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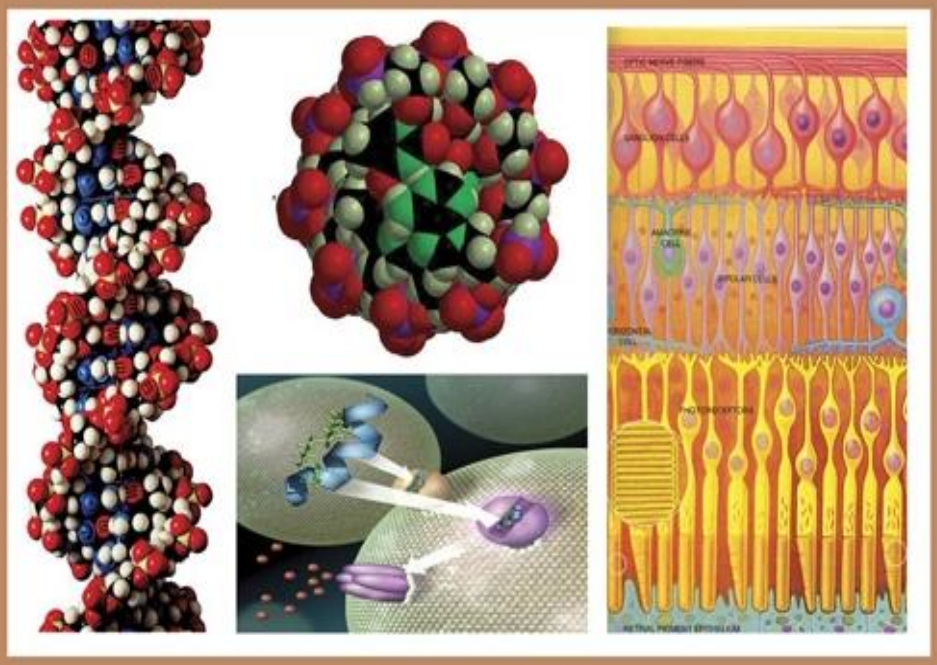

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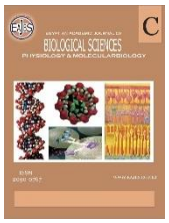

Regulation of Breast Cancer Stem Cells Associated-miRNAs by Ginger and Persimmon Extracts

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ABSTRACT

The ability of individual and combined extracts of ginger and persimmon to target BCSCs subpopulation and restore the expression of tumor suppressive miRNAs, miR-200c, miR-30a and miR-128, is evaluated in this study. Combined treatment led to decreasing $CD44^{+}$ $CD24^{-}$ subpopulation in MDA-MB-231 to a percent lower than both individual extracts, however, the combined treatment didn't affect the expression of pluripotency-associated genes, OCT3/4, SOX2 and NANOG, which are down-regulated as a consequence of both individual extracts treatment. Bioinformatics predicted DNMT3A as the target of miR-200C and miR-30a, and ABCB9 as the target of miR-128a. Both individual extracts exerted an activating effect on miRNAs (except persimmon extract on miR-30a) and an inhibitory effect on their targets DNMT3a and ABCB9 expression. Additionally, combined treatment keeps the activating effect of individual extracts on miRNAs, decrease the inhibitory effect of individual extracts on ABCB9, and reversed it into activating effect in the case of DNMT3a. Moreover, we found a significant decrease in miR-200c promoter methylation after individual treatments, which could represent a positive feedback loop between miR-200c and its target DNMT3a, however, this effect on methylation status is absent in the case of combined treatment. The methylation of LINE1, the index of global DNA methylation, was increased in response to ginger extract treatment, predicting increasing in genomic stability. Our finding highlighted the role of individual and combined aqueous ginger extract and methanolic persimmon extract to subdue BCSCs by modulating the expression of tumor-suppressive miRNAs, despite the variation of molecular genetics and epigenetic response of candidate genes.

INTRODUCTION

Cancer stem cells (CSCs), also known as cancer-initiating cells, represent a small proportion of cancer cells that have the ability to develop into other tumorigenic and non-tumorigenic cells. CSCs characterized by stem properties, including self-renewal and pluripotency (Lou, Weiyang, *et al.*,2018). The hallmark of breast cancer stem cells (BCSCs) is the up-regulation of surface marker CD44 and down-regulation (or absence) of CD24 (Shackleton, Mark, *et al.*,2006).

The abundance of BCSCs in poorly differentiated and highly malignant breast cancer tissues is higher than in differentiated, low-grade malignancies (Pece, Salvatore, *et al.*,2010). MicroRNA (miRNAs), a group of non-coding RNA molecules that are involved in gene expression regulation at the post-transcriptional level, are dysregulated in human cancer acquisition of stem cell-like properties (Garg, Minal.,2015).

Aberrant DNA methylation at promoter-associated CpG islands may lead to inactivation of tumor suppressor genes and be involved in the initiation and progression of human cancer. Tumor suppressor miRNAs could be silenced by hypermethylation, resulting in the activation of target oncogenes. On the other hand, upregulation of oncogenic miRNAs due to hypomethylation is reported in various cancers. The factors involved in DNA methylation, including DNMT1, DNMT3A, DNMT3B, are also documented to be targets for some miRNAs (Saito, Yoshimasa.,2014).

OCT3/4, NANOG and SOX2 are a group of pluripotency regulators which reinforce each other's expression (Zhou, Ying, *et al.*,2017). These pluripotency-associated transcription factors are greatly involved in the development and malignant progression of various types of tumors, including breast (Ling, Gui-Qin, *et al.*,2012).

Several Natural compounds and its derivatives have been found to exhibit anticancer properties and used in clinical treatment, especially due to low side effects, through different mechanisms, including epigenetic regulation (Wang, Yujiong, *et al.*,2013) (Lin, Qian, *et al.*,2017). Ginger (Zingiber officinale L., Family Zingiberaceae) stem was reported to have more than 80 components in the volatile oil. In addition, Ginger contains a number of phytochemicals, and about 65 compounds were identified from essential oils (Leja, Katarzyna B., and Katarzyna Czaczyk.,2016) (Yashin, Alexander, *et al.*,2017). Anticancer activity of ginger was documented against many types of cancer, like colon and lung, mainly due to the presence of gingerols, paradols and shogaols (Sang, Shengmin, *et al.*,2009). Persimmon (Diospyros kaki) fruit is enriched with many bioactive compounds, including polyphenols, terpenoids, steroids, flavonoids, carotenoids, minerals, and dietary fiber (Karaman, Safa, *et al.*,2014) (Rates, Stela Maria Kuze.,2001). A number of these bioactive compounds have chemopreventive (Raskin, Ilya, *et al.*,2002) and anticancer properties (Bei, Weijian, *et al.*,2005).

This study is a step towards evaluating the impact of using a total extract of ginger and persimmon to regulate miRNAs dysregulated in BCSCs and their target genes with a role in mediating methylation and drug resistance. In addition, analyze the ability of these extracts to regulate the expression of OCT3/4, NANOG and SOX2.

MATERIALS AND METHODS

1-Extracts Preparation:

To prepare aqueous extract of ginger, 500 ml of distilled water was added to 100 gm of dry powder of the root parts of ginger (Zingiber officinale L., Family Zingiberaceae) and heated at 60 °C for 6 h. shaking was continued overnight then the supernatant was decanted, redistilled then dissolved in RPMI media at desired concentrations. Persimmon (Diospyros virginiana fruits) was extracted with methanol (10:1; volume for weight) for 6 h in a water bath at 45 °C. The methanol was evaporated and the crude extract was weighted and dissolved in RPMI media at the desired concentrations.

2-Cell Line Propagation And Treatment:

The human breast cancer cell line (MDA-MB-231) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, Md.) supplemented with 10 % fetal bovine serum (Gibco BRL, Gaithersburg, Md.), 1 % penicillin/ streptomycin (5 mg/mL each) and 2 % L-glutamin (2 mM) at 37 °C in a humidified

atmosphere of 5 % CO_2. Cells at approximately 80 % confluence were trypsinized, seeded and incubated at 37 ºC and 5 % CO_2 overnight. Cells then treated with different concentrations of methanolic extract of persimmon and water extract of ginger separately and in combination at different concentrations.

3-Bioinformatic Analysis:

Bioinformatics algorithm (http://www.microrna.org/microrna/home.do) was used to predict downstream targets of miR-200c, miR30a and miR-128. Mature sequences of miRNAs were downloaded from miRBase (http://www.mirbase.org) and the 3' UTR sequence of human DNMT3a and ABCB9 was downloaded from the Ensemble genome browser.

4-MTT Assay:

Cells were seeded in 96 well microplates (3 X 10^3 cells/well) in 100 µL RPMI-1640 culture medium and incubated at 37 ºC and 5 % CO_2 overnight. Cells were treated and re-incubated for 24 h, 100 µL of MTT [3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (0.5 mg/ml) solution were added to each well and incubated until purple formazan crystals appeared. The medium was discarded and 100 µL of DMSO was added to dissolve the crystals. The optical density (OD) of solubilized formazan was measured at 592 nm using an automated microplate reader. The absorbance is related to the relative number of viable cells. Results are expressed as a percent of untreated control.

5-Reverse Transcription and Real-time PCR;

Total RNA, containing miRNA, was extracted from treated cells using Direct-zol™ RNA MiniPrep (zymo research) according to the manufacturer's instructions. The extracted mRNA and microRNA were reverse transcribed into single-stranded complementary DNA (cDNA) using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and miScript II RT Kit (Qiagen), respectively. For mRNA expression, mixtures were prepared using maxima SYBR green qPCR master mix (Thermo Scientific) according to manufacturer. GAPDH was used as an internal reference gene to normalize gene expression. Primers were designed using free available Primer3 software (http:// bioinfo . ut.ee/primer3-0.4.0/primer3/). Expression analysis of miRNAs was carried out using miScript SYBR Green PCR Kit according to the manufacturer using included universal (reverse) primer, while forward primers were designed by an online miRNA primer design tool (http://genomics.dote.hu:8080/ mirnadesigntool/). Expression was normalized to RNU6B. Results were expressed as the ratio of reference to target gene using $2^{-\Delta\Delta Ct}$ method. Primers are listed in Table 1.

Table 1: Sequence of the primers used in the study.

Gene	Forward (5→3)	Reverse (5→3)
MiR-200c	TGGGTTAATACTGCCGGGTAAT	Universal
MiR-30a	TGTGTTGTGTAAACATCCTCGAC	Universal
MiR-128a	TGTTTTTTTTTCACAGTGAACCGG	Universal
OCT3/4	ATCAAAGCTCTGCAGAAAGAACTC	AATAGAACCCCCAGGGTGAG
SOX2	ATGGCAATCAAAATGTCCATTGTTT	TTTAAACAAGACCACAGAGATGGTT
NANOG	GAAAAGTCTTAAAGCTGCCTTAACC	GAAAAGTCTTAAAGCTGCCTTAACC
DNMT3A	TTTTAACTCTCGCCTCCAAAGAC	AGAGCTGCTGGTGTCCCC
ABCB9	CAGTCTCAGCTCCGACCTTG	CAAGGCCATCATCATCCACAG
MiR-200c M	GGGTTTTTTATATTTCGTTACGG	ACGATACGTCCGACGACTCG
MiR-200c U	GGGTTTTTTATATTTTGTTATGG	AAACAATACATCCAACAACTCACA
OCT3/4 M	TAGTTATTGGTTTGATTTTCGGAAC	ACGCTAACCTAAAACATAACACGTA
OCT3/4 U	ATTGGTTTGATTTTTGGAATGA	ACACTAACCTAAAACATAACACATA
SOX2 M	GTTTATTTATTTTTTTCGAAAAGGC	ATAAATTTCTAACGACCAATCAACG
SOX2 U	GTTGTTTATTTATTTTTTTTGAAAAGGTG	AAATAAATTTCTAACAACCAATCAACAC
NANOG M	AATTATATTTTTGATTTAAAAGTTGGAAAC	CAACTCAATCCAACAAAACGTT
NANOG U	AATTATATTTTTGATTTAAAAGTTGGAAAT	AACTCAATCCAACAAAACATT
LINE-1M	GAGGTATTGTTTTATTTGGGAAGC	TACTAACAATCAACGAAATTCCGTA
LINE1-U	AGGTATTGTTTTATTTGGGAAGTGT	TACTAACAATCAACAAAATTCCATA

M: methylated; U: unmethylated

6-Methylation Analysis:

DNA was extracted from treated cells, subjected to bisulfite modification by EpiTect Bisulfite kit (Qiagen). The converted DNA was used for methylation-specific PCR (MSP) and the percent of methylation was detected using CT method. Promoter sequences OF OCT3, SOX2 and NANOG were downloaded from the Ensemble genome browser. The promoter of miR-200c was obtained from mirstart database (http://mirstart.mbc.nctu.edu.tw/home.php). The sequence of LINE-1 was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Primers were designed using free online methprimer (http://www.urogene.org/methprimer) as listed in Table 1.

7-Flow Cytometric Analysis:

The cells were analyzed by fluorescence-activated cell sorting (FACS). Briefly, cells were trypsinized and washed by PBS. The cell pellet was re-suspended in 100 µL ice-cold PBS supplemented with 2% FBS. 20 µL of IOTest® CD44-FITC and IOTest® CD24-PE antibodies (Beckman Coulter, USA) were added to the cells and then incubated at room temperature for 25 minutes in the dark with mild agitation. The cells were rinsed twice with PBS, re-suspended in 0.5 mL of PBS supplemented with 2% FBS and analyzed by flow cytometry (Epics XL, Beckman Coulter). Unstained cells were used as the negative control.

8-Statistical Analysis:

Data were analyzed using SPSS 16.0 software and presented as mean ± standard error. Statistical significance of the data was analyzed by Independent Samples T Test. $P< 0.05$ was considered statistically significant.

RESULTS

1-Ginger and Persimmon Extracts Are Non-Cytotoxic against MDA-MB-231 cells:

The cytotoxic activity of the individual extracts was investigated using different concentrations (10, 30, 50, 80, and 100 µg of each extract), while for the combinations the following concentrations of ginger and persimmon extracts were used; 50:50, 40:60, 60:40, and 30:30, respectively; to treat MDA-MB-231 cells for 24, 48, and 72 hours. Results revealed that all treatments are non-cytotoxic and no decrease in cell viability was observed. For subsequent experiments, 80 µg of each extract was used and in the combined treatment we used a mixture of 30: 30 µg of each extract.

2-Ginger and Persimmon Extracts Decrease BCSCS Population:

MDA-MB-2331 treatment with

Ginger and persimmon extracts led to decreasing $CD44^+$ $CD24^-$ cells population from 64.4 in untreated control to 32.5, 49.9 and 24.2 in cells treated with ginger, persimmon, and combined extracts, respectively (**Fig. 1**).

3-Ginger and Persimmon Extracts Regulate Expression Of Pluripotency-Associated Genes:

Real-time PCR data revealed that the expression of OCT3/4, SOX2, and NANOG were inhibited in Ginger- and persimmon-treated cells (P=0.000, 0.005 and 0.008; P=0.000, 0.015 and 0.004, respectively), while no effect was observed after the combined treatment of both extracts (P>0.1) (Fig. 2)

4-Ginger and Persimmon Extracts Regulate Expression of miRNAs:

Cells treated with Ginger, persimmon and combined extracts showed a significant 3.5-, 7- and 4-fold increase of miR-200c expression (P=0.009, 0.005 and 0.008, respectively). MiR-30a expression was varied between treated cells, whereas ginger extract led to 50 % increase (P=0.049), persimmon extract exerted no change in the expression (P=0.432), and combined treatment displayed a 50 % increase (P=0.038). Expression of miR-128a showed 47%, 56% and 100% increase in response to ginger, persimmon and combined extracts treatments (P=0.02, 0.014and 0.004, respectively) (Fig. 3D).

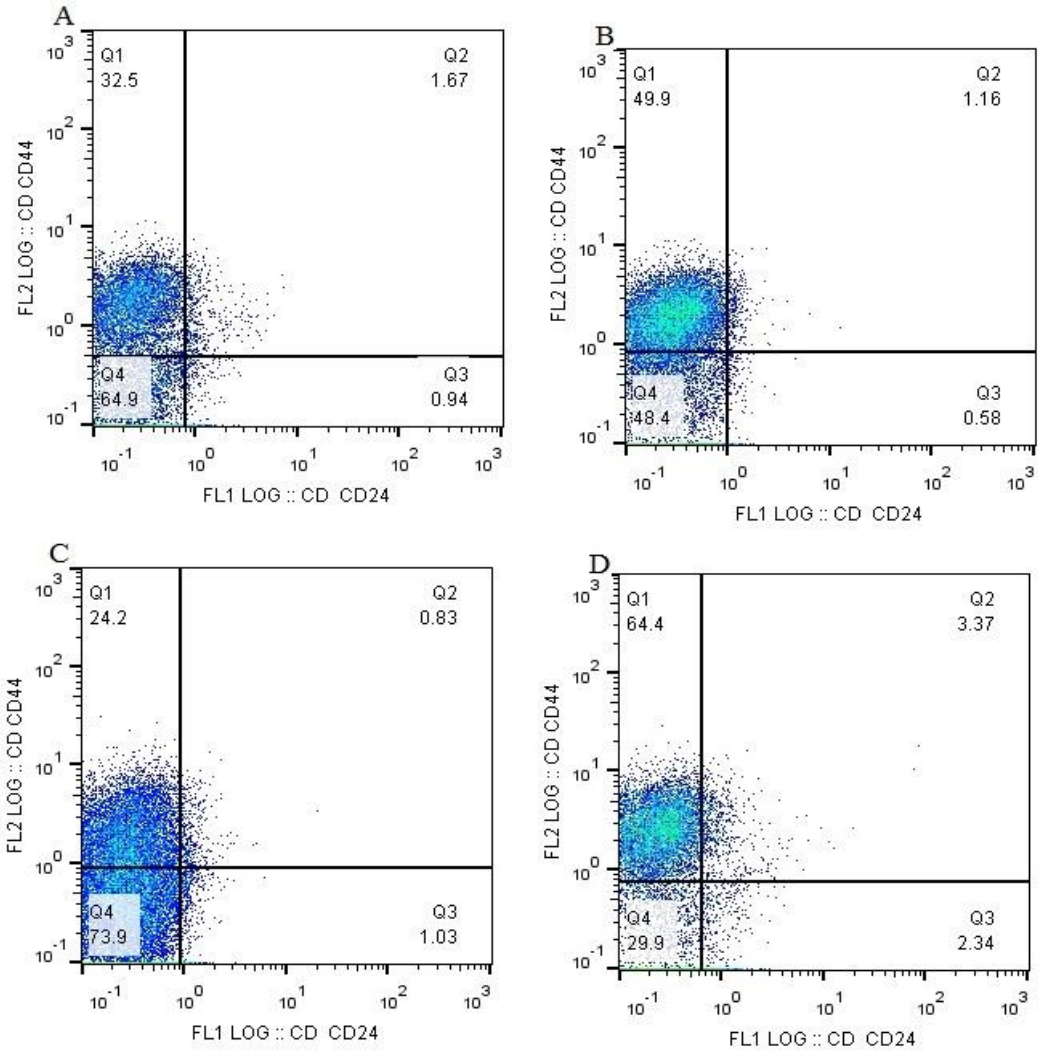

Fig.1: Flowcytometric analysis reveals decreasing CSCs population in response to the treatment of ginger (A), persimmon (B) and combined extracts(C) relative to untreated control (D).

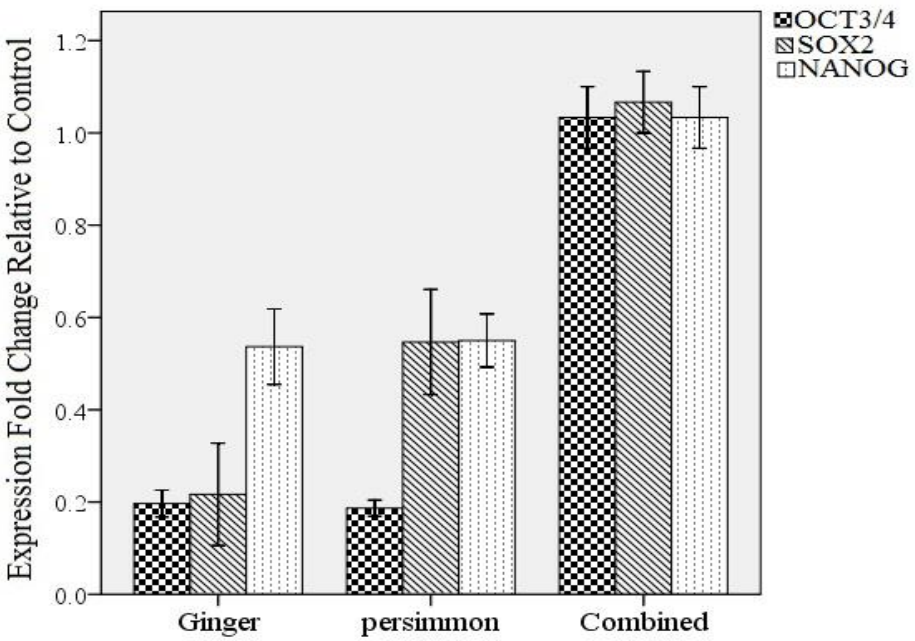

Fig. 2: Regulating expression of OCT3/4, SOX2, and NANOG genes after treatment with ginger and persimmon extracts.

5-Ginger and Persimmon Extracts Regulate the Expression of Drug Resistance- And Methylation-Associated Genes:

Bioinformatic algorithms predicted DNMT3a as a target of miR-200C and miR-30a, while ABCB9 was predicted as a miR-128a target (Fig. 3A-C). DNMT3A expression was suppressed in cells treated with ginger and persimmon extracts compared to untreated control (P=0.002 and 0.001, respectively); while the combined treatment displayed 3.4-fold increase in its expression (P=0.011). Treatment with ginger and persimmon extracts resulted in a significant decreasing the expression of ABCB9 gene (P=0.001); while the combined treatment displayed a decrease in the inhibitory effect of individual extracts (P=-0.032) (Fig. 3D).

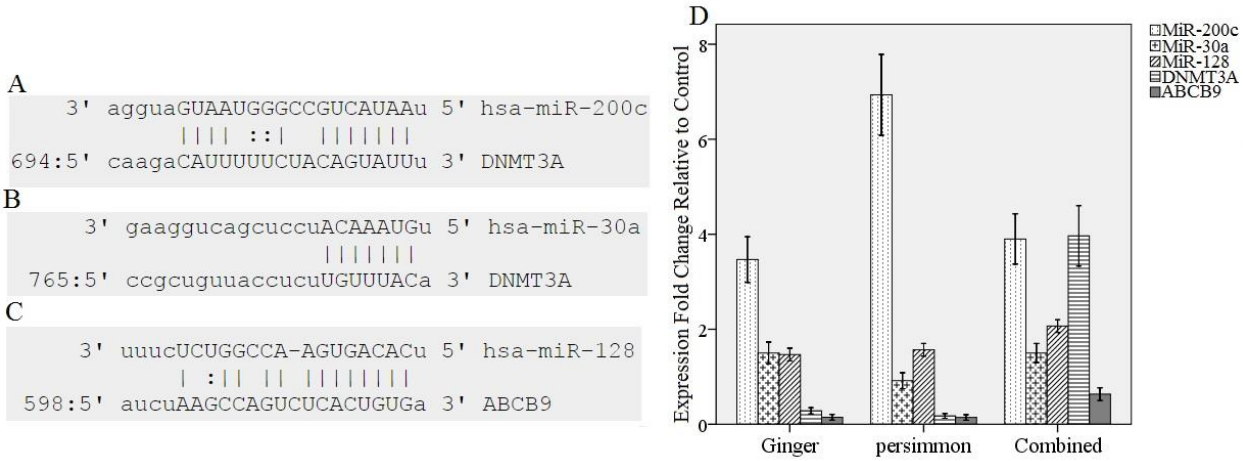

Fig. 3: A, B, and **C** show a schematic representation of bioinformatic analysis of predicted seed regions and binding sites of "miR-200c and DNMT3A" **(A)**," miR-30a and DNMT3A" **(B)** and "miR-128a and ABCB9" **(C). D** showing regulating the expression of miRNAs and their targets as a consequence of treating with ginger and persimmon extracts.

6-Ginger and Persimmon Extracts Regulate DNA Methylation Status:

MSP data revealed a significant decrease of miR-200C promoter methylation to 36 and 54% relative to control (100%) as a consequence of treatment with Ginger and persimmon extracts (P=0.001 and 0.005, respectively), while combined treatment showed no change in methylation status (P=0.225) comparing to control. Additionally, change in the methylation status of OCT3/4 and SOX promoter after different treatments were not significant (p>0.1), whereas NANOG promoter showed a significant increase after persimmon and combined extracts treatment (P=0.006 and 0.003, respectively) without significant change in ginger treated cells (P=0.095). The methylation status of LINE1 was increased in response to ginger treatment (p=0.005), unchanged significantly in response to persimmon (P=0.06), and decreased in cells with combined treatment (P=0.007) (Fig. 4).

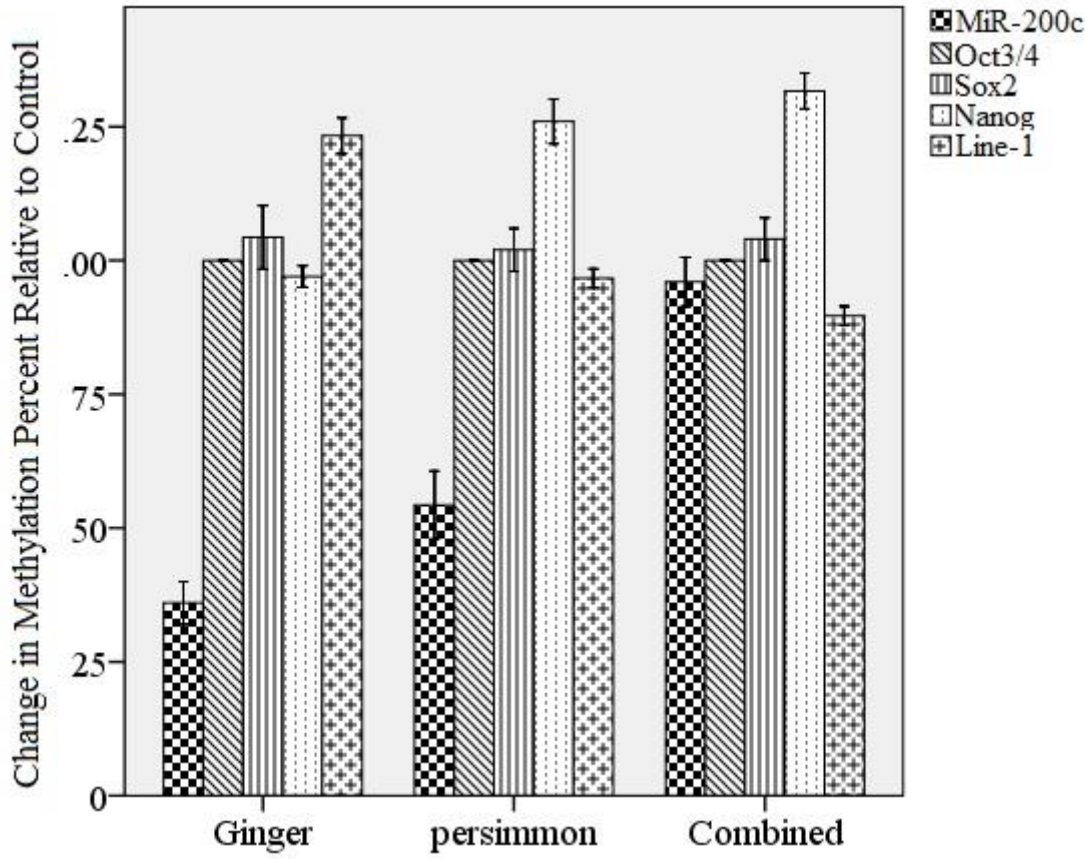

Fig. 4: Regulation of promoter-associated CpG islands of miR-200c, OCT3/4, SOX2 and NANOG, in addition to LINE-1, in response to treatment with ginger and persimmon extracts.

DISCUSSION

The efficacy of ginger (Akimoto, Miho, *et al.*,2015) and persimmon (Butt, Masood Sadiq, *et al.*,2015) as anticancer agents are previously reported. For example, 6-gingerol was found to enhance viability reduction induced by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in gastric cancer cells through inhibition of TRAIL-induced NF-κB stimulation (Ishiguro, Kazuhiro, *et al.*,2007). Also, carotenoid contents of persimmon were found to have chemoprotective effects against various types of cancer (Redpath, S.,

and A. P. George.,2008). Herein, we focused on the ability of ginger aqueous extract and persimmon methanolic extract to target BCSCs population in MDA-MB-231 cell line. This ability is manifested through flow cytometric data which revealed a significant decrease of $CD44^{+}CD24^{-}$ cells population in response to both individual extracts, and more effectively in response to combined extracts treatment without cytotoxic activity.

Generally, many mechanisms are involved in the regard of anticancer activities of natural agents, including, but not limited to, the normalization of altered gene expression levels. We focused on restoring the expression of tumor-suppressive miRNAs and their predicted targets which are involved in drug resistance or DNA methylation, and inhibiting pluripotency-related genes. Bioinformatic analysis predicted DNMT3A as a target of miR-200C and miR-30a, and ABCB9 as a target of miR-128a, therefore, we analyzed the effect of ginger and persimmon extracts on the expression of miR-200c, miR-30a and miR-128a and their targets DNMT3A and ABCB9. MiR-200c, miR-30a and miR-128 are previously identified as tumor suppressor miRNAs down-regulated in breast cancer (Korpal, Manav, *et al.*,2008) (Kawaguchi, Tsutomu, *et al.*,2017) by different mechanisms, including hyper-methylation of promoter-associated CpG island (Neves, Rui, *et al.*,2010). MDA-MB-231 cell line was observed to express a putative hypermethylator phenotype which coordinates with overexpression of total DNMT activity (Roll, J. Devon, *et al.*,2008). ATP binding cassette (ABC) B9 (ABCB9), a member of the ABC transporter family associated with drug accumulation in lysosomes (Moody, Hannah L., Michael J. Lind, and Stephen G. Maher.,2017), was reported to be regulated by miR-24 and play a role in mediating its chemo-sensitivity effect in breast cancer cells (Gong, Jian-Ping, *et al.*,2016).

Our results revealed that both individual extracts exerted an activating effect on miRNAs (except persimmon extract on miR-30a) and an inhibitory effect on their targets DNMT3A and ABCB9. On the other hand, combined treatment keeps the activating effect of individual extracts on the selected miRNAs, however, this differs regarding the candidate targets. Whereas combined extracts led to decreasing inhibitory effect of individual extracts in the case of ABCB9, it reversed this effect into up-regulation in the case of DNMT3A. This may be attributed to the degree of complexity of miRNAs and their mode of action, for instance, the fact that individual miRNAs have the ability to target several mRNAs and that individual mRNAs could be regulated by several miRNAs promote us to suggest that many other miRNAs that target candidate genes DNMT3A and ABCB9, directly or indirectly via transcription factors, could be regulated as a consequence of the treatment. Previous studies demonstrated that (6)-Gingerol, the polyphenolic alkanone with the most potent anticancer compound in ginger, acts as an up-regulator of miR-27b leading to the apoptosis of myeloid leukemia cells (Rastogi, Namrata, *et al.*,2014).

Decreasing DNMT3a expression after individual treatment with individual extracts makes us suggest decreasing the methylation status of miR-200c-promoter associated CpG islands, we found a significant decrease in methylation percent after individual treatments, which could represent a positive feedback loop between miR-200c and its target DNMT3a, however, this effect on methylation status of miR-200c was absent in the case of combined treatment despite increasing DNMT3A expression, reflecting that up-regulation of miR-200c after combined treatment is mediated through a mechanism other than modulating its promoter associated methylation. Worth mentions, miR-200c present as a cluster with miR-141 and transcribed by the same promoter, this suggests that miR-141 methylation status may also be regulated by the ginger and persimmon extracts. Down-regulation of OCT3/4, SOX2, and NANOG expression parallel with decreasing

DNMT3A in all treated cells, led us to suggest that DNMT3A have no role in their promoter methylation. Therefore, we studied methylation status of their promoters and our suggestion is confirmed regarding OCT3 and SOX2 promoter methylation which isn't changed. While methylation of NANOG is increased in the case of treatment with persimmon and combined treatment, suggesting an involvement of other methylation-mediating factors in this regulation.

Methylation of long interspersed nucleotide element 1 (LINE-1), which is spread ubiquitously throughout the genome and commonly used as an index of global DNA methylation, was documented to be over-methylated in normal tissue, reflecting the stability of the genome, and hypo-methylated in tumor (Pearce, Mark S., et al.,2012) (Nelson, Heather H., Carmen J. Marsit, and Karl T. Kelsey.,2011). Our results indicate an increase in the methylation percent of LINE-1 after ginger treatment; this effect is absent in persimmon-treated cells, while a significant decrease is displayed as a consequence of combined treatment. This variation in methylation status of LINE-1 is unmatched with DNMT3A expression in the case of individual extracts, reflecting that it couldn't be mediated by DNMT3A, and other contributors in the ginger and persimmon extracts are involved. However, in the case of combined treatment, the decrease of DNMT3A expression is synchronous with decreasing methylation percent of LINE-1.

Our finding highlighted the role of individual and combined treatment with aqueous ginger extract and methanolic persimmon extract to subdue BCSCs subpopulation by modulating the expression of tumor suppressive miRNAs, despite the variation in molecular response of candidate genes due to the complexity of the molecular genetics and epigenetic level.

Conflict of Interests: The authors declare that there is no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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