts on ALL cell lines were 300 µg/ml, 167 µg/ml and 70 µg/ml, respectively. Surprisingly, the methotrexate (MTX)-resistant sub-line showed more sensitivity to ginger. Combined treatment with ginger and MTX showed synergistic effects on CCRF-CEM, Nalm-6 and ALL primary cells. It was shown that ginger does not impair the high expression levels of ABCA2 or ABCA3 transporter genes in the ALL malignant cells, suggesting other molecular pathways involved in its anticancer potential.

Conclusion To the best of our knowledge, this is the first study that reveals the antileukemic effect of ginger extract on both, pediatric ALL cell lines and primary cells.

Keywords Childhood acute lymphoblastic leukemia · Ginger · Methotrexate · Multidrug resistance · Supplemental therapy

Introduction

Acute lymphoblastic leukemia (ALL) is a hematologic malignancy caused by impaired differentiation, proliferation, and accumulation of lymphoid T or B cell progenitors (Bhojwani et al. 2015; Hunger and Mullighan 2015). B-ALL is more prevalent and occurs more frequently in children (Paul et al. 2016). However, T-ALL demonstrates poor disease prognosis (Bhojwani et al. 2015). According to the published studies, the overall cure rate for newly diagnosed childhood ALL is improved substantially by 80% in developed countries (Kato and Manabe 2018; Ma et al. 2015). Nonetheless, disease recurrence has been always considered as a challenging problem regarding the cancer treatment (Housman et al. 2014). Multidrug resistance (MDR), is the major obstacle to effective chemotherapy, which may contribute to relapse (Lage 2008). It has been reported that adenosine triphosphate binding cassette (ABC) transporters, located on cellular membranes acting as drug pumps, contribute to drug resistance and subsequently treatment failure (Eichhorn and Efferth 2012). Two members of this family, ABCA2 and ABCA3, have been previously introduced as biomarkers for resistant pediatric ALL patients (Aberuyi et al. 2017; Rahgozar et al. 2014).

Methotrexate (2,4-diamino-*N*10-methyl propylglutamic acid, MTX), a well-known chemotherapy drug, inhibits dihydrofolate reductase and thymidylate synthase, two key enzymes in folate metabolism, resulting in impaired DNA/RNA biosynthesis and cell death (Abolmaali et al. 2013; Wijdeven et al. 2016; Wojtuszkiewicz et al. 2015). This mechanism affects both malignant and normal cells such as bone marrow haematopoietic cells, intestinal mucosal cells,

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and hepatocytes, in a non-selective way (Kelly and Owusu-Apenten 2015), therefore generating undesirable side effects during chemotherapy regimens (Fotoohi and Albertioni 2008). Considering the remarkable toxicity of cancer medications and the acquired resistance to chemotherapy drugs, tremendous attempts have been made to discover natural products with limited side effects and toxicity (Sheikh et al. 2017).

In the present study, cinnamon (*Cinnamomum aromaticum*), ginger (*Zingiber officinale*) and green tea (*Camellia sinensis*) were selected and examined on leukemic cell lines and patient primary cells, alone or combined with MTX. The wide range of antifungal, antibacterial, anti-diabetes, anti-oxidant and anticancer effects of the aforementioned herbal extracts were previously demonstrated (Baliga et al. 2011; Butt and Sultan 2011; Cabrera et al. 2006; Hamidpour et al. 2015; Rao and Gan 2014; Shen et al. 2012; Singh et al. 2011). However, their possible anti-proliferative effects on pediatric ALL remained to be determined.

Materials and methods

Herbal materials and chemicals

Extracts of cinnamon bark (batch number ZSKY20131112), ginger dried root (batch number ZSKY20140123) and green tea dried leaf (batch number ZSKY20141119) were purchased from Shaanxi Zhengsheng Kangyuan Bio-medical Co., Ltd (Shaanxi, China). Detailed information about the herbal extracts is summarized in Table 1. Methotrexate (MTX) was obtained from Santa Cruz Biotechnology, Inc (Heidelberg, Germany) and dimethyl-sulfoxide (DMSO) was purchased from Cinnagen (Tehran, Iran). Roswell Park Memorial Institute-1640 (RPMI1640), fetal bovine serum (FBS), and penicillin streptomycin (Pen Strep) were bought from Bioidea (Tehran, Iran). Phosphate buffered saline (PBS) was from Sigma-Aldrich (Munich, Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

Table 1 Characteristics of the herbal extracts

Product name	Latin name	Plant part used for extraction	Method of extraction	Extract solvent	Ingredients	Test method
Ginger oil/water soluble	*Zingiber officinale roscoe*	Dried root	Supercritical CO_2 extraction	99.9% food grade carbon dioxide	Gingerols (3%)	HPLC
					Heavy metals (NMT 10 ppm)	USP231
					Arsenic (NMT 0.5 ppm)	ICP-MS
					Mercury (NMT 0.05 ppm)	ICP-MS
					Lead (NMT 0.2 ppm)	ICP-MS
					Aflatoxin (NMT 8 ppb)	HPLC
Green tea 90% extract	*Camellia sinensis* O. Ktze.	Dried leaf	Hydro-alcoholic	Grain alcohol	Polyphenol (NLT 90%)	UV
					EGCG (NLT 40%)	HPLC
					Coffeine (NMT 8%)	HPLC
					Heavy metals (NMT 10 ppm)	Atomic absorption
					Arsenic (NMT1 ppm)	Atomic absorption
					Lead (NMT 1 ppm)	Atomic absorption
					Phosphate organics (NMT 1 ppm)	Gas chromatography
Cinnamon bark 50% polyphenols	*Cortex Cinnamomi Cassiae*	Bark	Remaceration and soxhletation	Ethanol and water	Polyphenol, flavones	UV
					Arsenic (NMT 2 ppm)	ICP-MS
					Lead (NMT 2 ppm)	ICP-MS

USP United States Pharmacopeia; *NMT* not more than, *ppm* parts per million, *ppb* parts per billion, *HPLC* high-performance liquid chromatography, *ICP-MS* inductively coupled plasma mass spectrometry, *NLT* not less than

bromide (MTT) was purchased from Atocel (Graz, Austria). L-Glutamine was from Gibco (Sao Paulo, Brazil) and FITC Annexin-V apoptosis detection kit with PI was bought from BioLegend (London, United Kingdom). Ficoll–Hypaque was from Inno-train (Kronberg, Germany) and TRIzol reagent was bought from Invitrogen (California, CA).

Cell lines and patient primary cells

CCRF-CEM (derived from a 4-year-old female with T-ALL) and Nalm-6 (derived from a 19-year-old male with pre-B-ALL) human cell lines were purchased from Pasteur Institute (Tehran, Iran). MTX-resistant sub-lines were generated in-house (Dabaghi et al. 2016). Briefly, CCRF-CEM cells were seeded into 25-cm^2 flasks and continuously exposed to stepwise concentrations of MTX from 5 nM to 1.2 µM. Cells were maintained in the same concentration of MTX for two or three passages allowing for reaching regular growth rate. After full growth recovery, the concentration of MTX was increased by twofold each time.

Cell lines were grown in RPMI1640 supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) 100 IU/ml penicillin and 100 µg/ml streptomycin. RPMI-1640 was additionally supplemented with 1% (v/v) L-glutamine for Nalm-6. Patients primary cells were cultured in RPMI-1640 supplemented with 20% FBS and 1% L-glutamine.

Patient and control sampling

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the ethics committee of the University of Isfahan (agreement number 94/31540). Samples were collected with full written informed parents' consents in compliance with the ethical protocols and standards of Sayed-ol-Shohada Hospital (Isfahan, Iran). All children diagnosed with ALL who were hospitalized from June 2017 to April 2018 were included in the study except those with Philadelphia-positive leukemia, infantile leukemia, mixed-lineage leukemia and Burkitt-type leukemia. Patients were treated based on the Australian and New Zealand Children's Cancer Study Group ALL study protocol 8 (http://www.anzctr.org.au/trial_view.aspx?ID=1568). Cases without any symptoms of cancer (including ALL) were selected as age-matched controls. Two to five milliliters of bone marrow aspirate or peripheral blood was collected in heparin and sent on ice to the Cellular and Molecular Biology laboratory of University of Isfahan. Mononuclear cells (MNCs) were isolated by Ficoll–Hypaque density gradient centrifugation method, in accordance with the manufacturer's instructions.

Cell treatment

Cell lines and control MNCs were seeded into flat bottom 96-well microtiter plates in triplicate at a density of 15×10^4 cells per well suspended in 100 µl FBS supplemented media. To assess the effect of plant extracts, on malignant cell lines, 50 µl of freshly made increasing concentrations of cinnamon (300–1000 µg/ml), ginger (133–233 µg/ml) and green tea (50–150 µg/ml) extracts were added to the cells, separately. All the plant extracts were completely soluble in the supplemented media. Combination treatment was performed by the addition of 25 µl of the aforementioned plant extracts to equal volumes of MTX (0.5 µM).

In vitro proliferation assays

In order to assess the viability of cell lines followed by treatment with herbal extracts, MTT colorimetric assay was applied. After 48 and 72 h incubation, 10 µl of 5 mg/ml MTT solution was added to each well. Following 3 h incubation at 37 °C, the supernatant was removed and the formazan precipitate was solubilized by addition of 100 µl DMSO to each well. Absorbance values were measured at 492 nm using a Stat Fax-2100 microtiter plate reader (Palm City, FL).

Cell viability ratio was calculated by the following formula:

$$\text{Cell viability ratio (\%)} = 100\times\frac{\text{optical density (OD) of treated cells} - \text{OD of blank}}{\text{optical density (OD) of control cells} - \text{OD of blank}}.$$

The half-maximal concentrations of proliferation inhibition (IC_{50}) were determined from the viability curves.

Apoptosis assay

Mononuclear cells were isolated from whole blood/bone marrow aspirate samples of 12 children with acute lymphoblastic leukemia by Ficoll–Hypaque. Cells were plated at a seeding density of 25×10^4 cells per well and treated with 167 µg/ml ginger, 0.1 µM MTX either as single agents or in combination. After a 48 h incubation, cells were collected and washed with PBS supplemented by 0.5% FBS, then centrifuged at 200 rcf for 10 min. Supernatant was discarded and cell pellet was resuspended in 100 µl of cold 1× Annexin-V-binding buffer. In a flow cytometry tube, 5 µl of FITC conjugated Annexin-V and 10 µl of PI were added to the cells, in turns. Cells were incubated for 15 min at room temperature in the dark. 400 µl

of ice cold 1× binding buffer was added followed by fluorocytometric analysis. Data acquisition was performed by a BD FACSCalibur Flow Cytometer (London, UK). All data were analyzed using Cell Quest Pro (BD Biosciences, San Jose, CA) and FlowJo softwares (Tree Star Inc., Ashland, OR).

Real-time PCR

Total RNA was extracted from treated cell lines as well as isolated MNCs from patients' samples using TRIzol reagent, according to the manufacturer's instructions. PrimeScript™ RT reagent Kit (Takara, Japan) was utilized to reversely transcribe total RNA into cDNA which was amplified using real-time quantitative PCR on a Chromo4™ system (Bio-Rad, Foster City, CA). A list of the applied primers is mentioned in Table 2. Briefly, 1 µl of 0.4 ng/µl of the synthesized cDNA, 0.4 µl of 100 nM each forward and reverse primers, 5 µl of SYBR green Master Mix and 3.2 µl of ddH_2O were added to the real-time tubes in a final volume of 10 µl. Subsequently qRT-PCR was performed in duplicate according to the following protocol included 40 cycles for steps 2–4: 1—preincubation at 95 °C (5 min), 2—denaturation at 95 °C (20 s), 3—annealing at 60 °C (30 s) and 4—product expansion at 72 °C (25 s). Data were normalized by *GAPDH* as the internal control gene. All relative expression levels were calculated and reported using the $2^{-\Delta\Delta C_t}$ method.

Combination index

Combination index (CI) was measured to determine the combination effect based on IC_{20}, IC_{30}, IC_{50} and IC_{70} values achieved from herbal extract and MTX alone and combination treatment. Combination index was calculated according to the following equation:

$$\mathrm{CI} = \left(D_x\right)_{\mathrm{com1}} / \left(D_x\right)_1 + \left(D_x\right)_{\mathrm{com2}} / \left(D_x\right)_2,$$

where $\left(D_x\right)_{\mathrm{com1}}$ (or $\left(D_x\right)_{\mathrm{com2}}$) is the dose-caused x% proliferation inhibition for drug 1 (or 2) in the combination, and $\left(D_x\right)_1$ (or $\left(D_x\right)_2$) is the dose of drug 1 (or 2) alone, causing the same % inhibition. The obtained CI was then used to identify the outcome of herbal/chemical drugs combination as additive ($0.9 < \mathrm{CI} < 1.1$), synergistic ($\mathrm{CI} < 0.9$), or antagonistic ($\mathrm{CI} > 1.1$) effects.

Statistical analysis

Data were conducted using GraphPad Prism 5.01 and 6.01 Software (GraphPad, San Diego, CA). The statistical significance of differences between two sets of data and various treatment groups were estimated using unpaired nonparametric *t* test and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, respectively. The receiver operating characteristic (ROC) curve analysis was employed for examining the statistical significance of observed mRNAs expression differences, and also to select the cut-off points. The correlation between parameters of interest was analyzed through Spearman's coefficient calculation. Statistical significance for all tests was considered as P values < 0.05. All results were expressed as mean ± standard error of the mean (SEM).

Results

Effect of the herbal extracts on CCRF-CEM viability

CCRF-CEM cell line was chosen as a T-ALL subtype model according to its poor response to treatment. Plates were coated with 15×10^4 cells per well in triplicates. Increasing concentrations of cinnamon (100, 400, 800, 1000, 1500, 2000, and 3000 µg/ml), ginger (17, 33, 83, 167, 233, 283, 333, and 667 µg/ml) and green tea (10, 20, 50, 100, 150, 200, and 400 µg/ml) were added, separately. Cell viability

Table 2 Primer sequences of *ABCA2*, *ABCA3*, *ABCB1* and *GAPDH*

Gene	Primer sequence (5′ to 3′)	Primer length (base pairs)	Amplicon length (base pairs)	T_m	GC%
ABCA2	F:CCGCACCATCCTTCTGTCCACCCACC	26	263	70.79	65.38
	R:TGCGGATGAACTGGGACACCTGGAGC	26		70.31	61.54
ABCA3	F:GGCCATCATCATCACCTCCCACAGCA	26	277	66.7	57.7
	R:AGCGCCTCCTGTTGCCCTTCACTCTG	26		68.7	61.5
ABCB1	F:CTCATGATGCTGGTGTTTGG	20	102	53.4	50
	R:TGGTCATGTCTTCTTCCAGA	20		52.3	50
GAPDH	F:GCCCCAGCAAGAGCACAAGAGGAAGA	26	106	68.64	57.69
	R:CATGGCAACTGTGAGGAGGGGAGATT	26		66.38	53.85

F forward, *R* reverse, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, T_m melting temperature

was assessed using MTT assay after two different time points of 48 h and 72 h, in order to evaluate time-dependency for the possible cytotoxicity of the applied herbal extracts, and 72-h time point incubation was selected accordingly (Fig. 1a–c, respectively). Cinnamon, ginger and green tea extracts showed significant toxicity on CCRF-CEM cell line in a dose-dependent manner, and the percent cell line viability decreased from 74.342 ± 3.905% to 0.68 ± 0.049%, 89.675 ± 3.426% to 3.781 ± 1.032% and 64.175 ± 9.923% to 7.17 ± 10.417% (mean ± SEM, $n = 9$), respectively. The half-maximal concentrations of proliferation inhibition (IC_{50}) after 72 h incubation were 300 µg/ml, 167 µg/ml, and 70 µg/ml for cinnamon, ginger, and green tea, respectively (Fig. 1a–c).

Effect of the herbal extracts on normal mononuclear cells

To investigate whether the inhibitory effect of the herbal extracts was selective on CCRF-CEM, fresh normal peripheral mononuclear cells (MNCs) were exposed to the extracts and cell viability was assessed. Cinnamon extract did not display any significant effect on MNCs from 400 to 1000 µg/ml in comparison with the control group ($P = 0.1344$ and $P = 0.1039$, respectively). Cells stayed viable while treated by 167 µg/ml ginger and 70 µg/ml green tea, by 94.26% ($P = 0.06$) and 90% ($P = 0.44$), respectively. Collectively, normal MNCs were not significantly sensitive to proliferation inhibition induced by 50% inhibition concentration of the herbal extracts (Fig. 2a–c).

Effect of the herbal extracts/methotrexate on CCRF-CEM

The cytotoxic effect of the herbal extracts in combination with a commonly used chemotherapeutic agent, methotrexate, was evaluated. The anti-proliferative effect of ginger extract/MTX combination showed significantly higher growth inhibition effect compared with MTX alone [38.71 ± 0.67%, 35.96 ± 2.558% and 30.77 ± 1.381%

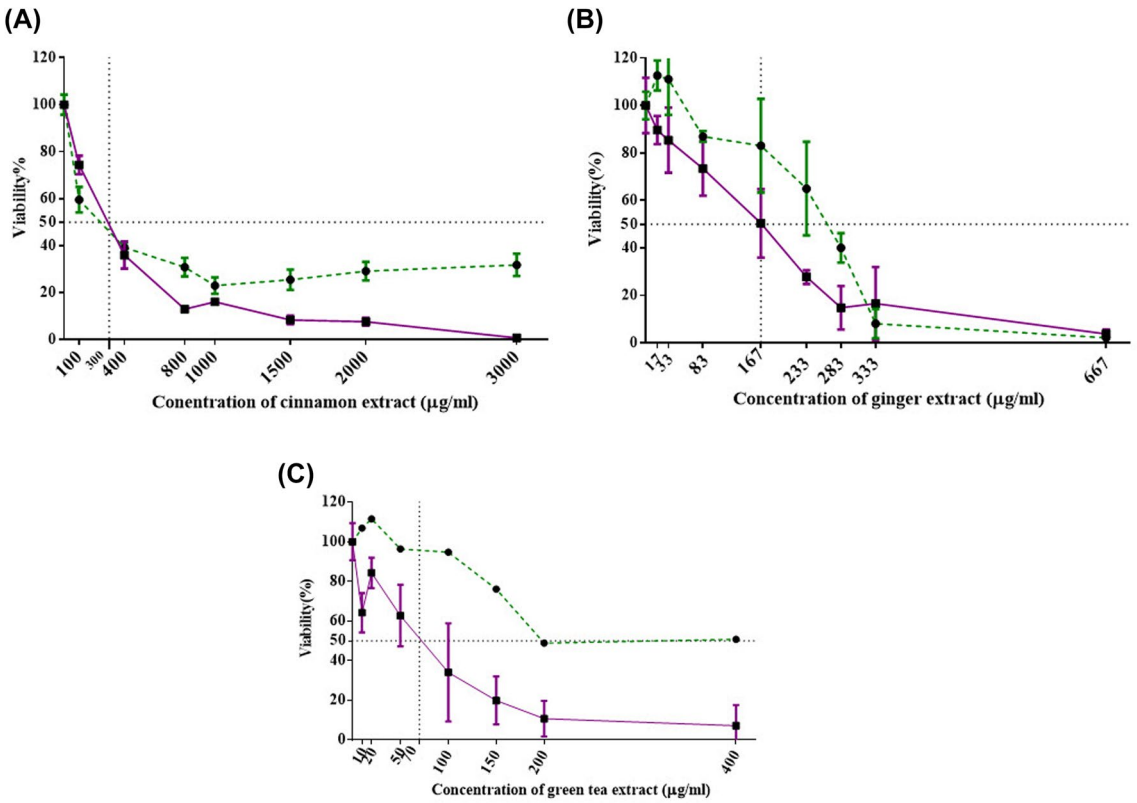

Fig. 1 Cinnamon, ginger and green tea extracts showed anti-proliferative activity on CCRF-CEM cell line. Human T-ALL cell line (CCRF-CEM) was treated with increasing concentrations of cinnamon (**a**), ginger (**b**), and green tea (**c**) extracts for 48 h and 72 h, separately. The percentage of cell viability was significantly decreased compared with the untreated cell line in a time and dose-dependent manner. Values are mean ± SEM of three independent experiments. Each experiment was performed in triplicate. Dotted crossed lines demonstrate the half-maximal concentrations of proliferation inhibition (IC_{50}) of the cinnamon (**a**), ginger (**b**) and green tea (**c**) extracts. (Circles) 48 h incubation, (squares) 72 h incubation. The 72 h incubation time was selected due to the higher cytotoxic effect of the plant extracts on the CCR-CEM cell lines

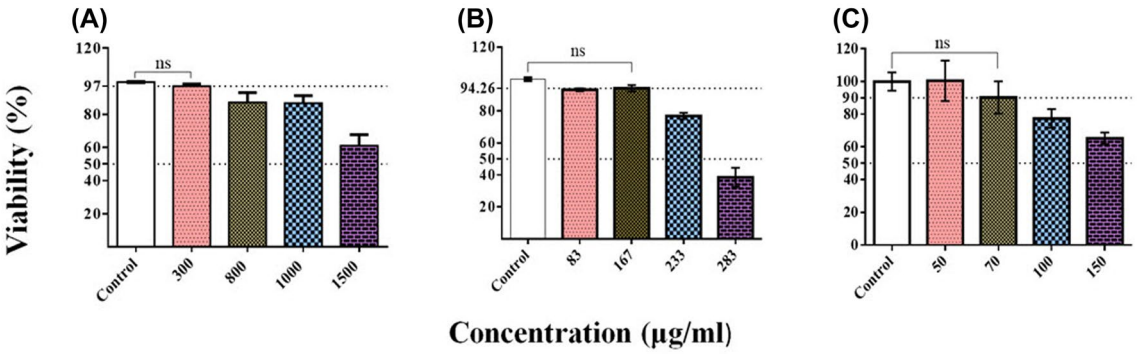

Fig. 2 Percent viability of normal mononuclear cells (MNCs) in the presence of cinnamon, ginger and green tea extracts. Normal peripheral mononuclear cells were isolated from 4 volunteers using Ficoll. Cells (15×10^4 cells/well) were seeded into 96 well flat bottom microwell plates and treated with cinnamon (**a**), ginger (**b**) and green tea (**c**) extracts for 72 h. For the control group, cells were incubated with RPMI-1640 alone. Reduced percent viability of MNCs treated by 50% inhibition concentration of cinnamon, ginger and green tea was negligible and equal to 2.7%, 5.74% and 10%, respectively. On the other hand, IC_{50} of cinnamon, ginger and green tea extracts on MNCs were considerably high compared with the toxicity of these herbal extracts on CCRF-CEM and equal to > 1500 µg/ml, 267 µg/ml, and > 150 µg/ml, respectively. Values are mean ± SEM of three independent experiments. Each experiment was performed in triplicate

(for 133, 167 and 233 µg/ml ginger combined with 0.5 µM MTX) vs 55.7 ± 0.912% (for 0.5 µM MTX alone) (mean ± SEM, $P = 0.0007$)] (Fig. 3b). However, the outcome of cinnamon and green tea extracts in combination with MTX were not significantly different with that of MTX alone [59.63 ± 9.971%, 58.389 ± 0.619% and 46.437 ± 3.142% (for 300, 600 and 1000 µg/ml cinnamon combined with 0.5 µM MTX) vs 54 ± 2.718% (for 0.5 µM MTX alone) (mean ± SEM, $P > 0.05$)] and [46.663 ± 3.575%, 48.768 ± 3.851% and 37.051 ± 2.345% (for 50, 70 and 150 µg/ml green tea combined with 0.5 µM MTX) vs 60 ± 6.675% (for 0.5 µM MTX alone) (mean ± SEM, $P > 0.05$)] (Fig. 3a, c). The calculated CI value for ginger/MTX was 0.725, demonstrating a synergistic interaction between these two compounds.

Effect of the herbal extracts on MTX-resistant sub-lines

The effect of extracts on 0.6 µM and 1.2 µM MTX-resistant sub-lines was evaluated. Interestingly, it was demonstrated that although the increasing concentrations of MTX (up to 1.2 µM) had no effect on the viability of the CCRF subline, MTX-resistant cells showed more sensitivity to the increasing concentrations of the ginger extract compared with the non-resistant CCRF-CEM cell line in a dose-dependent manner. However, treating the CCRF sub-lines with increasing concentrations of MTX showed approximately 100% viability on the other words, the IC_{50} of ginger for 0.6 µM and 1.2 µM MTX-resistant CCRF-CEM was relatively lower than that for CCRF-CEM (117 µg/ml and 84 µg/ml vs 167 µg/ml, respectively) (Fig. 4b). Results indicated that the cinnamon extract growth inhibitory effect on CCRF-CEM and the related resistant sub-lines was not significantly different ($P = 0.06$ and $P = 0.41$ for 0.6 µM and 1.2 µM MTX-resistant cells, respectively) (Fig. 4a).Green tea extract was shown to be more toxic for CCRF-CEM cells rather than the resistant sub-lines (Fig. 4c).

According to the results demonstrating the sensitivity of drug-resistant sub-cell lines to the ginger extract and the synergistic interaction effect between ginger extract and MTX on the malignant CCRF-CEM viability, ginger was selected among the other plant extracts for further experiments.

Effect of ginger extract on Nalm-6 viability alone and in combination with MTX

The anti-proliferative effects of the single and combined administration of ginger extract and methotrexate on the B-ALL cell line, Nalm-6, was evaluated. 15×10^4 cells/well were seeded on microtiter plates and treated with two concentrations of 133 µg/ml and 167 µg/ml ginger extracts alone and combined with different concentration of MTX including 0.1 µM and 0.5 µM MTX. After 96-h incubation, cell viability was assessed using MTT assay. Results showed that the anti-proliferative effect of MTX/ginger extract was significantly more than that of MTX alone. In other words, cell viability reduced down to 60.3 ± 7.99 and 39.92 ± 1.11% in the presence of 0.1 µM MTX combined with 133 µg/ml, and 167 µg/ml ginger, respectively, compared with 91.87 ± 6.74% when cells were treated with MTX alone. Application of 0.5 µM MTX showed decreased percent viability when cells were treated with a combination of MTX and ginger (52.27 ± 1.752% and 36.36 ± 0.458% for 133 µg/ml and 167 µg/ml ginger, respectively) compared with MTX alone (79.65 ± 6.11%) (mean ± SEM, $n = 6$) (Fig. 5). The calculated combination index from dose–effect data of single

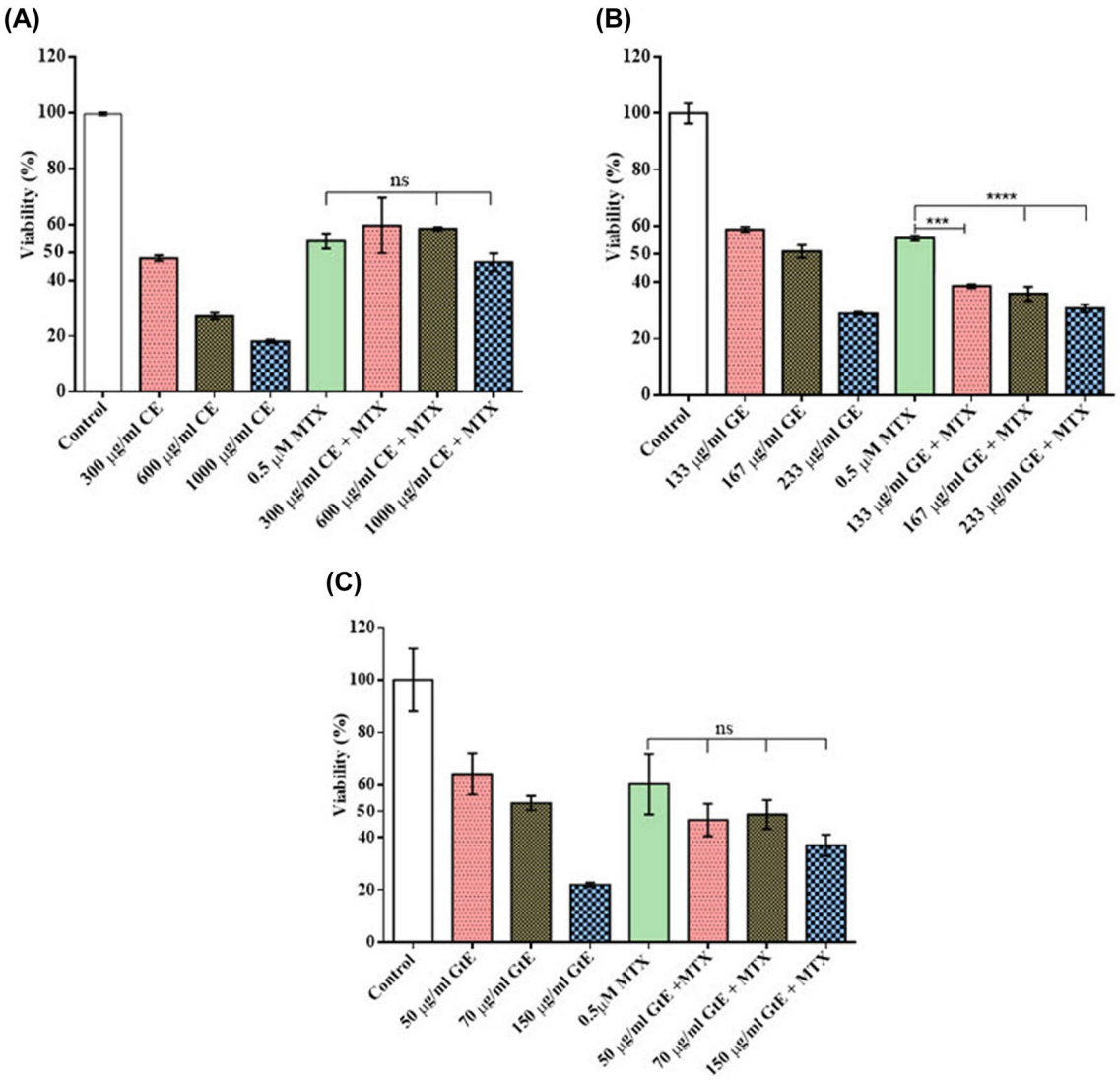

Fig. 3 Combination of ginger extract, but not cinnamon or green tea, with methotrexate showed a synergistic anti-proliferative effect on CCRF-CEM. Plates were coated with 15×10^4 CCRF-CEM cells/well. Several ratios of the IC_{50} values of cinnamon (**a**), ginger (**b**) and green tea (**c**) alone and in combination with 0.5 µM, the 50% inhibition concentration of MTX, were added to the wells, separately, and incubated for 72 h. Control cells were treated with RPMI-1640 alone. Percent viability was then assessed using MTT assay. Values are mean ± SEM of three independent experiments. Each experiment was performed in triplicate. *CE* cinnamon extract, *GE* ginger extract, *GtE* green tea extract, *ns* not significant, ***$P<0.001$, and ****$P<0.0001$

and combined drug treatments was 0.896, showing a synergistic cytotoxicity for ginger and MTX on Nalm-6.

Effect of ginger extract combined with MTX on pediatric ALL samples

The effect of 167 µg/ml ginger combined with 0.1 µM MTX was investigated on the fresh samples collected from 7 de novo and 5 relapsed childhood ALL. The rationale for selecting 0.1 µM MTX instead of 0.5 µM was to avoid the possible side effects of the chemotherapy drug on patients, as much as possible. Malignant cells were isolated from whole blood/bone marrow of patients, at diagnosis, using density gradient Ficoll media. 25×10^4 cells were seeded into the microtiter plates and treated with ginger, MTX and combination of ginger/MTX for 48 h. Percent apoptosis was then calculated using Annexin-V/PI double staining and flow cytometry analysis. Increased apoptosis was observed in 11 out of 12 treated sample patients compared with untreated samples ($26.9\pm5.409\%$ vs $8.132\pm0.949\%$, mean ± SEM, $P=0.002$) (Fig. 6a–d and Table 3).

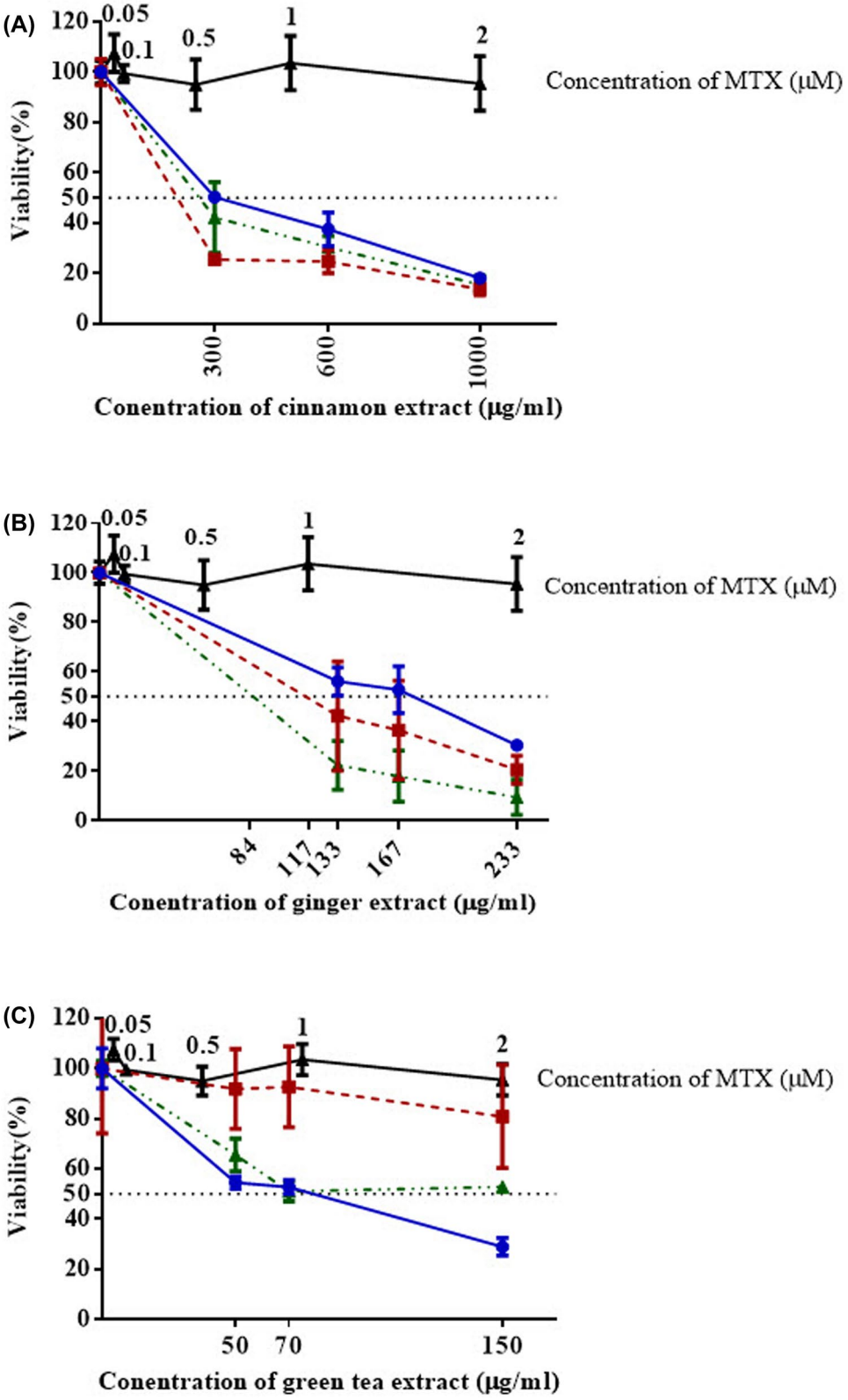

Fig. 4 The effect of cinnamon, ginger and green tea extracts on 0.6 µM and 1.2 µM MTX-resistant CCRF-CEM sub-lines. Methotrexate resistant CCRF-CEM sub-cell lines in addition to their parent lines were treated with increasing concentrations of cinnamon (**a**), ginger (**b**) and green tea (**c**) extracts. Viability was assessed using MTT assay. Resistant sub-lines showed higher sensitivity to the ginger extract, but not to cinnamon or green tea. Values are mean ± SEM of three independent experiments. Each experiment was performed in triplicate. (Circles) CCRF-CEM, (squares) CCRF-CEM sub-line resistant to 0.6 µM MTX, (triangles) CCRF-CEM sub-line resistant to 1.2 µM MTX. Solid black lines (from **a** to **c**) demonstrate the resistance of CCRF-CEM sub-line to the increasing concentrations of MTX (0–2 µM)

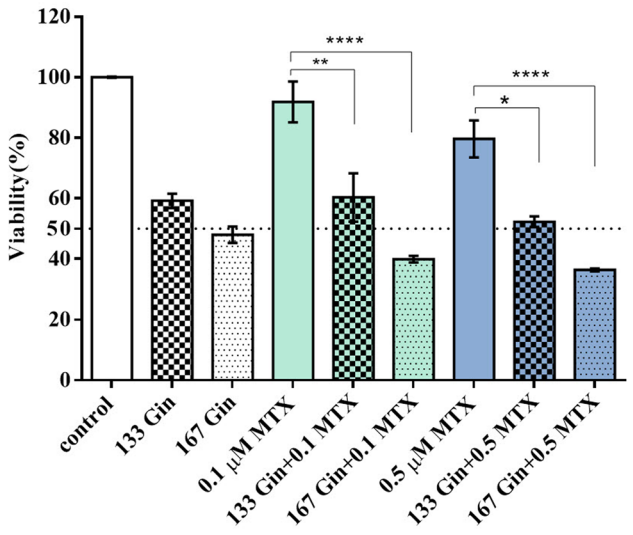

Fig. 5 Effect of ginger extract on Nalm-6. Cells were seeded on 96 microwell plates and incubated with 0.1 and 0.5 μM MTX alone and combined with 133 μg/ml and 167 μg/ml ginger extract for 96 h. Cell viability was then measured using MTT assay. Data showed that the cytotoxic effect of MTX alone was significantly less than that of the MTX/ginger extract combination. Values are mean ± SEM of two independent experiments. Each experiment was performed in triplicate. *$P < 0.01$, **$P < 0.01$, ****$P < 0.0001$

Effect of ginger extract on the mRNA expression profile of multidrug resistance genes

To investigate the possible mechanism through which ginger may exert its toxic effect on drug resistance, MTX-resistant CCRF-CEM, and Nalm-6 cells were seeded into 6-well plates and treated with 167 μg/ml ginger for 72 h and 96 h, respectively. Total RNA was isolated and real-time PCR was performed. Treated cells showed increased expression levels for *ABCA2* in MTX-resistant CCRF-CEM and Nalm-6 cells, compared with the untreated cell lines by 2.679- ($P < 0.05$) and 11.475-fold ($P < 0.01$), respectively (Fig. 7a, b).

Furthermore, the possible correlation between the expression levels of *ABCA2/ABCA3* and response to treatment was evaluated in the above-mentioned pediatric ALL samples. The mRNA expression levels of *ABCA2* and *ABCA3* were assessed at the onset of patients diagnosis, before cells were exposed to ginger/MTX (Table 3). The cut-off points of 0.543 and 1.533, were selected for the relative expression levels of *ABCA2* and *ABCA3*, based on the ROC curve analyses. Spearman's coefficient test displayed no significant correlation between the expression levels of neither of those genes and percent apoptosis induced by ginger/MTX treatment ($P = 0.248$ and $P = 0.946$, respectively) (data not shown).

Discussion

The current project demonstrated encouraging antitumor impacts on CCRF-CEM cell lines applied by cinnamon, ginger, and green tea extracts (Fig. 1a–c). A large body of experimental evidences had previously shown different biological activities of these herbal medicines including antioxidant, antimicrobial, anti-inflammatory, anticancer and anti-allergic potentials (Baliga et al. 2011). However, this is the first report to exhibit the antiproliferative impact of these phytopharmaceuticals on pediatric T-ALL cell lines. Interestingly, these herbal extracts showed no effect on healthy mononuclear cells (Fig. 2a–c), suggesting selective cytotoxicity against the tumor cells.

Combined treatment of ginger with MTX increased the cytotoxic effect of MTX by 1.54-fold for the T-ALL malignant cell lines (Fig. 3b) and 2.3-fold for B-ALL cells (Fig. 5), while the cytotoxic activity of this herbal extract in normal mononuclear cells was negligible (Fig. 2). MTX is a substantial component of nearly all the chemotherapeutic regimens (Kodidela et al. 2014). However, the poor pharmacokinetic and adverse side effects attributed to this drug has limited its therapeutic outcome (Cheung-Ong et al. 2013; de Beaumais and Jacqz-Aigrain 2012; Tian and Cronstein 2007). Therefore, identifying plant extracts, such as ginger, which show synergistic interactions with MTX may be used as a cost-effective, phytonutrient-based supplement of cancer therapy, offering the advantage of less toxicity and more antitumor efficacy (Saxena et al. 2016). On the other hand, neither cinnamon, nor green tea showed increased cytotoxicity followed by combined treatment with MTX (Fig. 3a, c). This may be due to the anti-oxidative properties of their polyphenol compounds (Dugoua et al. 2007; Mahbub et al. 2017), which may interrupt the release of the free radicals induced by MTX or deactivate the MTX-derived reactive agents contributing to decreased cytotoxic effect of MTX.

Fascinatingly, combination of ginger with MTX showed novel and auspicious results when applied on ALL patient primary cells. Eleven out of 12 patient samples showed 1.2–16.5% increased apoptosis compared with the untreated samples (Fig. 6a). Some of the aforementioned patients were those with recurrent relapses and multidrug resistance complexities (patient number 10 and 11). These results were supported by the in vitro experiments performed on the in-house developed MTX-resistant T-ALL sub-lines (Fig. 4). Data showed a dose response decreased viability of the resistant cells upon treatment with increasing concentrations of ginger extract. More interestingly, the amount of ginger required for 50% proliferation inhibition of 1.2 μM MTX-resistant cells was considerably less than that needed for the equal percent inhibition of 0.6 μM

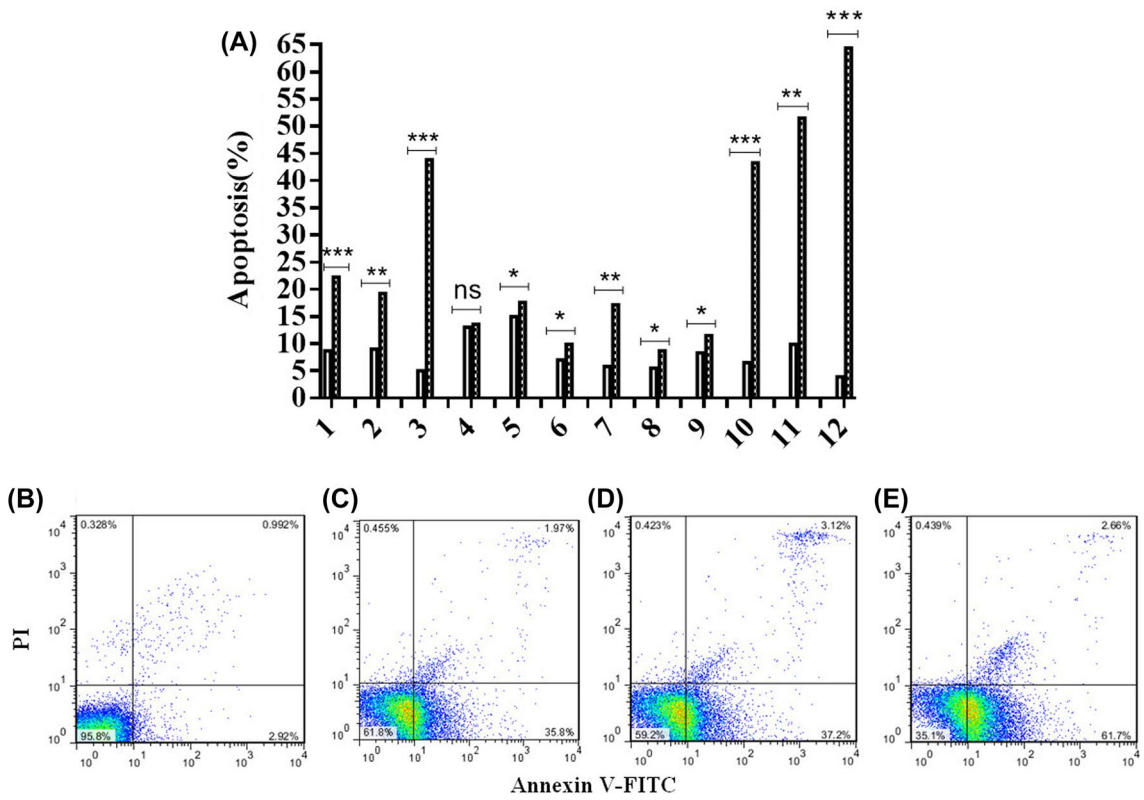

Fig. 6 Treatment of ginger and methotrexate on ALL primary cells. Malignant cells were isolated from patients' whole blood/bone marrow, at diagnosis, using Ficoll gradient media. Cells were, then, seeded into 96 microtiter plates followed by incubation with a 167 µg/ml ginger extract and 0.1 µM MTX either as single agents or in combination for 48 h. Apoptosis was analyzed using Annexin-V/PI double staining and flow cytometry analysis. **a** Sample number 1, 6, 7, 10, and 11 were isolated from patients with relapsed ALL and sample number 2, 3, 4, 5, 8, 9, and 12 are those isolated from new case ALLs, at diagnosis. (Rectangles) untreated cells, (filled rectangles) treated cells with combined ginger extract and MTX, *ns* not significant, $*P<0.05$, $**P<0.01$, $***P<0.001$. **b–d** A representative example for demonstrating the effect of MTX alone and in combination with ginger extract on patient primary cell apoptosis (see Table 3, case number 12). The percent apoptosis induced by the combined treatment of MTX and ginger extract was significantly higher than that induced by MTX alone in the ALL primary patients ($P<0.05$). **b** Untreated cells, **c** cells treated with ginger alone, **d** cells treated with MTX alone, **e** cells treated with the combination of ginger extract and MTX

MTX-resistant sub-line (84 µg/ml vs 117 µg/ml, respectively, $P<0.01$). In other words, the more resistant the cell was to the chemotherapy drug, the more sensitive it was to the medicinal herb. These data introduce ginger as a promising candidate for improved combination therapies in ALL, especially for those patients who show resistance to chemotherapy.

To identify the possible targets through which ginger may exert its cytotoxic effect on drug-resistant cells, the expression profiles of the ABC transporters, ABCA2 and ABCA3 were analyzed after treating the resistant sub-lines with ginger extract. The rationale for selecting the aforementioned drug efflux pumps was our previous studies on multidrug resistant genes identifying *ABCA2* and *ABCA3* as biomarkers which may be responsible for MDR in pediatric ALL (Aberuyi et al. 2017; Rahgozar et al. 2014). Expected increased expression levels of the above-mentioned genes were measured in the MTX-resistant sub-line (data not shown). However, surprisingly, results showed that treatment with the ginger extract was contributed to elevated, but not decreased, expression levels of *ABCA2* in the B or resistant T cell lines, by 11.475- and 2.679-fold, respectively (Fig. 7a, b). It is suggested that the altered cytoplasmic level of *ABCA2* or *ABCA3* transcripts may be considered as a defending response of the cells, in order to efflux ginger as a recognized toxic material. Interestingly, a possible compensatory effect between ABCA2 and ABCA3 transporters was previously reported in pediatric ALL patients (Aberuyi et al. 2017), which may explain the reason for the overexpression of only one of these related genes in the treated cell lines. No correlation was found between the aforementioned genes expression profiles and percent apoptosis followed by ginger/MTX treatment in the ALL patient primary samples (Table 3). Further experiments are required to determine the mechanism through

Table 3 Data obtained from patient primary cells exposed to a combination of 167 µg/ml ginger and 0.1 µM methotrexate

Case no.	Patient's condition	Source (WB/BM)	Sex	Age (year)	WBC > 50,000/µl	ALL subtype	Apoptosis followed by ginger/MTX treatment (%)	mRNA expression levels before ginger/MTX treatment	
								ABCA2	ABCA3
1	Relapsed	BM	F	8	+	Pre-B	22.225	2.205338	1.028827
2	New case	BM	M	3	+	T	19.280	5.049014	4.30496
3	New case	PB	M	4	−	T	43.870	2.950583	18.77837
4	New case	PB	M	0.7	+	Pre-B	13.580	0.379192	0.133139
5	New case	BM	M	11	−	Pre-B	17.560	0.211833	0.105185
6	Relapsed	PB	M	12	−	Pre-B	9.880	2.498392	3.401097
7	Relapsed	PB	F	12	+	Pre-B	17.120	1.05336	0.4383
8	New case	PB	M	12	−	Pre-B	8.690	0.350139	0.76897
9	New case	BM	M	4	−	Pre-B	11.500	1.760518	1.625631
10	Relapsed	PB	F	1.5	−	Pre-B	43.260	1.73628	1.169588
11	Relapsed	BM	M	6	−	Pre-B	51.475	3.472561	3.012581
12	New case	PB	M	3	+	T	64.360	0.688248	0.146198

F female, *M* male, *PB* peripheral blood, *BM* bone marrow

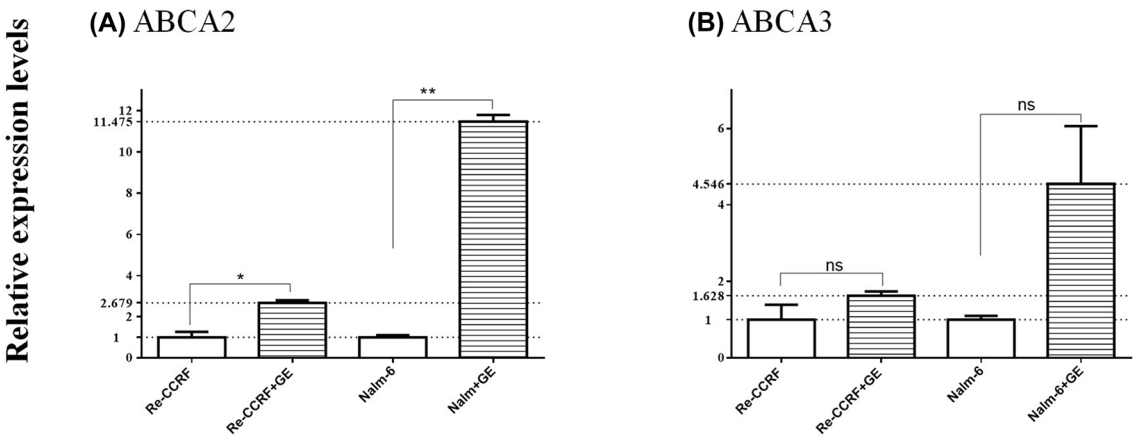

Fig. 7 Relative expression levels of *ABCA2* and *ABCA3* genes in ginger treated T and B-ALL cell lines. The MTX-resistant CCRF-CEM and Nalm-6 cell lines were treated with 167 µg/ml ginger extract and the mRNA expression levels of *ABCA2* and *ABCA3* multidrug resistant genes were assessed using real-time PCR. Interestingly, results showed that the expression levels of *ABCA2* was elevated, but not decreased, in the treated T and B cells, compared with the untreated cell lines (2.679- and 11.475-fold, respectively). Re-CCRF = 1.2 µM MTX-resistant CCRF-CEM, *GE* ginger extract, *ns* not significant, $^{*}P < 0.05$, $^{**}P < 0.01$

which ginger may exert its antiproliferative and antidrug resistance effect on the malignant lymphoblastic cells.

In conclusion, the present study demonstrated the intriguing in vitro antileukemic effect of ginger extract on B and T-ALL cell lines in addition to the patients' primary cells. Cinnamon and green tea extracts showed similar effects on CCRF-CEM. Results for the combined treatments of CCRF-CEM and Nalm-6 cell lines revealed 0.725 and 0.896 CI values, respectively, for ginger/MTX, demonstrating a synergistic interaction between MTX and ginger, but not the two other phytopharmaceuticals. Interestingly, ginger exhibited higher antiproliferative impact on the drug-resistant leukemic sub-lines compared with the sensitive parent cells. Moreover, it was revealed that ginger did not exert its negative effect on drug resistance by modifying the expression levels of the *ABCA2* and *ABCA3* drug efflux genes, suggesting other molecular pathways involved in its anticancer potential. To the best of our knowledge, this is the first report to validate the antitumor impact of ginger on childhood ALL and may definitely pave the way for the development of improved leukemia therapies. Future studies will explore the possible mechanism through which ginger may apply its toxic effect on the malignant cells.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Aberuyi N, Rahgozar S, Khosravi Dehaghi Z, Moafi A, Masotti A, Paolini A (2017) The translational expression of ABCA2 and ABCA3 is a strong prognostic biomarker for multidrug resistance in pediatric acute lymphoblastic leukemia. Onco Targets Ther 10:3373–3380. https://doi.org/10.2147/ott.S140488

Abolmaali SS, Tamaddon AM, Dinarvand R (2013) A review of therapeutic challenges and achievements of methotrexate delivery systems for treatment of cancer and rheumatoid arthritis. Cancer Chemother Pharmacol 71:1115–1130. https://doi.org/10.1007/s00280-012-2062-0

Baliga MS, Haniadka R, Pereira MM, D'Souza JJ, Pallaty PL, Bhat HP, Popuri S (2011) Update on the chemopreventive effects of ginger and its phytochemicals. Crit Rev Food Sci Nutr 51:499–523. https://doi.org/10.1080/10408391003698669

Bhojwani D, Yang JJ, Pui CH (2015) Biology of childhood acute lymphoblastic leukemia. Pediatr Clin N Am 62:47–60. https://doi.org/10.1016/j.pcl.2014.09.004

Butt MS, Sultan MT (2011) Ginger and its health claims: molecular aspects. Crit Rev Food Sci Nutr 51:383–393. https://doi.org/10.1080/10408391003624848

Cabrera C, Artacho R, Giménez R (2006) Beneficial effects of green tea—a review. J Am Coll Nutr 25:79–99. https://doi.org/10.1080/07315724.2006.10719518

Cheung-Ong K, Giaever G, Nislow C (2013) DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. Chem Biol 20:648–659. https://doi.org/10.1016/j.chembiol.2013.04.007

Dabaghi M, Rahgozar S, Moshtaghian J, Moafi A, Abedi M, Pourabutaleb E (2016) Overexpression of SORCIN is a prognostic biomarker for multidrug-resistant pediatric acute lymphoblastic leukemia and correlates with upregulated MDR1/P-gp. Genet Test Mol Biomark 20:516–521. https://doi.org/10.1089/gtmb.2016.0031

de Beaumais TA, Jacqz-Aigrain E (2012) Intracellular disposition of methotrexate in acute lymphoblastic leukemia in children. Curr Drug Metab 13:822–834. https://doi.org/10.2174/138920012800840400

Dugoua J-J, Seely D, Perri D, Cooley K, Forelli T, Mills E, Koren G (2007) From type 2 diabetes to antioxidant activity: a systematic review of the safety and efficacy of common and cassia cinnamon bark. Can J Physiol Pharmacol 85:837–847. https://doi.org/10.1139/Y07-080

Eichhorn T, Efferth T (2012) P-glycoprotein and its inhibition in tumors by phytochemicals derived from Chinese herbs. J Ethnopharmacol 141:557–570. https://doi.org/10.1016/j.jep.2011.08.053

Fotoohi AK, Albertioni F (2008) Mechanisms of antifolate resistance and methotrexate efficacy in leukemia cells. Leuk Lymphoma 49:410–426. https://doi.org/10.1080/10428190701824569

Hamidpour R, Hamidpour M, Hamidpour S, Shahlari M (2015) Cinnamon from the selection of traditional applications to its novel effects on the inhibition of angiogenesis in cancer cells and prevention of Alzheimer's disease, and a series of functions such as antioxidant, anticholesterol, antidiabetes, antibacterial, antifungal, nematicidal, acaricidal, and repellent activities. J Tradit Complement Med 5:66–70. https://doi.org/10.1016/j.jtcme.2014.11.008

Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, Sarkar S (2014) Drug resistance in cancer: an overview. Cancers (Basel) 6:1769–1792. https://doi.org/10.3390/cancers6031769

Hunger SP, Mullighan CG (2015) Acute lymphoblastic leukemia in children. N Engl J Med 373:1541–1552. https://doi.org/10.1056/NEJMra1400972

Kato M, Manabe A (2018) Treatment and biology of pediatric acute lymphoblastic leukemia. Pediatr Int 60:4–12. https://doi.org/10.1111/ped.13457

Kelly T, Owusu-Apenten RK (2015) Effect of methotrexate and tea polyphenols on the viability and oxidative stress in MDA-MB-231 breast cancer cells. J Appl Life Sci Int 2:152–159. https://doi.org/10.9734/JALSI/2015/14142

Kodidela S, Chandra PS, Dubashi B (2014) Pharmacogenetics of methotrexate in acute lymphoblastic leukaemia: why still at the bench level? Eur J Clin Pharmacol 70:253–260. https://doi.org/10.1007/s00228-013-1623-4

Lage H (2008) An overview of cancer multidrug resistance: a still unsolved problem. Cell Mol Life Sci 65:3145–3167. https://doi.org/10.1007/s00018-008-8111-5

Ma X et al (2015) Rise and fall of subclones from diagnosis to relapse in pediatric B-acute lymphoblastic leukaemia. Nat Commun 6:6604. https://doi.org/10.1038/ncomms7604

Mahbub A, Le Maitre C, Haywood-Small S, Cross N, Jordan-Mahy N (2017) Dietary polyphenols influence antimetabolite agents: methotrexate, 6-mercaptopurine and 5-fluorouracil in leukemia cell lines. Oncotarget 8:104877–104893. https://doi.org/10.18632/oncotarget.20501

Paul S, Kantarjian H, Jabbour EJ (2016) Adult acute lymphoblastic leukemia. Mayo Clin Proc 91:1645–1666. https://doi.org/10.1016/j.mayocp.2016.09.010

Rahgozar S et al (2014) mRNA expression profile of multidrug-resistant genes in acute lymphoblastic leukemia of children, a prognostic value for ABCA3 and ABCA2. Cancer Biol Ther 15:35–41. https://doi.org/10.4161/cbt.26603

Rao PV, Gan SH (2014) Cinnamon: a multifaceted medicinal plant. Evid Based Complement Altern Med 2014:642942. https://doi.org/10.1155/2014/642942

Saxena R, Rida PC, Kucuk O, Aneja R (2016) Ginger augmented chemotherapy: a novel multitarget nontoxic approach for cancer management. Mol Nutr Food Res 60:1364–1373. https://doi.org/10.1002/mnfr.201500955

Sheikh BY, Sarker MMR, Kamarudin MNA, Ismail A (2017) Prophetic medicine as potential functional food elements in the intervention of cancer: a review. Biomed Pharmacother 95:614–648. https://doi.org/10.1016/j.biopha.2017.08.043

Shen Y, Jia L-N, Honma N, Hosono T, Ariga T, Seki T (2012) Beneficial effects of cinnamon on the metabolic syndrome, inflammation, and pain, and mechanisms underlying these effects—a review. J Tradit Complement Med 2:27–32

Singh BN, Shankar S, Srivastava RK (2011) Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol 82:1807–1821. https://doi.org/10.1016/j.bcp.2011.07.093

Tian H, Cronstein BN (2007) Understanding the mechanisms of action of methotrexate: implications for the treatment of rheumatoid arthritis. Bull NYU Hosp Jt Dis 65:168–173

Wijdeven RH, Pang B, Assaraf YG, Neefjes J (2016) Old drugs, novel ways out: drug resistance toward cytotoxic chemotherapeutics. Drug Resist Updates 28:65–81. https://doi.org/10.1016/j.drup.2016.07.001

Wojtuszkiewicz A et al (2015) Methotrexate resistance in relation to treatment outcome in childhood acute lymphoblastic leukemia. J Hematol Oncol 8:61. https://doi.org/10.1186/s13045-015-0158-9